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INHIBITION STUDIES OF CATECHOL-O-METHYLTRANSFERASE

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Thesis

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To Dr. A. D'Iorio who proposed,
directed and encouraged this work,
my most profound gratitude and
appreciation are due.

TABLE OF CONTENTS

F O R E W O R D

The work presented in this thesis was initiated as an attempt to explore *at* the enzyme level the well known potentiating action of thyroxine on the physiological and biochemical function of adrenaline and noradrenaline. It is not a study of the more general interrelationship between the thyroid and the adrenal glands.

One could conceive of two possible distinct ways by which the action of adrenaline is potentiated in the hyperthyroid animal. Firstly, the potentiation might be due to a hypersensitivity to the catecholamine imparted by the thyroid hormone - such as, for example, facilitated access to and responsiveness of receptor sites. This mechanism would not account for the observed potentiation *at* the biochemical level such as the adrenaline-induced hyperglycemia. Secondly, the observed phenomena could be accounted for by a diminished rate of destruction of the catecholamine. This could probably account for both the physiological and metabolic effects. Lastly, both hypersensitivity and impaired rate

of destruction might be responsible.

The mode of inactivation of catecholamines has become known only in recent years. Two main enzymatic systems are recognized as being responsible for the biological destruction of catecholamines: monoamine oxidase and catechol-O-methytransferase. The first has been known since 1928; the second only since 1957.

The possibility of a diminished rate of destruction of catecholamines being due to inhibition of monoamine oxidase by thyroxine failed to be substantiated, since such inhibition could not be demonstrated in vitro. A full account of this is given in the Introduction.

The possibility of direct inhibition of the second enzymatic system mentioned above, namely, catechol-O-methyltransferase, sparked the present investigation. It must be noted that when these studies were initiated our knowledge of this enzyme was not a closed chapter insofar as its properties per se and its precise physiological significance are concerned. At the outset of this work and during its course the behaviour of the enzyme against a variety of compounds, only remotely or not at all connected with thyroid activity or even thyroid hormone structure, was deemed necessary and proved to be helpful and indeed instructive.

Inevitably then, this work spontaneously expanded into a study, however modest, of an enzyme as yet inadequately known.

I N T R O D U C T I O N

S E C T I O N I

THYROXINE POTENTIATION OF CATECHOLAMINE EFFECTS
A REVIEW OF THE LITERATURE

The potentiating action of thyroxine on the effects of catecholamines, primarily of adrenaline, manifests itself in a variety of ways, all of which are ascribable to an exaggerated response of virtually all the effects of adrenaline as a result of the hyperthyroid state of the animal organism. Conversely, reports on the diminished effects of adrenaline in the hypothyroid state have also appeared.

The extensive literature relating to this subject goes as far back as 1908 when EFFINGER et al. (1908) observed in dogs an adrenaline mydriasis after thyroid feeding. Total thyroidectomy on the other hand diminished and sometimes abolished adrenaline induced glucosuria in dogs. The increased sensitivity of the iris to adrenaline (mydriasis) was also noted and reported much later (BERGWALL AND KUCHINSKY 1931). Similar effects on carbohydrate metabolism were noted by BURN and MARKS (1925). These investigators report experiments on both thyroidectomized and thyroid-fed animals. Thyroidectomy results in a decreased

hyperglycemic response to adrenaline, whereas thyroid feeding, so long as the glycogen store of the liver is not diminished, leads to an increased hyperglycemic reaction to adrenaline. Prolonged thyroid feeding (more than two weeks) results in a decreased hyperglycemic response due to depletion of the liver glycogen store, as was shown by CRAMER and KRAUSE (1913).

A more detailed study was reported in 1953 by [illegible] (1953). He established the dose-response curve in normal rabbits for both adrenaline and noradrenaline in relation to the hyperglycemic effect. The response bore a linear relation to the logarithmic dose. The two responses were parallel but noradrenaline was found to have 18% only of the hyperglycemic effect of adrenaline. The mean hyperglycemic response, expressed as the percentage blood sugar increase, to 60µg/kg adrenaline was increased after two weeks of thyroid feeding (0.2gm daily) to a level corresponding to 85µg/kg adrenaline before thyroid feeding. In parallel experiments with noradrenaline the mean hyperglycemic response to 250µg/kg of the latter was increased during the first three days of thyroid feeding to a greater extent than was the adrenaline response, but at the end of two weeks it was less than the initial response. By seven days of thyroid feeding

the increase in the adrenaline effect was the greater. COMSA (1950) has also reported that in thyroidectomized guinea pigs the blood sugar response to an injection of adrenaline was diminished compared to that of intact animals, and that injection of thyroxine restored the normal response to the amine. The same effects were noted by SPINKS and BURN (1952) in thyroid-fed and thyroidectomized rabbits.

JOSUE (1903) first demonstrated the sclerogenic effects of repeated intravenous injections of adrenaline. MIKULICICH and OESTER (1951), OESTER et al. (1955), DAVIS and OESTER (1952) and OESTER (1959) have reported that thyroxine as well as triiodothyronine treatment of rabbits results in a higher percentage and a more severe grade of aortic sclerosis than in control animals. Even toxic doses of thyroxine alone failed to produce arteriosclerosis and in thyroidectomized rabbits there was no evidence of arteriosclerosis on intravenous injections of adrenaline (OESTER 1959).

The alterations induced in the aortic walls of rabbits and enhanced by thyroxine treatment have been extensively studied by LORENZEN (1959, 1961, 1961a, 1961b, 1961c, 1962). The connective tissue forms the supporting fra-work of blood vessels. The connective tissue is composed of three parts - namely, ground substance,

fibers and cells. The ground substance is essentially composed of the nonsulfated mucopolysaccharides, hyaluronic acid and chondroitin, and the sulfated polysaccharides chondroitin sulfates, keratosulfate and heparitin sulfate. The collagen fibers predominate in connective tissue and collagen is unique in containing a high percentage (about 14%) of hydroxyproline. Changes in the connective tissue of the aortic walls of rabbits were assessed by LORENZEN on the biochemical level by the content of hexosamine and hydroxyproline (per mg of dry tissue) and the in vivo uptake of S^{35}-sulfate injected on the last day of the treatment (counts/100 sec./mg dry tissue). He found that daily injections of adrenaline for 15 days resulted in an increased hexosamine and calcium content and S^{35}-sulfate uptake and a decreased collagen content of the aortic wall. Simultaneous treatment of the animals with L-thyroxine intensified these changes (LORENZEN 1959, 1961). However, injection of L-thyroxine into male albino rats for two weeks also resulted in changes of the same nature as those observed following injection of adrenaline and L-thyroxine simultaneously. The author considers these changes to be due to a thyroxine-induced sensitization to endogenous adrenaline (LORENZEN 1961a). In another series of experiments (LORENZEN

1961b) he divided the rabbits into three groups: 1- Injected with adrenaline for two weeks and with L-thyroxine for another two weeks. 2- Injected with adrenaline for two weeks and physiological saline for another two weeks. 3- Untreated controls. The alterations in the adrenaline-thyroxine-treated group were not so pronounced as previously observed after simultaneous injections of the two hormones. But the thyroxine injections in this group brought about a clear-cut increase in hexosamine content and S^{35}-sulfate uptake as compared with the untreated controls. In contradistinction the adrenaline-saline-treated rabbits did not differ significantly from the controls. The conclusion is drawn that L-thyroxine administered in continuation of adrenaline treatment is able to maintain the adrenaline-induced alterations by probably sensitizing the arterial wall to endogenous adrenaline. It was further demonstrated ([illegible] 1962) that thyroidectomy per se caused no alterations in the aortic walls whereas it inhibited the adrenaline - induced alterations. Finally, it was established ([illegible] 1961c) that L-thyroxine also enhances the adrenaline - induced alterations in the aortic walls of rabbits.

The well known but poorly understood calorigenic

action of adrenaline resulting from an increase in the respiratory metabolism has also been shown to depend on the thyroid status of the animal. [illegible] et al. (1936) observed that the rise in oxygen consumption in normal rats following adrenaline injection was augmented after thyroid feeding, while in thyroidectomized animals a decrease of oxygen uptake was observed. Dessicated thyroid fed to thyroidectomized rats re-established the normal response to adrenaline. Furthermore, [illegible] (1942) established that a rise by dinitrophenol or fall by fasting in RMR does not bring about a similar increase or decrease in the response to adrenaline, indicating, according to the author, that the effect is specific for thyroxine. A number of investigators have confirmed the above initial observations on the calorigenic effects of adrenaline (SCHAEFFER and THIBAULT 1945, 1945a; [illegible] 1946; HORSFALL 1954; [illegible] et al. 1956; SWANSON 1956). The effects were evaluated either as oxygen consumption or heat output in rats, dogs and humans. Particularly detailed and illuminating are the experiments performed by SWANSON (1956). In intact rats doses of adrenaline of 40, 80 and 120 ug caused a marked increase in oxygen consumption, with a maximum observed after one hour. The magnitude, not the duration, of the response was found to vary directly with the dose of

adrenaline. The oxygen consumption of thyroidectomized rats was not affected by adrenaline. In thyroidectomized rats, two weeks after operation the oxygen consumption had fallen to 0.81 of that of the controls. Thyroxine injections after this period (daily, 3, 12, 24 and 48 µg) caused a rise in the oxygen consumption which reached a plateau in 5 days above that of the intact animals. This oxygen consumption varied linearly as the logarithm of the thyroxine dose. On adrenaline administration to these thyroidectomized, thyroxine-treated animals the oxygen consumption still varied linearly as the log dose thyroxine but the slope was approximately double. Finally, the sensitivity to adrenaline was so increased by thyroxine that a dose of adrenaline (100 µg/100 gm body weight) tolerable to intact or thyroidectomized rats was fatal to those receiving more than 3 µg of thyroxine. The unexpected observation in this work was that in the absence of endogenous or exogenous thyroxine, adrenaline could not exert a calorigenic effect. RIES (1942) and SCHAEFFER and THIBAULT (1945) did obtain increases in oxygen consumption of 43.4% and 8%, respectively, in their thyroidectomized rats. SIMSON attributes these effects to the poor control of dietary iodine and thyroxine, which might result in a thyroxine intake large

enough to induce an adrenaline effect on oxygen consumption. THIBAULT (1948) in her experiments included also noradrenaline but the effect on oxygen consumption was weaker and more transient than that observed with adrenaline. THIBAULT (1948a) also found that thyroxine augments the inhibitory effect of adrenaline on rabbit intestine, provided that the former is added to the Tyrode solution one hour before adrenaline. She further observed that intestine from a thyroidectomized rabbit was almost unresponsive to adrenaline, whereas intestine from a thyroxine treated rabbit was more sensitive than normal.

The effects of adrenaline on the heart have also been reported to be potentiated by thyroxine. OSWALD (1915) reported that injection of iodothyroglobulin into animals results in a faster pulse and higher blood pressure on injection of adrenaline. BAAR (1943) observed that thyroxine-treated rats show a cardiac accumulation of injected adrenaline with death ensuing in a few minutes. Increased accumulation of endogenous adrenaline has been observed by [illegible] et al. (1959) in rats made thyrotoxic by iodinated casein. BAAR (1944) further reported that the lethal myocardial concentration of adrenaline is distinctly lowered in rats by pretreatment with thyroxine and elevated by pretreatment

with uracil. In experiments with ten healthy young males (suffering from psychoses) he found that a daily dose of 0.4 gm of thiouracil for three months resulted in a significant diminution of the sensitivity of the heart to adrenaline as assessed by the reduced capacity for acceleration of the heart rate (RAAB 1945). Experiments with swine carotid arteries were conducted by SMITH (1953, 1954). Swine carotid arteries from thyroidectomized animals responded with a shorter vasoconstriction to adrenaline. The reaction was restored by addition of crystalline thyroxine to the perfusate (SMITH 1953). Similarly, thyroxine in concentrations of 5 parts/100 million immediately increased the sensitivity of the isolated swine carotid arteries to L-adrenaline (SMITH 1954). The sensitivity was demonstrated by an elevation in the percentage of vasoconstriction and a prolongation by 200-300 % in the reaction time to L-adrenaline. Thyroxine prolonged the constriction of the vasa vasorum induced by L-adrenaline by over 100 % even though thyroxine itself did not produce constriction of either the arterial wall or the vasa vasorum. The effects of intravenous infusion of noradrenaline on the systemic blood pressure and heart rate were studied in patients during varying states of thyroid function by SCHNECKLOTH et al. (1953). In four patients with thyrotoxicosis a

diminution of from 48 to 30% in the pressor response to noradrenaline was observed on return to the euthyroid state. The amplitude of the contractions of the isolated hearts from cats and guinea pigs was studied by HOFFMAN et al. (1942). In thyroidectomized animals the amplitude as well as the frequency was reduced. Similar observations concerning the effects of thyroxine on the response of the cardiovascular system to adrenaline, as exemplified above, have been reported by a number of investigators (ASHER and FLACK 1910; SCHERMAN 1927; EPPINGER and LEVINE 1934; LEWIS and McEACHERN 1931; SMILY and CUTLER 1936; MACKAY SAUER and BROWN 1935; WISE and HOFF 1938; ADRIAN and YOUMANS 1940; STURMAN and WEEK 1942). Some publications relating to the same effects could not be obtained either in the original or in abstract form but are given in the References (SANTESSON 1919; LEVY 1922; SADAN 1922; KÖNIG 1928, LUTZIN 1930). In effect, the hypersensitivity of the heart to adrenaline in the hyperthyroid state was developed as a diagnostic test for hyperthyroidism (Goetsch test) in a study of about 300 cases of thyroid disease by GOETSCH (1920). This investigator also confirmed that, in hypothyroidism, there is an increased tolerance to administration of adrenaline. LEVINE et al. (1930) reported that injected adrenaline will

initiate an attack on patients suffering from angina pectoris, whereas in none of the control healthy patients did the pain occur. SHAMBAUGH and CUTLER (1934) experimenting with dogs (in which evidence of pain was used as criterion) found that thyroidectomy resulted in the prevention of the painful response. BLUMGART et al. (1933) and LEVINE et al. (1933) applied subtotal thyroidectomy in the treatment of severe angina pectoris. MAAR (1947) suggested and applied thyroidectomy or thiouracil treatment to patients with angina pectoris and noted that the drug would free the patients of their symptoms, provided the treatment had been applied for sufficiently long periods.

Detailed measurements on thyrotoxic animals before and after total sympathetic block were performed by BRESSTER et al. (1954). Mongrel dogs were made thyrotoxic by the daily feeding of 0.8 gm/kg of dessicated thyroid for 3 weeks. The signs of thyrotoxicosis in 15 dogs manifested themselves in the following changes: 1) Elevation of blood PBI to 18 ug% 2) 76% increase in oxygen consumption, 3) 110% increase in cardiac index, 4) 85 beats/min. increase in heart rate, 5) no significant change in mean arterial pressure. Total sympathetic block by the epidural injection of 0.4% procaine resulted in a decrease of the mean values for oxygen consumption,

heart rate and cardiac index, which were not significantly different from the values found in β1 blocked euthyroid dogs. The infusion of L-adrenaline or L-noradrenaline in blocked thyrotoxic animals resulted in a return of the oxygen consumption, cardiac index and cardiac rate to values found in the unblocked thyrotoxic dogs. Greater increases in oxygen consumption, heart rate and cardiac index were obtained at an infusion rate of 0.15 µg/kg/min. and 1.0 µg/kg/min. for L-adrenaline and L-noradrenaline respectively, in thyrotoxic dogs than were obtained in euthyroid dogs. It is concluded that the calorigenic and cardiovascular effects of thyrotoxicosis are not due to an effect of thyroxine per se, but rather to a potentiation of the physiological effects of adrenaline and noradrenaline. In a more detailed account of this work two years later [illegible] et al. (1956) confirm their results and conclusions.

Spinal anesthesia has been suggested and applied as a means of anesthetizing that part of the sympathetic nervous system which innervates the adrenal glands and therefore reducing the secretion of adrenaline, both immediately before operation and as a result of the increase in circulating thyroxine, due to manipulation of the gland, in patients undergoing thyroidectomy. [illegible] (1944) used spinal anesthesia in twenty cases of bilateral subtotal

thyroidectomy in order to avoid the "thyroid storm". He reports that in all cases the operative and post-operative course was impressively smooth and that the blood sugar of these patients before, during and after thyroidectomy was normal. This, of course, was taken to indicate that no excess adrenaline was present in the circulation. Use of spinal anesthesia is also reported by KNIGHT (1945) with similar results.

Use has been made of the adrenergic blocking agent Dibenzyline in an attempt to block the metabolic effects of thyroxine with contradictory results. HOLTCAMP and HEMING (1953) and RAMEY (1955) report that in thyrotoxic animals administration of dibenzyline prevents the rise in oxygen consumption either in the presence or in the absence of administered adrenaline. SOMMERS et al. (1957) on the contrary, found no such effect. They further report that after bilateral adrenal demedullation the injection of a large dose of thyroxine with or without concurrent administration of dibenzyline, is followed by a significant rise in oxygen consumption. It is concluded that the calorigenic effects of exogenous thyroxine do not depend on the presence of medullary hormones. In agreement with these results are the experiments of SCHWARTZ et al. (1957) who showed that dibenzyline does not block the metabolic effects of

thyroxine and they believe that discrepant findings result from differences in the thyroid status of the experimental animals.

The adrenaline-induced release of free fatty acids was studied in vitro by [illegible] and SCHWARZ (1960). Rat epididymal tissue from intact animals responded to adrenaline with a significant release of free fatty acids. Fat pads removed from hypothyroid animals showed no increase in free fatty acid release whereas those removed from hyperthyroid animals displayed an exaggerated release. The enhancement of the adrenaline action was evident 3 hours after the intraperitoneal injection of triiodothyronine and optimal at 15 hours. Similar results are reported by [illegible] et al. (1962).

Thus, a review of the literature affords overwhelming evidence that there is a definite relationship between thyroid hormone and adrenaline, manifested in the potentiation of virtually all the effects of adrenaline by thyroxine.

S E C T I O N II

THE ENZYMATIC INACTIVATION OF CATECHOLAMINES

Cytochrome oxidase and phenolases

Adrenaline on standing is autooxidized giving rise first to a labile indophenol derivative, adrenochrome, which in alkaline medium undergoes a series of reactions leading to adrenolutin - a fluorescent material. This mode of oxidation seems unlikely to occur in the living organism, as pointed out by BLASCHKO (1952), because of the presence of reducing agents such as sulfhydryl groups and ascorbic acid. Reports to the contrary have appeared and as much as 50 µg/liter of adrenochrome in human plasma has been claimed to occur under normal conditions (HOFFER, 1958). However, SZARA et al. (1958) were unable to verify this finding by using a specific method for adrenochrome. A fluorescent material found in the plasma of both normal and schizophrenic subjects did not possess the activation and fluorescent spectra of authentic adrenochrome. SCHAYER et al. (1953) had already shown that formation of adrenochrome in vivo does not seem likely, since administration to rats of C^{14}-adrenochrome and C^{14}-adrenaline

leads to entirely different chromatographic patterns of the urine of the animals. Thus, rat urine containing the metabolite or metabolites of adrenochrome, on paper chromatography under nitrogen gives a single diffuse peak with an R_f of 0.1-0.4, whereas in air only a smear of radioactivity is obtained. On the other hand, paper chromatograms from adrenaline - injected rats show three distinct radioactive peaks, either in air or in nitrogen.

A number of investigators have shown that cytochrome oxidase converts adrenaline to adrenochrome in vitro (GREEN and RICHTER 1937; KEILIN and HARTREE 1938; BLASCHKO and SCHLOSSMAN 1940). IISALO and PEKKARINEN (1958) showed that both amine oxidase and cytochrome oxidase are active in bovine heart muscle in vitro. The action of the latter enzyme results in the formation of adrenochrome. Furthermore, it was shown (IISALO 1962) that the enzymatic metabolism of, not only large added amounts of adrenaline and noradrenaline, but also of the endogenous amines could be inhibited to a small but significant extent by cyanide. BACQ (1938) reported on a catechol oxidase system in smooth muscle tissue, except intestine, which converts adrenaline to adrenochrome in vitro. An enzymatic system was reported (SCHACTER 1950) to occur in human blood serum capable of oxidizing catechols

but not hydroquinone, homogentisic acid, adrenaline or dopa. Dopa oxidase was shown to metabolize adrenaline in the iris of pigmented rabbits but the enzyme was absent in albino rabbits (AVGINENT and POULEN 1952). However, BHAGVAT and RICHTER (1938) reported that in animal tissues the amount of phenolase is small and cannot have any biological significance in the inactivation of adrenaline. A phenolase type of activity resulting in the formation of adrenochrome is displayed by ferritin (GREEN et al. 1956). Ceruloplasmin has also been implicated. HOLMBERG and LAURELL (1951) found that in human plasma there is good correlation between ceruloplasmin content and ability to oxidize paraphenylenediamine and benzidine.

These early attempts to elucidate the mode of inactivation of adrenaline and noradrenaline were not accompanied by much convincing evidence that adrenochrome was indeed the main in vivo metabolite of adrenaline. In fact, it has been shown (AXELROD 1959) that whereas cytochrome oxidase metabolizes catecholamines faster than any known enzyme in vitro, it attacks them to a negligible extent in vivo. Once more enzymic reactions in vitro are shown here not to reflect the true metabolic events in vivo.

Adrenaline dehydrogenase

After injection of adrenaline into rabbits a crystalline substance was isolated from the urine having the characteristics of protocatechuic acid. No definite identification was made however (WEINSTEIN and MANNING 1937). This finding would indicate that the side chain of adrenaline can undergo oxidative degradation in vivo. However, SCHAYER (1951) was unable to identify protocatechuic acid in the urine of rats injected with adrenaline. IMAIZUMI and KAWAMOTO (1952) reported the existence of an adrenaline dehydrogenase in the plasma of the rabbit. The enzyme appeared to catalyze the reaction leading to the oxidation of the secondary hydroxyl group of the side chain to a keto group, thus forming adrenalone (IMAIZUMI et al. 1952). Enzymic conversion of adrenalone to adrenaline was later reported (IMAIZUMI et al. 1954). However, the evidence was indirect since the reduction product was simply shown to be a substance with pressor activity and no rigorous chromatographic criteria were applied. KITA (1957) extended these studies. After injection of adrenaline into rabbits the following substances were identified by paper chromatography: adrenaline, adrenalone, protocatechuic aldehyde, protocatechuic acid and noradrenaline.

Adrenaline and adrenalone after incubation with guinea pig liver slices gave identical products on chromatography. The products were identified as protocatechuic aldehyde, protocatechuic acid, 3,4-dihydroxyphenylglyoxal and an unknown compound. Adrenaline gave adrenalone but the latter never the former. This negative finding seems to contradict the postulated reversible character of the dehydrogenation reaction mentioned previously (MAIMONI et al. 1954).

Against the work of this group stand the investigations of WEIL-MALHERBE and BONE (1957) who were unable to detect adrenaline dehydrogenase activity in the plasma of man or rabbit, or the liver, heart, spleen and brain of the rat. No confirmation of the presence and biological function of adrenaline dehydrogenase has come from other investigators. Moreover, the established pathways of catecholamine metabolism which will be dealt with later in this discussion render the adrenaline dehydrogenase scheme untenable as a normally occuring pathway in vivo to any significant extent. Therefore, the metabolic scheme of the Japanese workers cannot be considered as established.

Conjugation

RICHTER (1940) and RICHTER and MacINTOSH (1941) reported that in man large doses of adrenaline administered

by mouth are mainly eliminated in the form of conjugated derivatives in the urine. The conjugation involved one of the phenolic hydroxyl groups and the derivatives were assumed to be sulfates. Since the adrenaline ester was found to be pharmacologically inactive, conjugation was considered to be the main physiological process of adrenaline inactivation. This type of experiments was extended to dogs and BAYER and SHAPIRO (1945) found that adrenaline administered subcutaneously or orally to dogs is excreted in conjugated form in the urine. The mode of elimination was found to be independent of the mode of administration. BAYER (1946) considered conjugation to be the principal mode of inactivation of phenolic sympathomimetic amines. Discrepancies from the work of the previous investigators were noted in the work of BOLLMAN et al. (1947) in which the amount of sulfate excreted after large doses of adrenaline were given by mouth to rats, was found to be insignificant, whereas 21% of the amine was excreted as glucuronide. From subsequent work it became evident that administration of large doses of adrenaline resulted in the excretion of conjugated forms (von EULER and LUFT 1951; von EULER et al. 1953; von EULER and ORVEN 1955) although the nature of these forms and the extent of their occurence was not clearly demonstrated. KHANJIAN et al. (1956) in studies with normal human urine found that noradrenaline

becomes conjugated to the extent of 60% as the glucuronide, not the sulfate, whereas adrenaline seemed to be excreted in the free form. The work of SCHAYER (1951, 1951a) however, indicated that conjugation has no physiological significance under normal conditions since after intravenous injection of small quantities of adrenaline, no conjugate could be detected in the urine of rats and only traces were detected in the urine of rabbits. Moreover, free adrenaline was found in the urine but was not significantly increased following acid hydrolysis. A small amount of ether extractable fraction (presumably sulfate) was found to be present in rat urine and increased after hydrolysis. However, after feeding of large amounts of adrenaline to a rat, a large amount of adrenaline was found to be conjugated in the urine. The present evidence, then, suggests that conjugation is not of major significance in the elimination of catecholamines, but it can assume a major role when large amounts are ingested.

Oxidative deamination:- Monoamine oxidase

The first demonstration that amines could be oxidatively deaminated was provided by HARE (1928). She showed that in mammalian liver an enzyme catalyzed the oxidative deamination of tyramine in a reaction that led to the formation of the corresponding aldehyde and the liberation of ammonia. She named the enzyme tyramine oxidase.

Later, HUGH and QUASTEL (1937) described the oxidation of a number of aliphatic amines by brain and other tissues (aliphatic amine oxidase) and BLASCHKO et al. (1937) an enzymic system inactivating adrenaline (adrenaline oxidase). The work of BLASCHKO et al. (1937a) established beyond any reasonable doubt that, tyramine oxidase, aliphatic amine oxidase and adrenaline oxidase represent one single enzyme. Similarly, KOHN (1937) demonstrated the identity of tyramine oxidase and adrenaline oxidase in pig liver. However, he found the following half saturating concentrations for the substrates: 5×10^{-4} M for tyramine, 5×10^{-3} M for hordenine and 1.5×10^{-2} M for adrenaline. From this high value for adrenaline KOHN concluded that the enzyme cannot be of physiological significance for the oxidation of adrenaline. However, as indicated by BLASCHKO (1952), the measurement of the oxidation rates of the catecholamines is inaccurate due to some oxygen being consumed in other reactions in the enzyme preparations. RICHTER (1937) found that the amine oxidase of guinea pig liver and intestine oxidized adrenaline in the same manner as other primary, secondary and tertiary amines, the product of the reaction being in each case an aldehyde and ammonia or a lower amine. ZELLER (1951) suggested that this enzyme be named monoamine oxidase in order to distinguish

it from diamine oxidase (histaminase). The distinction between the two enzymes is arbitrary to some extent since it is now recognized that monoamine oxidase acts on many diamines and diamine oxidase, on many monoamines (BLASCHKO 1963).

Evidence that monoamine oxidase (MAO) is involved in the inactivation of adrenaline was presented by SCHAYER (1951a), SCHAYER et al. (1952) and SCHAYER and SHILEY (1953) who showed, by making use of adrenaline - β -C^{14} and adrenaline-methyl-C^{14} that the hormone injected into rats is split at some point between the β-carbon and the methyl-carbon due to the action of MAO. An average of 61% of radioactivity was recovered from the urine of animals injected with N-methyl-C^{14}-adrenaline, whereas, pretreatment of the animals with the MAO inhibitor iproniazid (1-isonicotinoyl-2-isopropylhydrazine, "marsilid") raised the recovery of radioactivity to an average of 96%. That oxidative deamination was a normal process in the animal organism was further supported by the experiments of PELLERIN and D'IORIO (1955, 1957, 1957a) and SHAW et al. (1956, 1957) on the metabolic fate of injected 3,4-dihydroxyphenylalanine (dopa) with the resulting excretion of deaminated acidic compounds, such as homoprotocatechuic and homovanillic acids. Noradrenaline itself was shown to be metabolized to 3-methoxy-4-hydroxymandelic acid (VMA)

(ARMSTRONG and McMILLAN 1957, ARMSTRONG et al. 1957). SCHAYER et al. (1955) repeated their experiments with rats injected with α-C^{14}-noradrenaline, and found that after pretreatment of the animals with either iproniazid or p-tolylcholine ether bromide (another MAO inhibitor), one of the metabolites on the paper chromatograms was largely suppressed, indicating that MAO is involved in a major portion of noradrenaline metabolism in the rat. RESNICK et al. (1958) found that 65% of the metabolites of infused β-C^{14}-adrenaline have lost the methyl group, whereas iproniazid treatment increases two-fold the amount of infused adrenaline retaining the methyl group in the side chain. Inhibition of MAO by iproniazid in humans results in diminished amounts of VMA and 3,4-dihydroxymandelic acid excreted in the urine after infusion of 2-C^{14}-adrenaline (GOODALL et al. 1958). The significance of MAO in the metabolism of noradrenaline was also demonstrated after infusion of 2-C^{14}-noradrenaline (GOODALL et al. 1959). KIRSHNER et al. (1959) and KIRSHNER (1960) repeated and confirmed this type of experiments. The amounts of VMA and its conjugate and the conjugate of the 3-methoxy-4-hydroxyphenylglycol were greatly reduced in the urine of cats pretreated with iproniazid (KIRSHNER 1960). The excretion of VMA in humans was found significantly depressed in six healthy humans treated with iproniazid

(STUDNITZ 1959). The noradrenaline level of various tissues including brain was found to be increased in animals pretreated with iproniazid (SHORE et al. 1958 , PEKKANINEN et al. 1958). The elevation of noradrenaline levels after iproniazid treatment in animal tissues has been amply confirmed for the brain of the rabbit (SHORE et al. 1957; BRODIE et al. 1959; SPECTOR et al. 1958) and for the brain of the rat (PAASONEN and KARKI 1959; GREEN and ERICKSON 1960; KARKI et al. 1962). Higher levels of excreted catecholamines following iproniazid treatment have also been reported. Thus, in rats after a subcutaneous injection of iproniazid there was a significant increase in the excretion of administered adrenaline and noradrenaline (CRAWFORD and LAW 1958). Guinea pigs exposed to audiogenic and visual stimuli caused by the presence of a great number of dogs or treated with iproniazid (10-15 mg per day per animal) excreted increased amounts of adrenaline and noradrenaline. The increase was greater in the iproniazid treated than the psychic stress-submitted animals (PEKKANINEN et al. 1960).

From the experiments just mentioned it becomes evident that the role of MAO in the metabolism of catecholamines has been largely derived from tests with a specific MAO inhibitor. Apart from experiments primarily

designed to follow the metabolic fate and changes in the metabolism of catecholamines, a large number of investigations were mainly concerned with the modification of physiological and pharmacological effects of the amines in relation to iproniazid treatment. It is this approach that is mainly responsible for much confusion and dispute over the significance of MAO in catecholamine metabolism.

Thus, [illegible] et al. (1954) reported that in guinea pigs, injection of iproniazid (150umoles/kg) two hours before the subcutaneous administration of adrenaline produced a marked increase in the toxic manifestations of the amine, such as tachypnea, contraction of the massceters, shaking of the head, shivering and pilomotor stimulation, etc. In mice slow prolonged rate of absorption of adrenaline and noradrenaline resulted in an increased mortality and increased cataract - producing ability of adrenaline and noradrenaline in animals pretreated with the inhibitor (BOROWITZ and NORTH 1959). Iproniazid administration two hours prior to removal of the liver of cats or guinea pigs prevents the inactivation of adrenaline by the liver homogenates (GRIESBACH and WELLS 1956). In dogs iproniazid caused a total inactivation of MAO in the liver homogenate and 73% reduction in the brain homogenate. The degradation of 5-hydroxytryptamine, tyramine and adrenaline was inhibited from 92 to 100% by the homo-

genates of the treated animals (ZELLER et al. 1955). In trying to assess the significance of MAO in catecholamine metabolism a number of other MAO inhibitors such as cocaine, ephedrine, choline-p-tolyl ether bromide, harmaline and methylene blue have been used. Ephedrine and cocaine reduce the inactivation rate of adrenaline during its passage through the liver (DAWES 1946). Whereas ephedrine in concentrations up to 10^{-3} M has no effect in the perfused ear of the rabbit, an addition amounting to 10^{-5} M caused an increase in the adrenaline stimulation. The effect of ephedrine in increasing the adrenaline response of the cat's nictitating membrane had been observed many years before (GADDUM and KWIATKOWSKI 1938). Cocaine, harmaline and iproniazid in concentrations which increased the pressor effects of intravenously given adrenaline, increased the concentration of adrenaline in the plasma withdrawn from the aorta of the resting animal (EAKINS and LOCKETT 1961). Both ephedrine (BURN and ROBINSON 1951) and choline-p-tolyl ether bromide (GADDUM and HAMED 1954) when added in the perfusing fluid potentiate the constriction action of adrenaline and noradrenaline. Addition of ephedrine does not modify the constriction action of corbasil (which is not attacked by MAO) (BURN and ROBINSON 1951). Methylene blue potentiated the pressor action of adrenaline (PHILPOT and CANTORI 1949). In

rabbits after pretreatment with iproniazid the drop in adrenaline and noradrenaline levels due to reserpine is largely prevented (BURTNER et al. 1957).

However, reports that MAO inhibitors do not affect the catecholamine effects have appeared in the literature. Thus, GRIESEMER et al. (1953) found that whereas iproniazid potentiates the action of phenylethylamine and tyramine upon the nictitating membrane of the cat, it does not significantly modify the action of adrenaline. The same lack of effect as well as lack of pressor response to adrenaline in the spinal cat after iproniazid has been reported by BURN et al. (1954). Both iproniazid and isoniazid proved to be ineffective in the response of the nictitating membrane to the intra-arterial injection of noradrenaline and tyramine (KAKIJO et al. 1956). Blood pressure, pulse rate and plasma adrenaline and noradrenaline were not significantly changed after infusion of the amines in ten humans pretreated with iproniazid but there was an increase of conjugated urinary noradrenaline in five of them. (FRIEND et al. 1958). The lack of increased response of the nictitating membrane is reported by VARAGIC (1958). Inhibition of MAO in the cat with iproniazid does not increase the pressor response to adrenaline and noradrenaline but it does increase the excretion of free

adrenaline (CORTE and CRAIAN 1957). The heart contractile force was found to be increased by six MAO inhibitors to dopamine, tryptamine and tyramine but not to noradrenaline (GOLDBERG and SJOERDSMA 1955). In depressive patients during treatment with iproniazid (25-150mg daily for 8 to 33 days) there was no significant change in the excretion of adrenaline and noradrenaline (CARLSSON et al. 1959). Endogenous catecholamines were found not to be potentiated by iproniazid. In the cat stimulation of the postganglionic sympathetic nerves to the spleen results in an output of sympathin (noradrenaline) in the venous blood from the organ which was not altered after pretreatment of the animal with iproniazid (BROWN and GILLESPIE 1957).

Thus, the evidence on the significance of MAO in the metabolism of catecholamines seems to be contradictory. As noted previously, the bulk of the negative evidence is derived from tests on physiological responses to catecholamines. On the other hand, the significance of MAO on catecholamine metabolism was practically always confirmed whenever it was sought on the biochemical level, in terms of metabolites excreted in the urine.

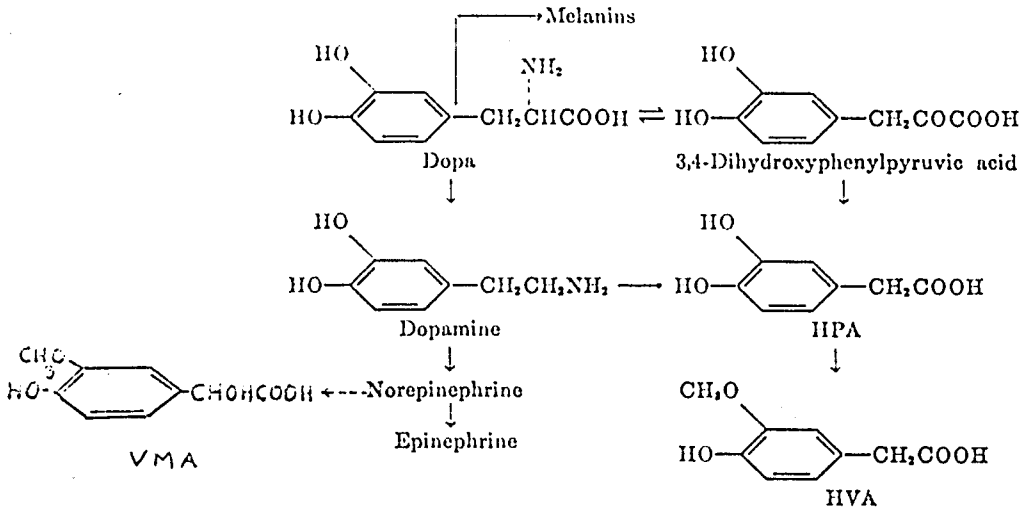
O-methylation.- Catechol-O-methyltransferase

The original observation of MacLAGAN and WILKINSON (1951) that 3,5-diiodo-4-hydroxybutylbenzoate is O-methylated in vivo is considered to be the first

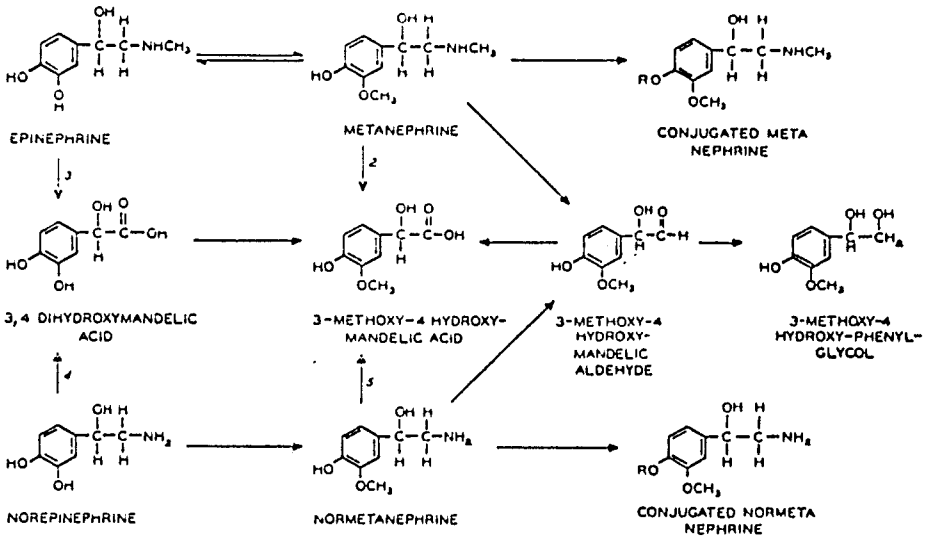
glimpse into a novel mechanism involving the O-methylation of the catechol ring, including catecholamines (PELLERIN et al. 1958; AXELROD 1959). However, recent work (TOMITA 1962; TOMITA and CHA 1963) indicates that the enzyme system responsible for the O-methylation of iodophenols is distinct from the enzyme system involved in the O-methylation of the catechol ring. In fact, as will be shown in this Thesis the model substrate of the former system inhibits the latter.

ARMSTRONG et al. (1956) in the course of an investigation on phenylketonuria discovered in human urine a wide variety of acids including such unusual ones as homovanillic acid, feruloylglycine, vanilloylglycine, vanillic acid, dihydroxyferulic acid and ferulic acid. All these substances are characterized by a catechol ring methylated in the meta-position. Excretion of homovanillic acid in rabbit urine following the oral administration of 3,4-dihydroxyphenylacetic acid had been reported a year earlier (BOOTH et al. 1955). This group of investigators (DENDS et al. 1957) further reported that oral administration to rats and rabbits of several catechol acids resulted in the urinary excretion of their m-methoxy derivatives. Thus, the possibility of biological O-methylation of phenolic substances, particularly of catechol acids, begun to receive the

attention of investigators in this field. ARMSTRONG et al. (1956) and SHAW et al. (1956, 1957) established that homovanillic acid is a normal constituent of human urine and that it is actually a metabolite of 3,4-dihydroxyphenylalanine (dopa). Homoprotocatechuic acid (HPA) was found to be the immediate precursor of homovanillic acid, since ingestion of the former resulted in the urinary excretion of increased amounts of the later. Scheme (I) was proposed to describe the possible pathways of dopa metabolism (solid arrows). This metabolic sequence was supported by earlier experiments (PELLERIN and D'IORIO 1955) demonstrating that injection into rats of DL-dopa-2-C^{14} results in the urinary excretion of hydroxytyramine (dopamine), 3,4-dihydroxyphenylpyruvic acid and 3,4-dihydroxyphenylacetic acid. The same substances plus noradrenaline were identified as metabolic products of dopa in bovine adrenal homogenates (PELLERIN and D'IORIO 1957). Homovanillic acid was also identified (PELLERIN and D'IORIO 1957a). Further evidence in favor of biological methylation of the catechol ring came from the discovery (ARMSTRONG and McMILLAN 1957; ARMSTRONG et al. 1957) that infusion of noradrenaline resulted in the excretion of 3-methoxy-4-hydroxymandelic acid (VMA) (Scheme I broken arrow). In these experiments it was established that both the oral and parenteral adminis-



Scheme I



Scheme II
Route of metabolism of adrenaline and noradrenaline

tration of noradrenaline as well as the condition of pheochromocytoma led to the excretion of VMA in the urine. The unanswered question at that time was whether methylation occured before or after the oxidative deamination of the noradrenaline side chain. The established steps in Scheme I leading clockwise from dopa to HVA were suggestive of the methylation step occuring after oxidative deamination of the side chain of dopa. Demonstration of an enzymatic system capable of methylating catechol acids in meta - position was soon reported by [illegible] and [illegible] (1957b, 1958). The enzyme system was found to be active in the soluble fraction of rat liver and kidney homogenates. Five substrates were tested among which 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxymandelic acid which were methylated to homovanillic acid (HVA) and VMA, respectively, already reported to occur in human urine (ARMSTRONG et al. 1956; SHAW et al. 1957). The system required methionine, ATP, magnesium and reduced glutathione. These experiments served also to demonstrate that the degree of methylation was dependent on the length and degree of saturation of the side chain, as well as the functional groups carried by it. Thus, the convergent reports pointing to the possibility of a biological O-methylation on the level of the catechol acids, were substantiated and the respons-

ible enzyme system was specified in terms of subcellular distribution and required cofactors.

In the same year AXELROD (1957) reported the partial purification from rat liver of an enzyme catalyzing the transfer of the methyl group from S-adenosyl-methionine to adrenaline. Oxidative deamination of the enzymatic product with a monoamine oxidase preparation yielded 3-methoxy-4-hydroxymandelic acid proving that adrenaline had been methylated in the meta- position. 3,4-dihydroxy-benzoic acid was also methylated by the enzyme, indicating its identity with the enzyme described by [illegible] and D'IORIO (1957b, 1958). The partially purified preparation required S-adenosylmethionine as the methyl group donor and magnesium. In crude preparations S-adenosylmethionine could be replaced by methionine and ATP.

The possibility of the biological methylation of catecholamines being established, further insight and understanding of its role and significance followed at a rapid pace. A study of the enzyme, 30-fold purified from rat liver by ammonium sulfate fractionation and adsorption and selective elution from calcium phosphate gel (AXELROD and TOMCHICK 1958), revealed that it is activated by Co^{++}, Mn^{++} and Mg^{++} and to a lesser extent by Zn^{++}, Fe^{++}, Cd^{++} and Ni^{++}. SH groups are apparently essential for activity since p-chloromercuribenzoate and iodoacetic acid inhibited

the enzyme. All catechol compounds were methylated in the meta position and therefore served as substrates, with catechol itself being the best substrate. No stereospecificity was noted.

The in vitro experiments were followed by experiments in vivo. Thus, the glucuronic acid conjugates of normetanephrine were identified in rat urine and the meta-methoxy derivatives of adrenaline, noradrenaline and dopamine were excreted in the urine after administration to rats of adrenaline, noradrenaline and dopamine (AXELROD et al. 1958). O-methylation was shown to be a major route of catecholamine metabolism in man (LABROSSE et al. 1958). Infusion of β-H^3-adrenaline led to free and conjugated metanephrine comprising 54% of the total radioactivity in the urine. One half of infused metanephrine itself was excreted as free and conjugated metanephrine and 25% as VMA. These results were interpreted to mean that the role of monoamine oxidase in adrenaline metabolism was mainly the deamination of metanephrine and that the principal way of adrenaline metabolism in man was O-methylation. Furthermore, large amounts of normetanephrine were identified in the urine of patients with pheochromocytoma (LABROSSE et al. 1958a). O-methylation was soon claimed to be the principal route of adrenaline and noradrenaline metabolism

in the rat (AXELROD et al. 1959a). 25% of infused noni-cotopic adrenaline was excreted in the urine as free and conjugated metanephrine. On the other hand about 1/3 of infused metanephrine was excreted as free and conjugated metanephrine, this presumably meaning that 2/3 were oxi-dized by monoamine oxidase. Accordingly, it was inferred that about 70% of administered adrenaline was converted to metanephrine, thus establishing the primary role of O-methylation. Similar results were obtained with norad-renaline. Of course the validity of these calculations was in large part dependent on the assumption that the relative magnitudes and priority of enzymatic attack by catechol-O-methyltransferase (COMT) and monoamine oxidase remained unchanged under normal and experimental condi-tions - the latter being set up by unphysiological doses of catecholamines. The experiments of LAROSSE et al. (1961) supported by those of KOPIN (1960) furnished much evidence that O-methylation is indeed of major importance in catecholamine metabolism. The presence of metane-phrine and normetanephrine was established by LAROSSE and KAM (1960) in normal human urine. A new metabolite of adrenaline was soon recognized and identified (AXELROD et al. 1959). It was shown to be 3-methoxy-4-hydroxy-phenylglycol sulfate and was excreted in rat urine after the intraperitoneal administration of H^3-adrenaline. It

was further shown that in man only about half of this metabolite is formed by the deamination of methylated catecholamines (KOPIN 1960). The possibility then existed that part of it was formed by prior deamination of catecholamines and this was shown to be so by the demonstration (KOPIN and AXELROD 1960) that 3,4-dihydroxyphenylglycol is indeed a metabolite of adrenaline, occuring in animals in which COMT is inhibited by pyrogallol.

The report on the occurence of a new metabolite of dopamine in the urine of rats injected with dopamine should be mentioned at this time (GOLDSTEIN et al. 1960). On the basis of indirect evidence it is believed to be 3-methoxy-4-hydroxyphenylethanol. The occurence and significance of this type of metabolite under normal conditions has not been established.

Inhibitors of COMT. Pyrogallol had been shown to prolong the response in vivo to adrenaline and sympathetic nerve stimulation (LAOG 1936). Pyrogallol is a substrate for COMT (AXELROD et al. 1960) and as such it would compete with catecholamines for the enzyme. It was indeed shown to inhibit the O-methylation of adrenaline and noradrenaline in vivo and in vitro. (AXELROD and LAROCHE 1959). Kinetic studies by CROUT (1961) revealed that it acts as a noncompetitive inhibitor and its Ki was calculated to be

8×10^{-6} M. A number of catechols, catechol itself, adrenalone, arterenone and gallic acid have been reported to inhibit the O-methylation of adrenaline to various degrees (AXELROD et al. 1960). Nicotinamide and glycocyamine (guanidineacetic acid) were also shown to inhibit the O-methylation of noradrenaline in vivo (UDENFRIEND et al. 1959). Tropolones were shown to be potent inhibitors of COMT in vitro by BELLEAU and BURBA (1961, 1963). Thujaplicin (4-isopropyltropolone) was shown to prevent O-methylation of catecholamines in vivo. (MUSACCHIO and GOLDSTEIN 1962). Dopacetamide has been described as a potent nontoxic inhibitor in vivo by CARLSSON et al. (1962). The α-propyldopacetamide is described as an even more potent inhibitor (CARLSSON et al. 1963). Quercetin has been shown to delay the disappearance and prolong the action of catecholamines in vivo (AXELROD 1960).

S E C T I O N III

CATECHOL-O-METHYLTRANSFERASE VS MONOAMINE OXIDASE

With the discovery of O-methylation as a major metabolic pathway for catecholamines the question arose as to the relative importance of COMT and MAO in the inactivation process.

LABROSSE et al. (1958) claimed, partly on negative evidence on the role of MAO, cited in a previous section, and partly on experimental data showing extensive O-methylation of administered adrenaline and a lesser extent of oxidative deamination of administered metanephrine into humans, that the principal route of catecholamine metabolism is O-methylation. This view was supported by the potentiating effects of pyrogallol on the action of adrenaline noted by BACQ (1936) many years previously. These effects were confirmed by BACQ et al. (1959) in vitro and AXELROD and LABROSSE (1959) and CROUT (1960) in vitro and in vivo. The wide occurence of COMT on the other hand was established by the work of AXELROD et al. (1959). The enzyme occurs in the central and peripheral nervous systems as well as in liver, spleen, thyroid, aorta and other tissues. The methionine activating

enzyme, responsible for the formation of the active methyl donor, was also detected in monkey brain.

It must be noted that metanephrine sensitizes the nictitating membrane of the cat to adrenaline (BACQ and RANSON 1961) and that the methylated derivatives of adrenaline, noradrenaline and dopamine exert some activity on smooth muscle and blood pressure (CHAMPAGNE et al. 1960). Pretreatment of the rabbit with 3-methoxy or 4-methoxy or 3,4-dimethoxyadrenaline sensitizes the animals to the hyperlacticemic response to adrenaline (D'IORIO and CHAMPAGNE 1962). It appears then that O-methylation largely suppresses the direct biological activity of catecholamines. However, O-methylated derivatives still retain some activity by sensitizing to the action of adrenaline.

Two minutes after the rapid intravenous administration of H^3-adrenaline and noradrenaline to cats, the corresponding methylated products, metanephrine and normetanephrine, were present in all tissues, and selectively so in the heart (AXELROD et al. 1961). The disappearance of H^3-adrenaline from plasma appeared to consist of two phases: an initial rapid fall concurrent with O-methylation, followed by a slow decline indicating a slow release from binding sites and parallel metabolism (AXELROD et al. 1959a). A variety of sympathomimetic

amines increased the rate of disappearance of injected adrenaline and noradrenaline in the whole mouse, presumably by preventing the binding of catecholamines and their subsequent metabolism (AXELROD and TOMCHICK 1960). It was further suggested that in rats MAO inhibitors elevate catecholamine concentration in certain tissues by simply blocking this slow release from the binding sites (AXELROD et al. 1961). The fate of H^3-adrenaline in the whole mouse ten minutes after administration was determined after pretreatment of the animals with quercetin (a COMT inhibitor) (AXELROD and TOMCHICK 1959). It was found that quercetin inhibited the disappearance of adrenaline in this time interval. Studies on the in vivo inhibition of COMT with pyrogallol revealed only a moderate increase in the half life of administered noradrenaline, not commensurate with the 98-100% inhibition of noradrenaline methylation concurrently observed in the whole animals. In the same series of experiments iproniazid produced no increase in the half life of adrenaline and no inhibition of the noradrenaline metabolism, as estimated by measurement of residual noradrenaline in the whole animal. The authors (UDENFRIEND et al. 1959) conclude that alternative routes of metabolism may become operative in the absence of COMT. The limited significance and inadequacy of interpretation of

some experiments is demonstrated by the work of CROUT (1961a) in which simultaneous inhibition of COMT and MAO produces only a moderate prolongation of cardiovascular effects, as assessed by arterial pressure and cardiac contractile force, induced by injected noradrenaline into anesthetized dogs. The prolongation of pharmacological action was correlated with a delayed metabolism of the injected noradrenaline from the circulation. To the author while these results indicate that metabolism by the two enzymes contributes to the removal of noradrenaline from the circulation, they further suggest that its physiological inactivation does not necessarily require the metabolic destruction of the amine by the enzymes. In an extensive series of experiments by KIRSHNER and coworkers (KIRSHNER et al. 1961; DE SCHAEPDRYVER and KIRSHNER 1961a, 1961b, 1961c) the fate of injected adrenaline-2-C^{14} in the cat was followed both in various tissues and in the urine at various time intervals. The main conclusion drawn from these experiments is that blockade of either MAO or COMT resulted in a compensatory hyperactivity of the alternative, intact, enzyme, whereas the simultaneous blockade of both enzymes was followed by the formation of a new basic catabolite and the increased production of unidentified acidic, mainly conjugated catabolites. Specifically in the heart,

sufficient amounts of both enzymes are present to compensate for the blockade of one or the other enzyme. In the liver blockade of either enzyme results in a decreased rate in the metabolism of adrenaline (1961c). While these experiments did not support any categorical statement as to the relative temporal preponderance of O-methylation over deamination, the reserved position is taken that in heart, liver and kidney MAO can initiate an independent inactivation of the amine (1961b). It has been shown (BRODIE et al. 1956, CHESSIN et al. 1957) that reserpine administration to rabbits pretreated with a MAO inhibitor produces excitation rather than sedation. Furthermore, it prevents the known depletion of noradrenaline and serotonin content of the brain. SHORE and BRODIE (1957) interpreted this effect as being due to inhibition of MAO acting on the amines released by reserpine. On the basis of these results MAO was proposed to be the enzyme mainly involved in the inactivation of noradrenaline and serotonin in the central nervous system (SHORE et al. 1957). PLETSCHER (1956) and GIARMAN and SCHANBERG (1959) on the other hand, held the opinion that MAO inhibitors prevent reserpine from releasing the amines from their binding sites. In such a case, the conclusion that MAO is the enzyme mainly responsible for the destruction of the amines in the brain would not be valid. However,

SPECTOR et al. (1960) presented evidence that MAO inhibitors do not prevent reserpine from releasing noradrenaline and serotonin but they do prevent inactivation of the released amines in the brain of rabbits. Moreover, pyrogallol at a dose of 15mg/kg given intraperitoneally to rabbits did not prevent reserpine from eliciting its normal sedative effects and the rapid decline of brain noradrenaline and serotonin. These investigators further postulate that MAO and COMT have different physiological roles, the former being responsible for the metabolism of noradrenaline and serotonin in the tissues where it may regulate the level of the amines and the latter for the inactivation after their release into the circulation. This postulate is supported by some recent work. CROUT et al. (1961) found that MAO activity in vitro is 3.6 and 5 times greater than COMT activity in the brain and heart, respectively, of the rat. Moreover, MAO inhibitors produce a significant increase in catecholamine content of brain and heart, while pyrogallol failed to do so. After pretreatment with iproniazid intravenously injected noradrenaline was markedly accumulated in the myocardium, whereas clearance of adrenaline from the plasma was only slightly prolonged. Pyrogallol treatment results in a severe impair ment in the metabolism of circulating noradrenaline. The experiments show that endogenous nora-

drenaline accumulates in the brain and heart when MAO is inhibited. Exogenous noradrenaline accumulates in the heart under the same conditions but neither of these effects is produced by a COMT inhibitor. To the authors the results suggest that oxidative deamination is the more significant pathway in the metabolism of noradrenaline in the brain and heart of the rat - this being compatible with the observation that O-methylation is the major pathway for circulating noradrenaline.

The presence of COMT in the heart (AXELROD et al. 1959) and the finding that H^3-normetanephrine is present in the heart shortly after the administration of H^3-noradrenaline (AXELROD et al. 1961a) are not considered conclusive evidence that O-methylation is the predominant metabolic pathway in the heart (KOPIN et al. 1962). Actually, CROUT et al. (1961)suggested that this H^3-normetanephrine may have been formed in the liver and kidney and then transported to the heart. The intracellular as well as intercellular distribution of MAO and COMT differ (MAO is a mitochondrial enzyme) and experiments based on relative activities in broken cells may lead to erroneous conclusions (KOPIN et al. 1962). It was shown that binding itself may be a means of inactivation of adrenaline. Thus in the isolated perfused heart of the rat 95% of bound tritium at any time after the perfusion with H^3-adrenaline

is unmetabolized adrenaline (KOPIN et al. 1962). KOPIN and GORDON (1962) showed that depletion of bound H^3-noradrenaline after reserpine treatment is effected intracellularly before release into the circulation. In a further series of experiments (KOPIN and GORDON 1963) evidence is presented that the products of H^3-noradrenaline in the rat are excreted in two phases. O-methylation is the major route of metabolism in the first phase of unbound noradrenaline. In the second slow phase, the metabolites excreted seem to support the view that bound (and presumably endogenous) noradrenaline is primarily metabolized by MAO.

In spite of contradictory claims on the relative importance of the two enzymes which are largely due to different systems studied and methodologies employed, a unified picture is emerging in which both enzymes appear to possess equally important places.

Scheme II summarizes the route of metabolism of adrenaline and noradrenaline.

S E C T I O N IV

ATTEMPTED INTERPRETATIONS OF THE POTENTIATING ACTION OF THYROXINE ON CATECHOLAMINE EFFECTS

The earliest attempt to rationalize the potentiation of the catecholamine effects by thyroxine on the chemical level was made by THIBAULT (1950). She obtained "active thyroxine" from thyroxine on incubation at 37° in Tyrode solution with intestinal tissue. When this active principle was added to a solution of adrenaline it could be shown that the oxidation of the latter to adrenochrome was slower than that of a control solution of adrenaline. She concluded that this antioxidant property of active thyroxine was, at least partly, responsible for the observed effects of potentiation. The significance of these experiments and their interpretation may become clear in the light of the experimental results of the present work.

BURN and SPINKS (1952) showed that thyroidectomy increases the amount of amine oxidase in the liver of rabbits and rats, whereas thyroid feeding decreases the amount of the enzyme in rabbits (rats were not studied). SPINKS (1952) further confirmed the experiments

with thyroid-fed rabbits. SPINKS and BURN (1952) gave a detailed account of their experiments. The rise in blood sugar caused by adrenaline was higher in thyroid-fed rats and this was explained by the decrease in amine oxidase elicited by thyroid feeding. The decreased response to adrenaline in blood-sugar rise in thyroidetomized rabbits was explained by the increased level of amine oxidase. This interpretation was challenged by WESTERMAN (1956) and HOLTZ et al. (1956) who found higher amine oxidase levels in the livers of thyroxine or triiodothyronine-treated guinea pigs and rats than in the livers of the control animals. However, the disagreement in the results of various investigators may be resolved if the sex of the animals is taken into account. SKILLEN et al. (1952) found that MAO activity is much lower in the heart of female rats than in the heart of male rats. Thyroid feeding increased the MAO levels in females but did not significantly affect the MAO activity in males. Finally, propylthiouracil treatment produced a significant decrease in male heart MAO activity but did not alter that of the females. TRENDELENBURG (1953) determined the amine oxidase activity in the livers of 14 control and 20 thyroid-fed rabbits and found an 11.4% fall in the level of the enzyme due to thyroid feeding - a small but significant difference. The amount of total fluorogenic

material (measured as adrenaline) in the plasma of hyperthyroid rats, 5 and 15 minutes after an intramuscular injection of adrenaline was greatly increased (LEDUC et al. 1955) indicating a lowered rate of destruction of the amine.

ZILM and LARDY (1959) found higher than normal levels of circulating adrenaline and noradrenaline in thyroid-fed rats. The MAO declined steadily during feeding and was restored to normal levels after discontinuing the treatment. Addition, however, of thyroxine, triiodothyronine and analogues to fresh mitochondria from normal rats did not result in inhibition of enzyme activity. However, these experiments showed that high levels of circulating catecholamines are associated with low levels of MAO activity.

The discovery that COMT is involved in a major portion of catecholamine metabolism prompted an investigation by D'IORIO and LEDUC (1960). This investigation revealed the following: 1) daily injection (three injections in all) of rats with a standard dose of 500 µg of thyroxine resulted in a 45% decrease in the activity of liver COMT as measured by the O-methylation of protocatechuic acid in vitro.. 2) The urinary metanephrine in the treated animals was substantially decreased. 3) Thyroxine added in vitro had no effect on

liver COMT activity. The last negative finding would have been decisive if thyroxine were even moderately soluble in neutral or near neutral solutions. But thyroxine is notoriously insoluble under these conditions and unless the solubility barrier was somehow, circumvented no final answer would be possible as to whether it could act directly as an inhibitor of the enzyme in vitro. It was Dr. D'Iorio's proposition that the next best thing to getting thyroxine into solution would be the use of more soluble structural analogues of the hormone.

EXPERIMENTAL AND RESULTS

S E C T I O N I

INHIBITION OF COMT BY 3,5-DIIODO-4-HYDROXY-BENZOIC ACID (DIHBA) IN A CRUDE PREPARATION

Experimental

In the initial experiments crude enzyme preparations were used. Male SPRAGUE-DAWLEY rats weighing 150-200 gm were stunned with a blow on the head and subsequently decapitated. The livers were quickly excised, chilled and homogenized in a glass homogenizer with an equal amount of ice-cold distilled water. The homogenates were placed in appropriate polyethylene tubes and centrifuged for hour at 20,000xg in a refrigerated SERVALL centrifuge. The supernatant was employed as a source of catechol-O-methyltransferase (COMT). The enzyme has been shown to be mainly present in this fraction (PEILLEUX and D'IORIO 1958). Protocatechuic acid (3,4-dihydroxybenzoic acid) was used as substrate according to the method of D'IORIO (1961). This is enzymatically methylated giving rise to vanillic acid (3-metoxy-4-hydroxybenzoic acid). Methionine-methyl-C^{14} serves as the precursor of the active

methyl group donor, S-adenosylmethionine (S-Ame) (CANTONI 1953). The enzyme responsible for the activation of the methyl group is the methionine-activating enzyme which requires ATP, magnesium and, for maximum activity, reduced glutathione (CANTONI 1951, 1953). The methionine-activating enzyme is also present in the supernatant fraction together with COMT and catalyzes the formation of S-Ame-methyl-C^{14} in the presence of the required cofactors. The COMT then catalyzes the transfer of methyl-C^{14} to protocatechuic acid with the formation of vanillic acid. It is obvious that the presence in this system of the methionine-activating enzyme is a prerequisite for the function of COMT. Hence, the rate of O-methylation as measured by the rate of vanillic acid formation is in fact the overall rate of the two enzymatic steps. Basically the reaction system consisted of the following:

Sodium phosphate buffer	pH 7.4 240 micromoles
Protocatechuic acid	15 micromoles (3.75×10^{-3} M)
Magnesium chloride	50 micromoles
Reduced glutathione	10 micromoles
ATP	9 micromoles
Methionine-methyl-C^{14} (0.97×10^{8} d.p.m./mg)	0.8 micromoles
Enzyme preparation	1.0 ml.

Water to make a volume of 4.0 ml.

The above mixture shall be designated as the

"complete system".

The mixture except the enzyme, placed in 25 ml Erlenmeyer flasks was preincubated for 3 minutes at 37° in a Dubnoff metabolic shaker and the reaction was initiated by the addition of the enzyme preparation. The reaction was quenched by the addition of 0.2 ml of concentrated HCl. The precipitated proteins were removed by centrifugation at 2,000 r.p.m. for 10 minutes. To the supernatant, transferred to 15 ml centrifuge tubes, sodium chloride was added to a final concentration of 10%. More protein was precipitated and it was again removed by centrifugation as previously and the pH was adjusted to 1.0 with 5N NaOH. The mixture was extracted 3 times with 3 ml of ethyl acetate each time and the extracts were combined. Under these conditions the vanillic acid is extracted quantitatively (95-100%) (D'IORIO 1961). The vanillic acid is very stable; however, the extracts in 15 ml centrifuge tubes, immersed in a water bath at 40-45°, were evaporated to dryness under a stream of nitrogen.

Paper chromatography. The dry residue was redissolved in 0.1 ml of ethyl acetate and 10 microliters were spotted on paper. Paper Whatman No. 1 can be used, but Mrs. Nissala in our laboratory found that neater chromatograms and rounder spots were obtained with Whatman diethylaminoethyl-

cellulose DE 20. This paper was employed throughout the chromatographic experiments. The solvent system was butanol: acetic acid: water, 7:1:2. Use was made of the descending technique. The paper was cut into 1.5 cm wide strips separated by a 1 cm gap and held together with a common head, which immersed into the solvent in the reservoir supplied all the strips with the eluant. The chromatograms were run usually overnight - an average of 15-16 hours. Under these conditons the solvent front migrated 35-40 cm. and this resulted in a very good separation of the three major radioactive peaks. A strip spotted with a mixture of protocatechuic and vanillic acids was at all times included in the runs. After drying in a fume hood the position of vanillic acid was identified by spraying with nitraniline reagent II as described by SHIMI (1960). The composition of the solution was as follows:

A.	p-nitroaniline	0.1% in N HCl
B.	Sodium nitrite	0.2% in water
C.	Potassium carbonate	10% in water

All three solutions were cooled to about 5° in the cold room. 10 ml of A and 10 ml of B were mixed and allowed to stand for 5 minutes. 25 ml of C was then added and the mixture, in a spray bottle, was immediately used. Vanillic acid gave an intense violet spot, protocatechuic acid a less intense spot.

The chromatograms after complete drying in the fume hood were ruled with a pencil, numbered and cut into 1 cm sections. These rectangular (1 x 1.5cm) sections were subsequently placed in glass vials containing 10 ml of scintillation mixture and counted in a Nuclear Chicago liquid scintillation counter. Removal of the papers from the vials resulted in background count only, indicating that the scintillation mixture did not elute vanillic acid from the paper. The scintillation mixture was made by dissolving 3 gm of 2,5-diphenylxazol (PPO) and 100 mg of 1,4-bis-2-(4-methyl-5-phenylxazolyl)- benzene (dimethyl-POPOP) in one liter of toluene.

Results

The typical complete mixture gave a chromatogram with 3 major radioactive peaks. The fastest moving component occupied the same place in the chromatogram as authentic vanillic acid. Admixture chromatography showed that this radioactive peak exactly coincides with the violet spot given by authentic vanillic acid with the p-nitroaniline reagent. The Rf is 0.76. Figure 1A represents one such typical chromatogram. The other two peaks have not been identified. Omission of protocatechuic acid from the incubation mixture results in complete elimination of the vanillic acid peak from the chromatogram (fig. 1B).

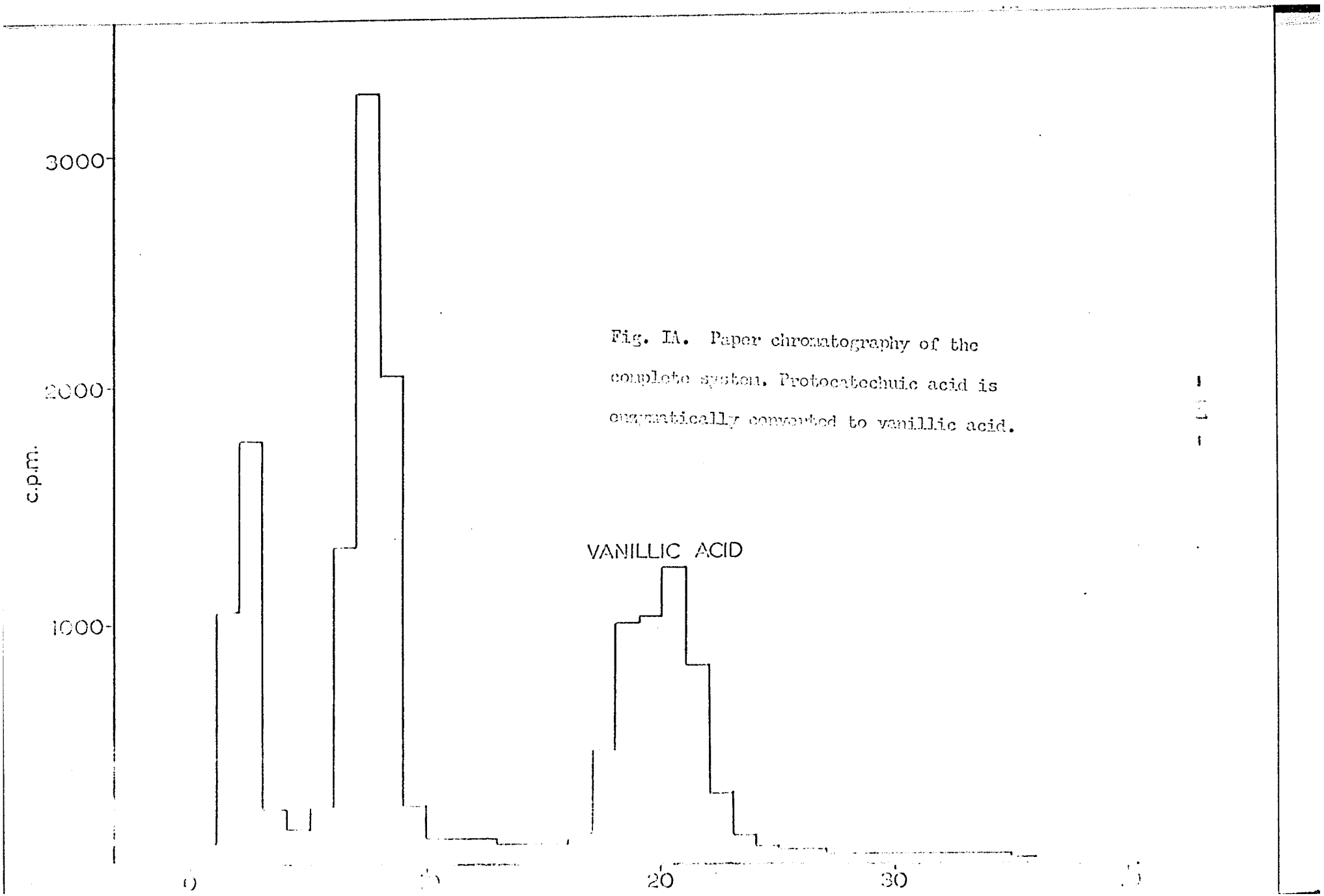


Fig. IA. Paper chromatography of the complete system. Protocatechuic acid is enzymatically converted to vanillic acid.

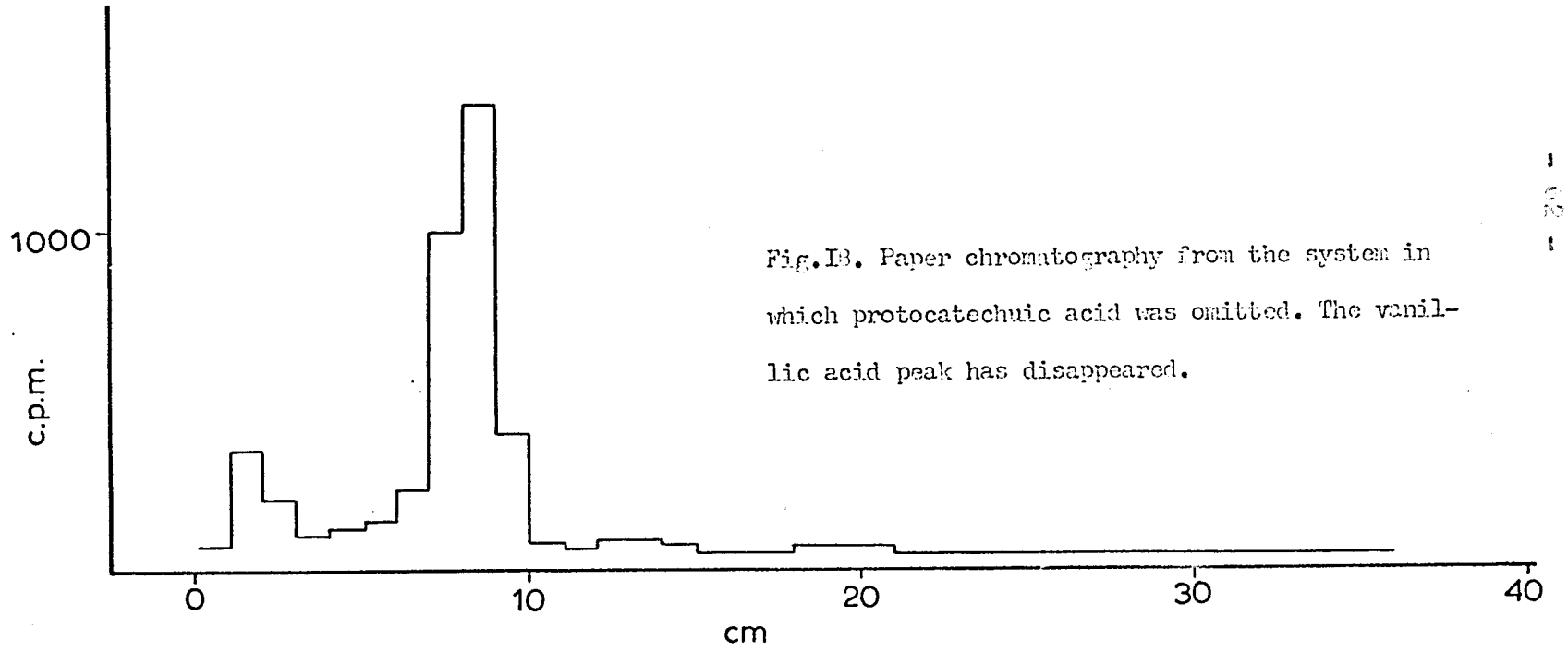


Fig.IB. Paper chromatography from the system in which protocatechuic acid was omitted. The vanillic acid peak has disappeared.

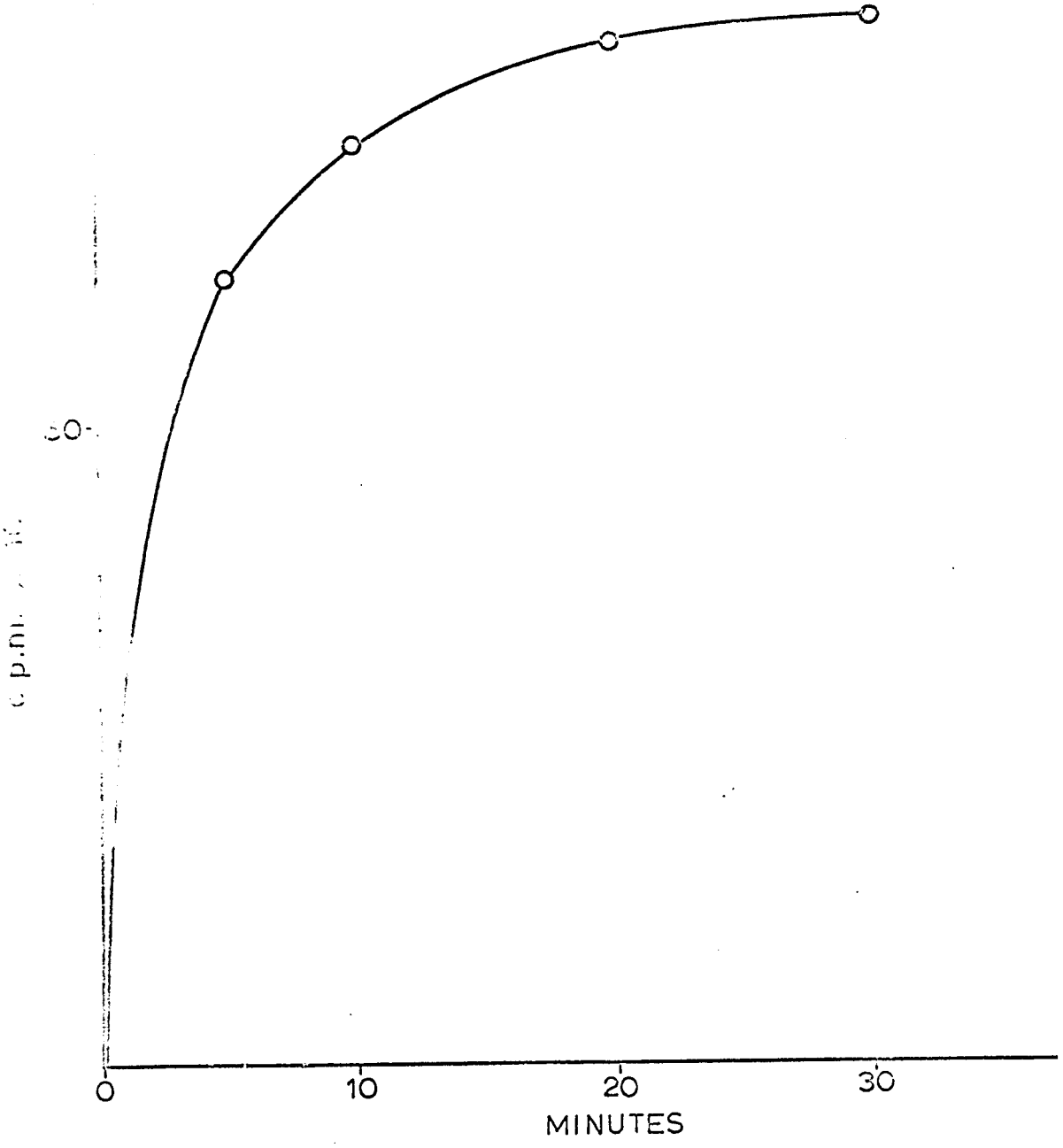


Fig. 2. Time course of the enzymatic methylation of protocatechuic acid to vanillic acid.

This clear-cut change in the profile of the chromatogram demonstrates the reliability and sensitivity of the method.

The time course of the reaction was then established as illustrated in fig. 2. The points are mean values from duplicate chromatograms and represent counts per minute of vanillic acid produced per 10 microliters of ethyl acetate extract. It is evident that the reaction is very fast in the first five minutes and it is practically completed by the end of 30 minutes.

Effects of 4-methyltropolone and 3,5-diiodo-4-hydroxybenzoic acid (DIHBA). In two separate experiments the effects of 4-methyltropolone and DIHBA (see addendum) on the activity of the enzyme were tested. Tropolone, a specific inhibitor of COMT (MULLEAU and BURTA 1961) was added dissolved in water to a final concentration of 4.6×10^{-3} M. DIHBA was added to a final concentration of 1.7×10^{-3} M dissolved in 0.1 ml of ethanol. An equal amount of ethanol was added to the control flasks. The incubation was carried out for 30 minutes. In table 1 it is shown that both substances display inhibitory action to the extent of about 35%. The experiments served to demonstrate in a qualitative fashion only, the inhibitory action of these coumpounds, since inhibition

Duplicate controls		Tropolone	Duplicate controls		Duplicate DHBA	
c.p.m.	Average c.p.m.	c.p.m.	c.p.m.	Average c.p.m.	c.p.m.	Average c.p.m.
5,575	5,768	3,839	7,822	8,623	5,736	5,602
5,962			8,424		5,468	

Table I - Effect of 4-methyltropolone and DHBA on the production of vanillic acid (c.p.m. per 10 microliters of ethyl acetate extract) after 30 minutes of incubation.

was measured at the end of 30 minutes when the enzymatic reaction was already terminated (fig. 2). The difference in the activities of the control experiments is, of course, due to the fact that they represent activities of two different enzyme preparations in two separate experiments.

Time course of the inhibition. It was next decided to follow the time course of the inhibition and to this end the reaction was followed at 5,10,20 and 30 minutes. The DIHBA was added this time to a final concentration equal to substate concentration, i.e., 3.75×10^{-3} M. Chromatograms were run in duplicate and the results are illustrated in table 2 and figure 3.

Effect of substrate/inhibitor ratio on the rate of the reaction. In fig. 4 the effect of DIHBA is demonstrated at three levels of concentration , i.e., 3.75×10^{-3} M, 3.75×10^{-4} M and 3.75×10^{-5} M. It is seen that DIHBA at 3.75×10^{-4} M which is ten times less than the substrate concentration, still exerts considerable inhibition. However, at a concentration of 3.75×10^{-5} M DIHBA does not affect the activity of the enzyme.

Reversibility of inhibition. Another experiment was

Time minutes	Controls c.p.m. of vanillic acid		DIHBA c.p.m. of vanillic acid	
	Duplicates	Average	Duplicates	Average
5	9,108 7,750	8,429	251 222	236
10	10,064 -	10,064	898 809	853
20	12,946 12,370	12,658	2,078 -	2,078
30	14,046 -	14,046	2,915 3,117	3,016

Table 2 - Production of vanillic acid expressed as c.p.m. per 10 microliters of ethyl acetate extract as a function of time in the absence and presence of DIHBA.

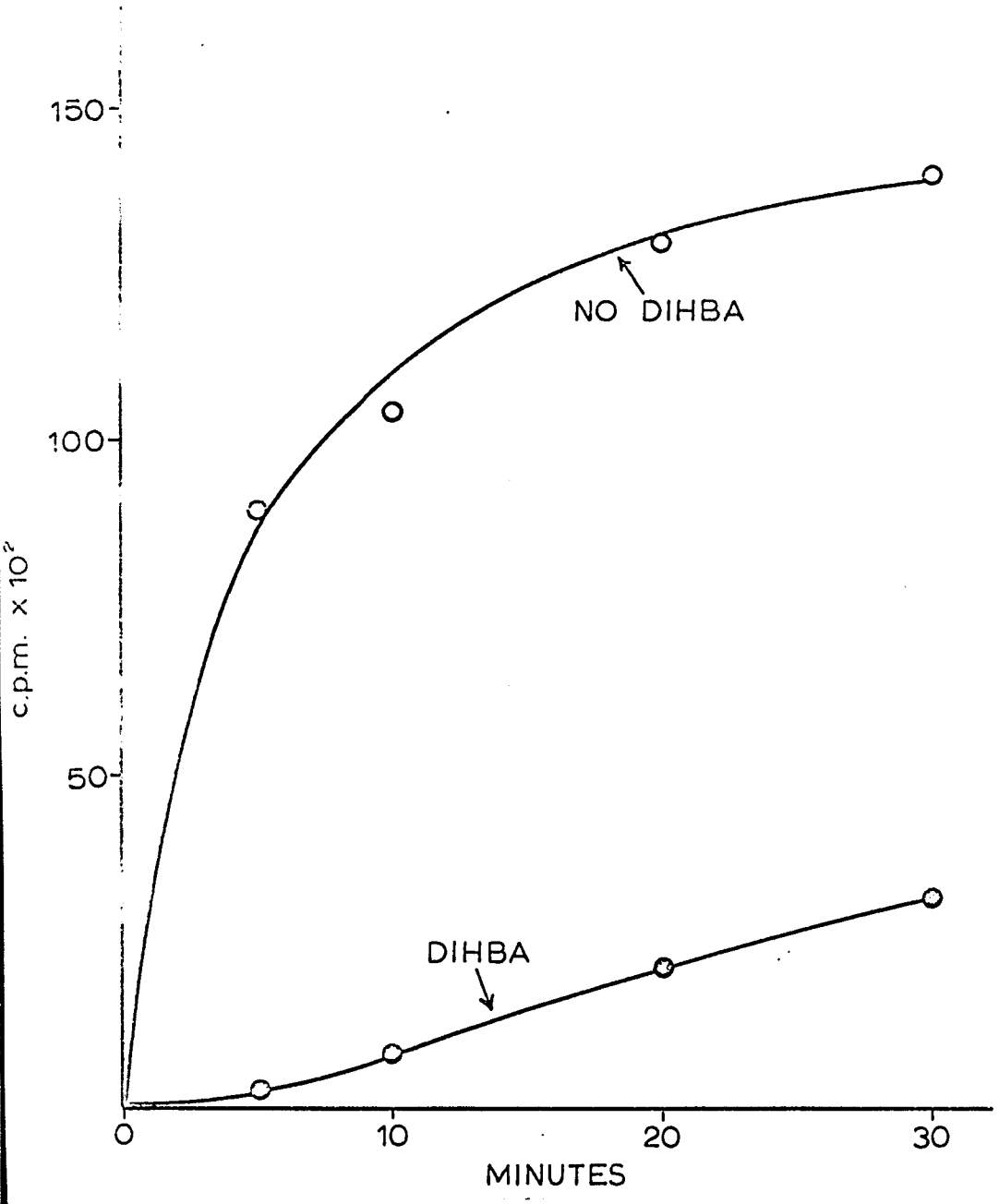


Fig.3. Inhibition of vanillic acid formation expressed as counts per minute in the presence of 3.75×10^{-3} M DIHBA.

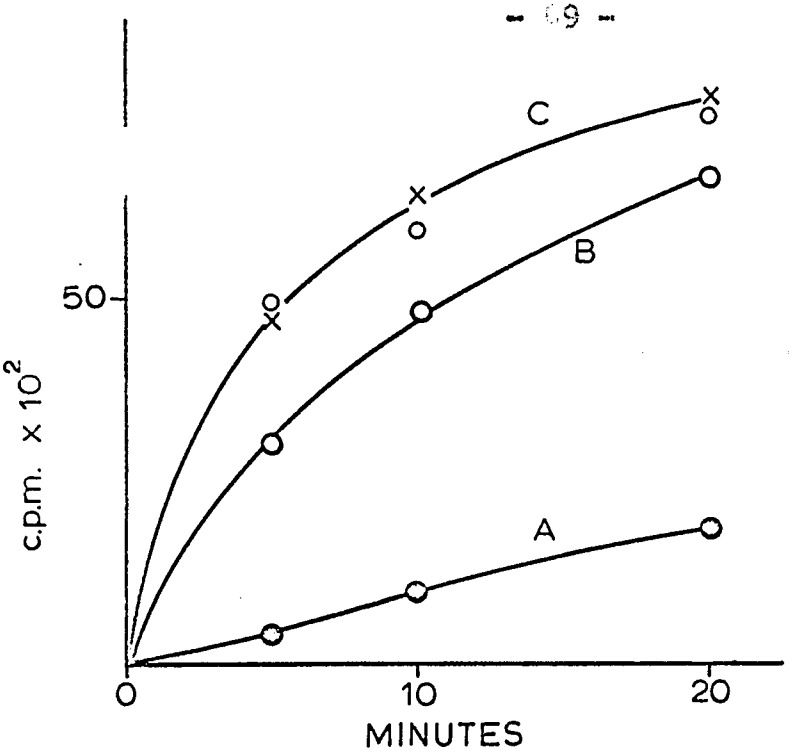


Fig.4. Inhibition of vanillic acid formation at three levels of DHBA. Curve A, $3.75X10^{-3}M$; Curve B, $3.75X10^{-4}M$; curve C, $3.75X10^{-5}M$. The open circles in curve C represent activity of the control.

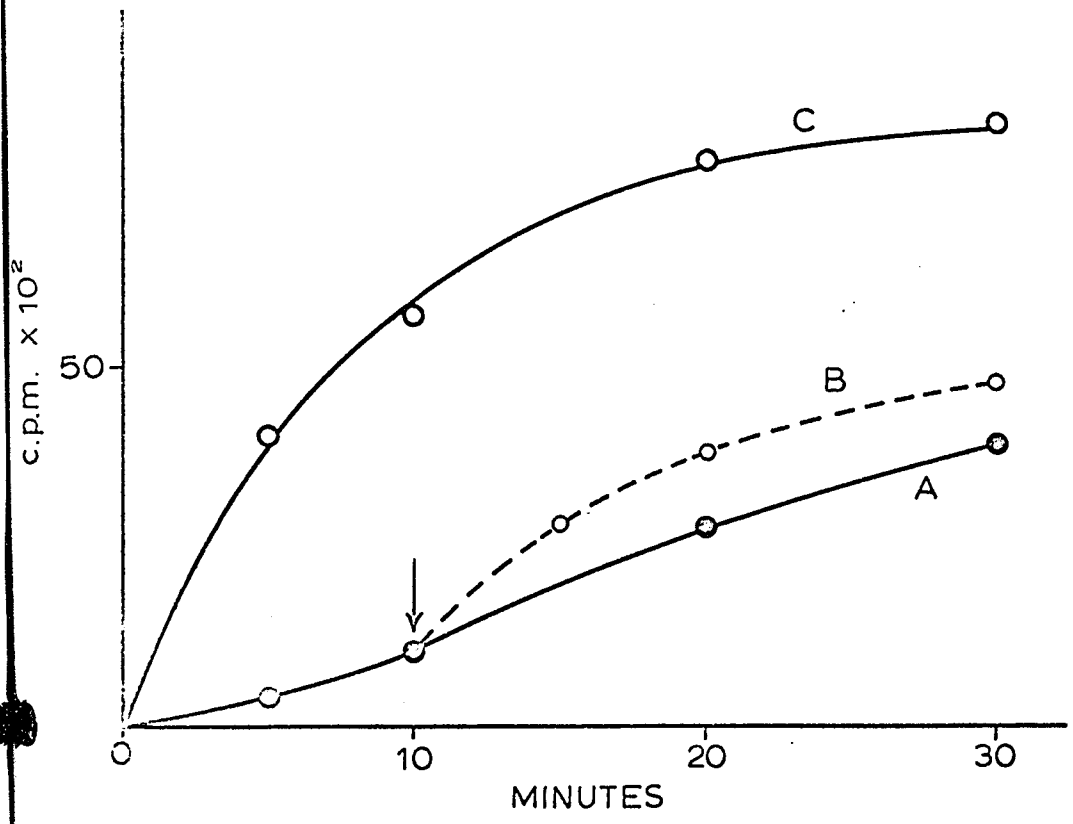


Fig.5. Partial reversal of inhibition by excess substrate. Curve C is for control. At the arrow the substrate concetration was raised

designed in order to see whether the inhibition could be reversed by excess substrate (protocatechuic acid). The reaction was initiated with equimolar concentrations of substrate and DIHBA, present at 3.75×10^{-3} M, but after 10 minutes an appropriate amount of substrate in a volume of 0.1ml was added. This addition brought the substrate concentration to 4.1×10^{-2} M (9-fold higher than originally). As is seen in fig. 5 the inhibition, even with this rather moderate excess in substrate, was partially reversed indicating that substrate and inhibitor were competing for the same site on the enzyme.

Possible O-methylation of DIHBA. TOMITA (1962) reported that a soluble enzyme from rat liver catalyzes the transfer of the methyl group from methionine to DIHBA in the presence of ATP at pH 6.0. If such were the case it should be possible to isolate 3,5-diiodo-4-methoxybenzoic acid (O-methyl-DIHBA) with labelled methyl group. This should show up in the chromatograms as a new radioactive peak - provided it was extractable with ethyl acetate. O-methyl-DIHBA was not available at the time and its solubility properties were unknown. However, it was decided to perform the usual ethyl acetate extraction but carry out the reaction, not only at the customary pH 7.4, but also at pH 6.0. The experiment was designed as is

outlined below:

pH. 6.0 and pH 7.4

a- Complete system

b- Complete system + DIHBA 3.75×10^{-3} M

c- Complete system - substrate

d- Complete system - substrate + DIHBA 3.75×10^{-3} M

At pH 6.0 the DIHBA was partly in suspension. The incubation was maintained at 37° for one hour. All eight chromatograms obtained from these systems were scanned for radioactivity in the liquid scintillation counter in the usual manner, in search of a probable new radioactive peak. On the assumption that O-methyl-DIHBA was present and extractable by ethyl acetate it should appear as a new radioactive peak in system b or system d or both, at pH 6.0 and possibly at pH 7.4. Inhibition at pH 7.4 would not necessarily imply the presence of O-methyl-DIHBA. A non-productive enzyme-DIHBA complex, not ultimately leading to O-methylation, might prevail at pH 7.4. If the presence of O-methyl-DIHBA were confirmed at pH 6.0 but not at pH 7.4 it would mean that DIHBA acts merely as competitive substrate in the former but as a true inhibitor at the latter pH.

In particular, a new radioactive peak in the chromatograms derived from system d should differentiate them from the chromatograms derived from system c.

Fortunately, the chromatograms from system c were very "clean" in that they contained only two unidentified radioactive peaks. Therefore, the presence of a new peak from system d due to the presence of O-methyl-C^{14}-DIHBA would be easily detectable.

The radioactive methionine used in these experiments ("MERCK") had an activity of 0.5 mc per 11.35mg. Since 1mc=2.22×10^9 d.p.m. it can be calculated that 0.3 micromoles added to the medium (cf. experimental part) correspond to 1.17×10^7 d.p.m. Since the efficiency of the liquid scintillation counter is well above 60% it can be calculated that $1.17 \times 0.6 \times 10^7 = 0.702 \times 10^7 = 7{,}020{,}000$ c.p.m. were actually added to the mixtures. Assuming a 0.1% incorporation as reported by SOMIYA, approximately 7,000 c.p.m. would be due to O-methyl-C^{14}-DIHBA. Only 1/10 of the ethyl acetate extract was applied on the paper; therefore, on the assumption that 50% of O-methyl-DIHBA had been extracted one would expect a novel radioactive peak totalling no less than 350 c.p.m. No such peak appeared in the chromatograms either from incubations at pH 6.0 or pH 7.4. However, this experiment could not be considered as conclusive evidence that under the described experimental conditions no methylation of DIHBA occurred, since the extractability of the methylated derivative

with ethyl acetate was questionable.

Use of ether extraction. MACLAGAN and WILKINSON (1951) had reported that they extracted the O-methyl-DIHBA from the acidified urine with ether. A small sample of the authentic compound was kindly provided to us by Dr H.A. LARDY and a test showed that it is quite soluble in ether. On the basis of the above information and solubility test a new experiment was designed. A new batch of methionine-methyl-C^{14} was employed (MERCK SHARPE and DOME OF CANADA, 0.5mc per 6.87 mg.). 0.27 micromoles of this and 3.0 micromoles of nonradioactive methionine were added to the mixtures. This addition corresponded to $3.9x10^{6}$ c.p.m. On the same assumption of 0.1% incorporation into O-methyl-DIHBA, the latter would give $3.9x10^{3}$ c.p.m. on total extraction with ether. Both substrate and DIHBA were added to the mixtures at pH 6.0 and pH 7.5, so that the speculated O-methylation of DIHBA could be correlated with the parallel inhibition of vanillic acid formation. All solutions except the methionine ones were adjusted to pH 6.0 or pH 7.5 before mixing. The experiment is outlined below:

Complete system at pH 6.0 and pH 7.5

Complete system + DIHBA ($3.75x10^{-3}$ M) at pH 6.0 and pH 7.5

Again, at pH 6.0 the DIHBA was partly in suspension.

The reaction was followed up to 30 minutes and fig. 6 illustrates the time courses of the inhibited and uninhibited reactions at the two acidities. The entire lengths of the chromatograms derived from the mixtures were counted and again no novel peak was noted in the strips corresponding to the mixtures containing the DIHBA after 30 minutes of incubation. The mixtures which had been incubated for 30 minutes and extracted as usually with ethyl acetate were reextracted three times with 3 ml of ether. The combined extracts were evaporated to dryness under a stream of nitrogen and redissolved in a small volume of ether. The entire volume of ether was spotted on the paper and chromatographed in the usual manner. All chromatograms derived from either pH in the presence or the absence of DIHBA showed a single radioactive peak with Rf= 0.30. It was concluded that under the experimental conditions described previously no methylation of DIHBA occured. From fig. 6 it is calculated that after 5 minutes of incubation a 82% inhibition is obtained at pH 7.5. At pH 6.0 the inhibition is 86%. The difference in the percentage inhibitions is not significant but the fact that DIHBA was only partly in solution at the lower pH may indicate that the inhibition is favored at this pH.

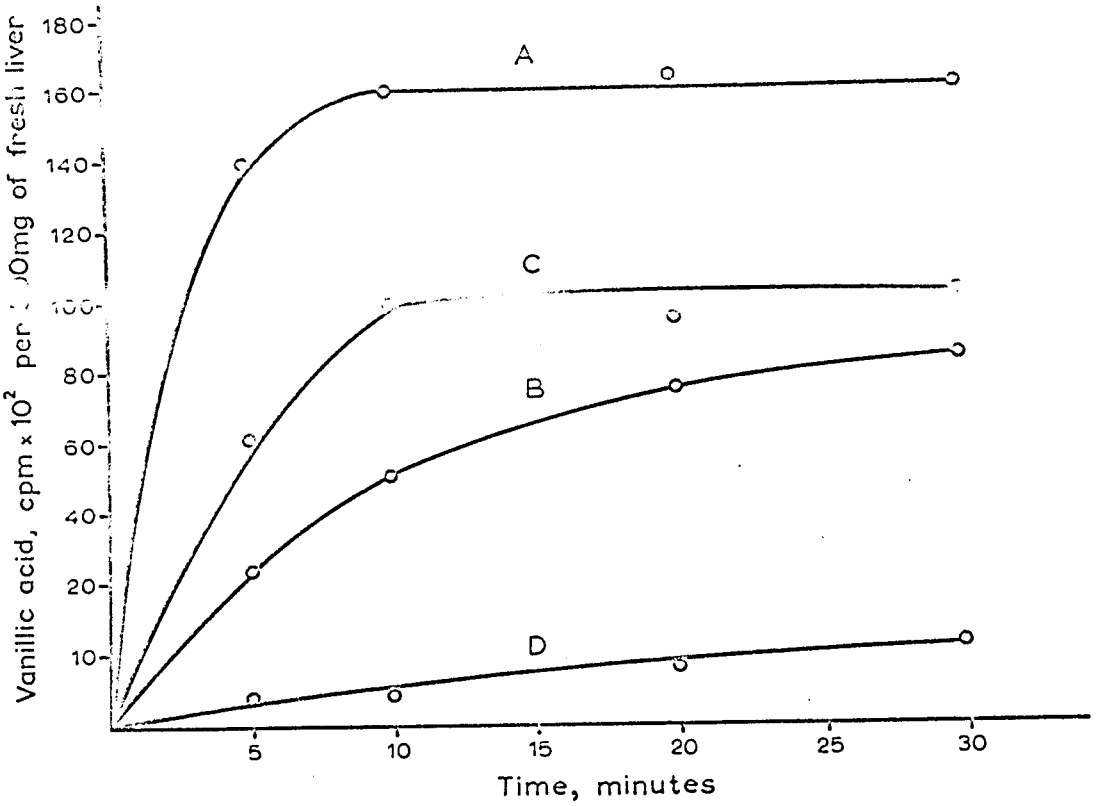


Fig.6. Inhibition of vanillic acid production by DIHBA. Curve A, control, pH 7.5; B, control+DIHBA, pH 7.5; C, control, pH 6.0; D, control+DIHBA, pH 6.0.

Attempted total reversal of inhibition by excess substrate. It has been shown that a 9-fold increase in the substrate concentration at pH 7.4 (fig. 5) reversed, partially, the inhibition. It was decided to test whether a 100-fold increase in substrate concentration would reverse the inhibition at pH 6.0. The conditions were as in the previous experiment except that 0.4 micromoles of methionine-methyl-C^{14} and 2.0 micromoles of nonradioactive methionine were added. The reaction was followed up to 30 minutes. The experiment is outlined below:

Complete system

Complete system + DHBA 3.75×10^{-3} M

Complete system + DHBA + excess substrate

No vanillic acid was produced in the mixtures with the excess substrate. Substrate inhibition and impurities at this very high concentration (10^{-1} M) for protocatechuic acid were probably responsible for the effect. Table 3 summarizes the results.

Time minutes	Complete system	Complete system + DIHBA	Complete system + DIHBA + Excess substrate
	c.p.m. of vanillic acid/10 microliters of extract		
5	5,560	240	nil
10	7,093	426	"
20	8,502	893	"
30	8,700	1,300	"

Table 3 - Production of vanillic acid in the presence and absence of DIHBA and in the presence of excess substrate (10^{-1} M) at pH 6.0.

Discussion. The experiments described in this section clearly demonstrate the inhibitory action of DIHBA upon the crude system from rat liver. It will be shown later in this work that this action stems from the inhibition of the second enzyme of the system, namely, catechol-O-methyltransferase (COMT). It was not possible to demonstrate the presence of O-methyl-DIHBA. TOMITA (1962) reported a 0.1% incorporation of C^{14} into O-methyl-DIHBA per 10 mg of protein. In our experiments about 80 mg of protein per flask were present.

SIMON et al. (1959) demonstrated that para O-methylation of 3,4-dihydroxyacetophenone in vitro is favored at a higher pH than that for meta methylation. This finding does not speak in favor of methylation of DIHBA (it being p-methylation) since the inhibition seems to be favored at lower pH.

S E C T I O N II

EXPERIMENTS IN VIVO

At this point it was thought that the assay method for COMT was not suitable for further insight into the nature of the inhibition described in the previous section. The formation of S-adenosylmethionine (S-Ame) was occuring simultaneously with O-methylation and one could not distinguish, by observing the overall inhibitory effect, between an inhibition of the methionine-activating enzyme and COMT. The possibility also existed that both enzymes were inhibited. The dependence on the methionine-activating enzyme could most conveniently be circumvented by the use of S-Ame-methyl-C^{14} but this was not available at the time. The fluorometric method of AXELROD (1962) was adopted with minor modifications and is described below.

COMT assay in rat liver

Reagents

L-Adrenaline bitartrate in water. The solution was prepared weekly and kept divided in small volumes in the deep freezer.

Sodium phosphate buffer, pH 7.9, 0.5M

Borate buffer, pH 10.0, 0.5M

Magnesium chloride 0.1M

Hydrochloric acid 0.1N

S-adenosylmethionine in water. The solution divided into small portions was kept in the deep freezer.

Metanephrine hydrochloride in water.

Procedure

The reaction mixture was prepared by mixing in 40 ml glass-stoppered centrifuge tubes, 100 micromoles of phosphate buffer, 20 micromoles of magnesium chloride, 0.3 micromoles of S-Ame, 0.6 micromoles of adrenaline bitartrate and 0.8 ml of rat liver homogenate prepared as described in the preceding section. The final volume was 2.0 ml. The reaction was initiated by the addition of the enzyme and carried out at 37°for 30 minutes in a water bath. One ml of borate buffer was added to stop the reaction. Blanks were prepared by omission of S-Ame from the incubation mixture. A known amount of metanephrine. HCl (100 micrograms) was carried through the procedure. The mixtures were extracted by shaking for 30 minutes in a wrist-action shaker with 20 ml of a mixture consisting of two parts of iso-amyl alcohol and

three parts of toluene (FISHER CERTIFIED). The tubes were then centrifuged for 5 minutes at 1,500 r.p.m. and 15 ml aliquots of the solvent mixture were transferred to similar clean tubes and were reextracted with 4 ml of 0.1N HCl for 5 minutes with the shaker. The supernatant solvent phase was removed by aspiration and discarded. The clear aqueous phases contsining the enzymatically formed metanephrine were transferred to clean test tubes. The metanephrine was measured in an AMINCO-BOWMAN spectrofluorometer at 335 mμ after excitation at 285 mμ. The overall recovery of metanephrine was 60%.

The linearity of the fluorescent response with the amount of metanephrine is demonstrated in fig. 7 in which arbitrary fluorescent units are plotted versus known amounts of metanephrine. HCl per ml of 0.1N HCl. This linear response covers practically the entire response range of the instrument.

One unit of COMT is defined as the amount of enzyme which produces one micromole of metanephrine in 30 minutes at 37°.

Monoamine oxidase (MAO) assay in rat liver. The recently devised rapid spectrophotometric method by WEISSBACH et al. (1960) was used. The method measures the rate of dis-

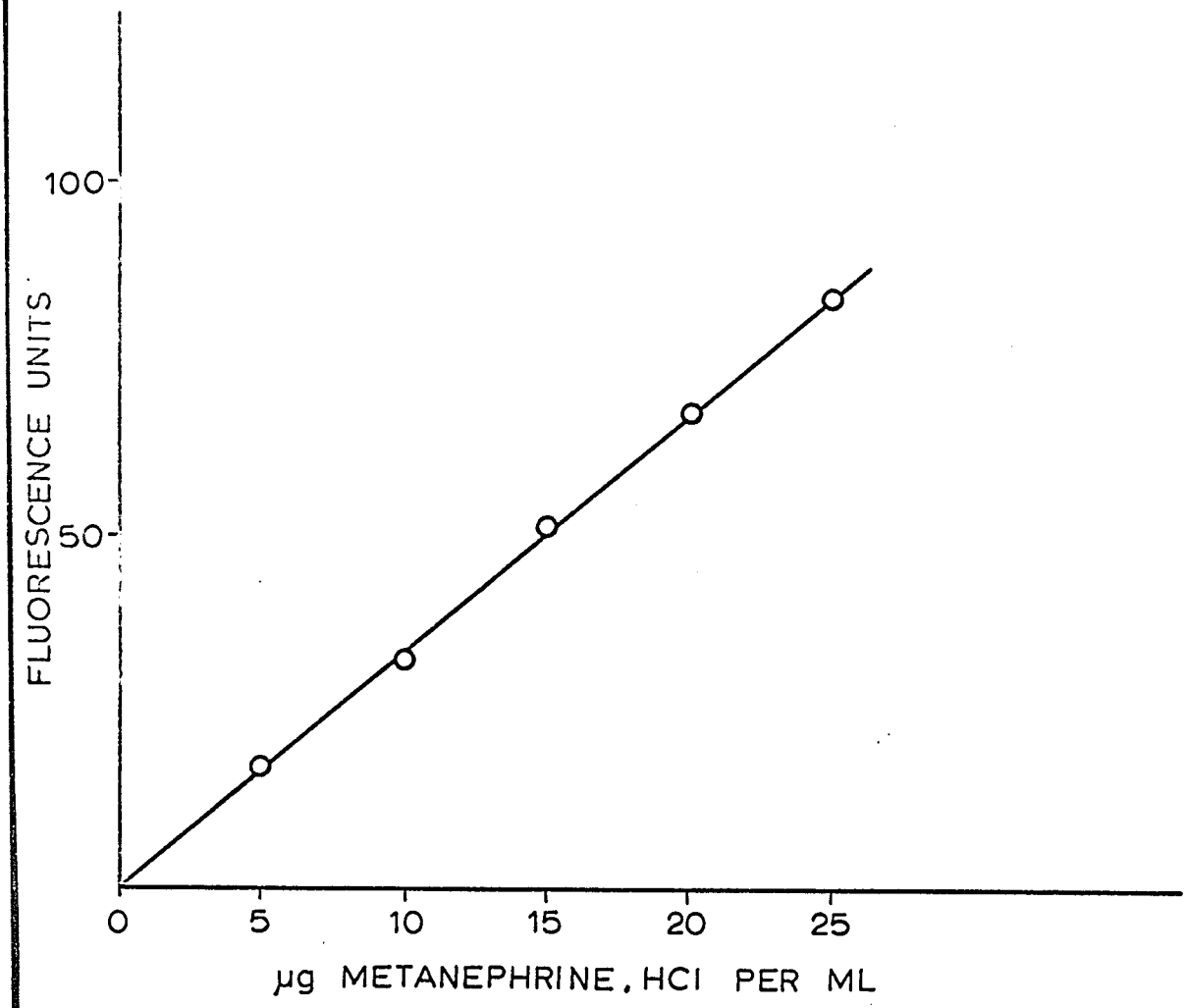


Fig.7. Fluorescence response to metanephrine in 0.I HCl. Excitation at 285mµ, emission at 335mµ.

appearance of kynuramine due to its enzymatic oxidation by MAO to the corresponding aldehyde. Kynuramine absorbs at 360 mμ and its rate of disappearance bears a linear relationship within a wide range to the rate of decrease in optical density at this wavelength.

Reagents

Sodium phosphate buffer 0.5M, pH 7.4

Kynuramine. 2MM in water. The solution divided into small portions was kept in the deep freezer.

Procedure

Rat liver was homogenized with 5-6 volumes of water and centrifuged in a refrigerated centrifuge for 10 minutes at 1,500 r.p.m. to remove cell debris. The supernatant is used as the enzyme preparation. In a square silica cuvette with a 1 cm light path the following were added: 150 micromoles of phosphate buffer, 0.3 micromoles of kynuramine and water to make a volume of 2.9 ml. The reaction was initiated by the addition of 0.1 ml of the enzyme preparation. A blank in a similar cuvette was prepared by omitting kynuramine. The reagents and water were at 30° at the time of mixing. The enzyme preparation was kept in ice. A Beckman DU model spectrophotometer

was employed to follow the reaction. The cell compartment was kept at 30° with a water jacket of circulating water from a large reservoir kept at 30°. A small motor was used to circulate the water. Readings of optical density were taken every minute, or every two minutes more conveniently. With some practice and a stopwatch very good timing can be obtained. The mixing was obtained by two rapid inversions of the cuvette. During the first two minutes after the addition of the enzyme the readings are erratic because of coarse particles settling in the cuvette, but beyond this short time the decrease in optical density is linear with time for a period of at least 10 minutes. This is illustrated in figure 8. At all times the linearity of the reaction was confirmed. The decrease in optical density was calculated for a period of 10 minutes starting from the third minute of the reaction.

One unit of MAO is defined as the amount of enzyme which causes a fall of 0.001 unit of optical density per minute at 30°.

Protein determination. The method of LOWRY et al. (1951) was employed.

Reagents

Reagent A 2% Na_2CO_3 in 0.1N NaOH

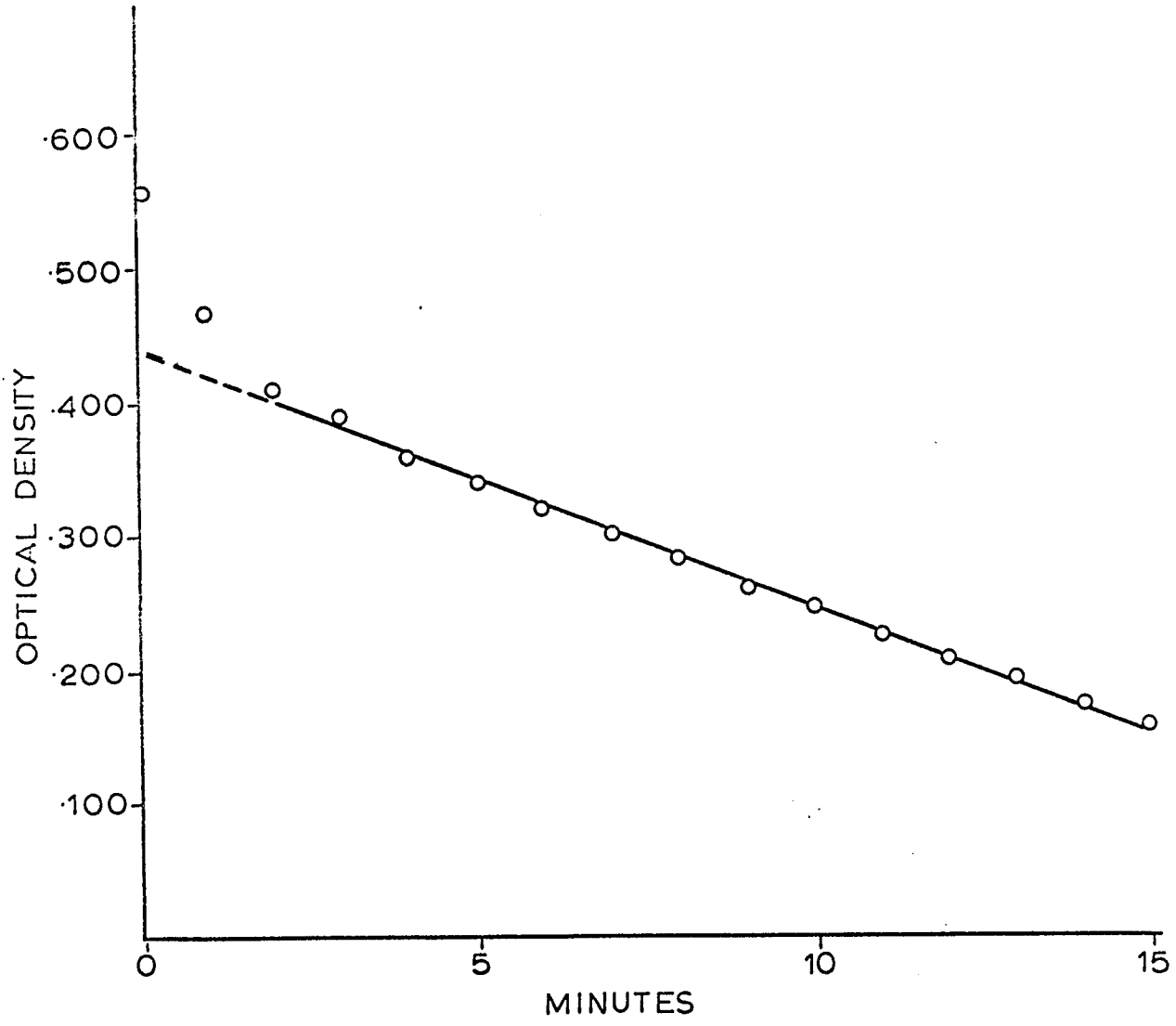


Fig.8. Rate of decrease in optical density due to the action of rat liver monoamine oxidase on kynuramine.

Reagent B 1% $CuSO_4.5H_2O$

Reagent C 2% potassium tartrate

Reagent D 1N phenol reagent

Procedure

50 ml of reagent A, 0.5 ml of reagent B and 0.5 ml of reagent C are mixed. 5 ml of this solution is mixed with 1.0 ml of protein solution and the mixture is allowed to stand for 10 minutes at room temperature. 0.5 ml of phenol reagent is added quickly and the solution is mixed thoroughly . After 30 minutes the optical density is measured in a BAUSCH and LOMB Spectronic 20 spectrophotometer.

A standard curve was prepared by the same procedure with crystalline bovine albumin freshly prepared.

Experiments with rats. Male SPRAGUE-DAULEY rats weighing 170-194 gm were divided into the following groups:

1- Untreated control group

2- Injected once with 2ug/gm body weight of L-thyroxine sodium and sacrificed 24 hours later

3- Injected twice with thyroxine with a 24 hours interval and sacrificed 48 hours after the first injection.

Thyroxine in M/15 phosphate buffer was solubilized by the addition of a few drops of 0.1N NaOH .

Results. Table 4 summarizes the results obtained for the liver levels of COMT and MAO in the three groups.

Statistical analysis (t test) of the results concerning COMT shows that the difference between the untreated animals and the animals injected once with thyroxine is not significant. However, the difference between the untreated and the twice injected animals is significant for $P < 0.01$.

For MAO the differences of both treated groups from the control group are significant for $P < 0.01$.

Discussion. These results confirm the experiments of various investigators mentioned in the Introduction regarding reduced levels of MAO in hyperthyroid animals. The results similarly confirm the experiments of D'IORIO and LEDUC (1961) qualitatively but not quantitatively. The reduction of COMT although significant is small compared with the 45% reduction reported by D'IORIO and LEDUC and seems to be in better agreement with the report of WURTMAN et al. (1963) on a small but significant decrease of COMT after large doses of thyroxine. The assay method employed by D'IORIO and LEDUC as well as

Rat No.	Control		One injections		Two injections	
	COMT Units $x10^{-4}$	MAO Units	COMT Units $x10^{-4}$	MAO Units	COMT Units $x10^{-4}$	MAO Units
1	16.7	4.31	18.0	3.63	15.5	1.86
2	17.8	4.87	21.0	3.66	9.7	2.38
3	17.9	4.15	20.0	4.00	13.4	2.85
4	14.7	5.97	19.0	4.42	12.2	2.95
5	20.2	4.67	-	-	11.3	3.62
6	15.9	4.91	-	-	-	-
Average:	17.2	4.81	19.5	3.93	12.4	2.73
S.D.	1.89	0.38	1.66	0.38	2.19	0.66

Table 4 - Levels of MAO and COMT in untreated control and thyroxine-treated rats. Units are expressed per mg protein.

the fact that their animals received three instead of two large injections of thyroxine might be responsible for the quantitative difference between their and our experiments. As will be shown later in this work thyroxine itself cannot be expected to be a good inhibitor of COMT in vivo but its analogues and metabolites are good inhibitors of the enzyme. For any significant direct inhibition of COMT in vivo, conditions must be ensured whereby these metabolites would accumulate to amounts sufficient to cause inhibition. Furthermore one should be able to distinguish between effects caused by direct inhibition and reduced protein synthesis in the hyperthyroid state. It follows that in vivo experiments of this kind could not be fruitful and meaningful unless they are preceded by experiments in vitro with a view to exploring and assessing the potentialities of the thyroxine-like type of molecules to act as inhibitors of the enzyme. That such potentialities are residing in these molecules was indicated by the inhibitory action of DIHBA. The remainder of this work is mainly concerned with the study of such expectations.

S E C T I O N III

STUDIES IN VITRO WITH IODOPHENOLS

The inhibitory effects of DIHBA on COMT described in detail in section I presented us with certain problems well-worth investigating. Should this inhibition be demonstrated, by the use of an appropriate enzymatic assay method, to be specific for COMT, then a number of iodophenols, or halophenols in general, might be expected to act in a similar way.

It was decided to adopt the assay method of AXELROD described in the previous section, which makes use of adrenaline as substrate and S-adenosyl-methionine (S-Ame) as the methyl group donor. This method does not depend on a prior enzymatic step since the methyl group donor is provided as such in the medium. Thus, the use of the crude enzyme preparation could be abandoned. Instead, a partially purified preparation of COMT was used at all times. The purification procedure was that described by AXELROD and TOMCHICK (1958) and is described below for 50 gm of liver.

Partial purification of COMT. Four adult male rats were

stunned with a blow on the head, decapitated and exsanguinated. The livers were excised and placed in ice-cold isotonic KCl. All operations were carried out at 0-3°,. 50 gm of liver was minced with scissors and homogenized with 200 ml of isotonic KCl. The homogenate was centrifuged for one hour at 40,000x g. The clear supernatant was transferred to a beaker and its pH was adjusted (pH meter) to 5.0 with 1N acetic acid. After centrifugation, the clear supernatant (S1) was transferred to a beaker. To 175 ml of the supernatant S1 30.8gm of ammonium sulfate were added (0-30% saturation) slowly under constant stirring with a magnetic stirrer in the cold room. The mixture was allowed to stand for 15 minutes and was then centrifuged. The clear supernatant (S2) was transferred and the precipitate was discarded. To 172 ml of supernatant S2 21.8 gm of ammonium sulfate were added as above (30-50% saturation). After 15 minutes the mixture was centrifuged and the new supernatant was discarded. The precipitated protein was dissolved in 25 ml of water and the solution was dialyzed in a dialysis bag against running tap water in the cold room overnight. The protein was then lyophilized for 8-10 hours. The preparation kept in the deep freezer (-20°) was stable for several months. No attempt was made at this stage for further purification. Four to five-fold purification was

achieved by this procedure.

Time course of the reaction. When activity was plotted versus time of incubation, under the conditions described in section II, a curve similar to those obtained with the crude preparations (section I, fig. 2) was established. By the end of 30 minutes the reaction was generally completed.

Conditions. Since iodinated phenols are not very soluble at the pH of the reaction (7.9) they were added dissolved in 0.1 ml of NaOH. This addition raised the pH to 8.4 without affecting the activity of the enzyme. The pH optimum for GOMT is broad, extending from 7.5 to 8.2 according to AXELROD and TOUCHICK (1958). DIDBA was added dissolved in 0.1 ml of ethanol. Accordingly, 0.1 ml of 0.1N NaOH or 0.1 ml of ethanol was added to the control tubes. Two blanks were prepared by omission of S-Ame. One blank contained in addition the substance under examination. At no time were the two blanks found to be different.

Unless specifically stated the final concentrations in terms of molarities in the incubation mixtures were as follows:

adrenaline bitartrate 3×10^{-4} M

S-adenosylmethionine	1.5×10^{-4} M
Magnesium chloride	1×10^{-2} M
phosphate buffer	5×10^{-2} M

6-10 mg of enzyme, dry weight, was generally added dissolved in 0.5 ml of water. The reaction was initiated by the addition of the enzyme (unless it is otherwise stated), preincubated at 37° for 2 minutes prior to its addition.

The activity was expressed in arbitrary fluorescence units.

Linearity of the reaction. The reaction was linear with time up to 5 minutes, that is, the activity was proportional to time for this time interval. The conditions were as described previously in the presence of 0.1 ml of 0.1N NaOH and 6 mg of enzyme (fig. 9). This length of time was used in the kinetic experiments designed to measure initial velocities.

Effect of DIHBA. The inhibitor was added in a final concentration of 10^{-4}M. A plot similar to figure 3 was obtained. This experiment clearly established that DIHBA is a specific inhibitor of COMT. However, the enzyme was only partially purified and the presence of iodophenol-O-methyltransferase could not be excluded. A straight-

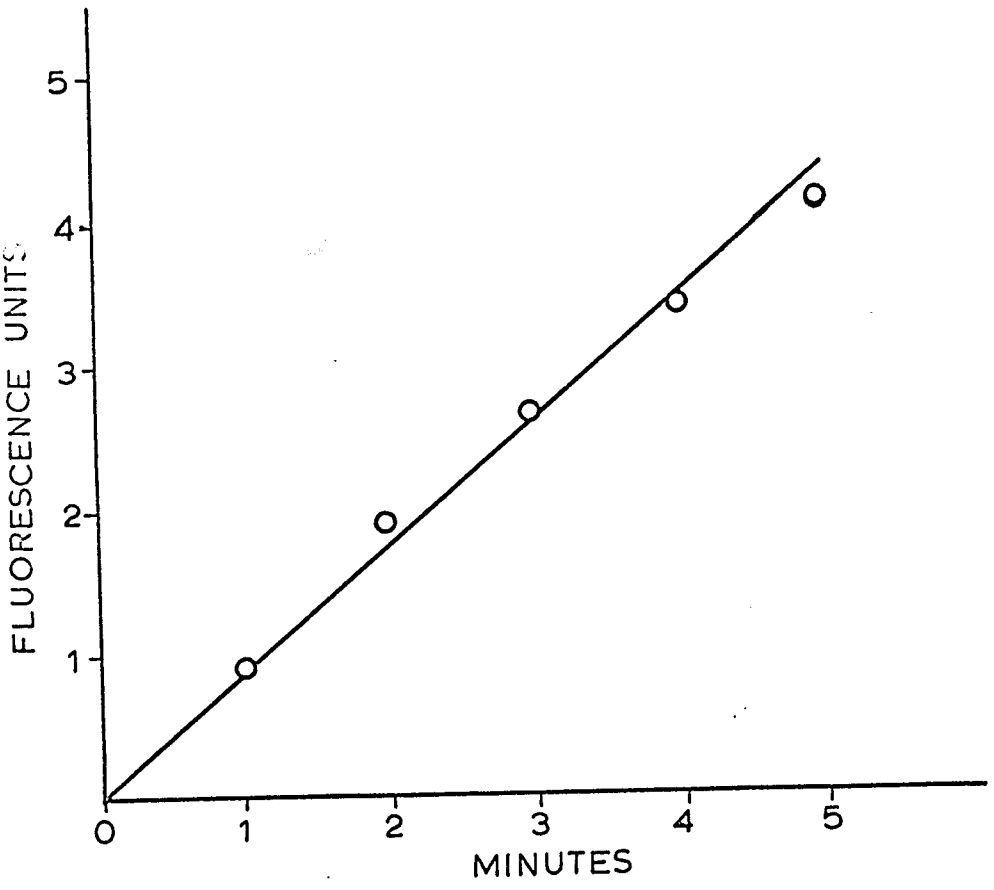


Fig.9. Rate of metanephrine formation by a partially purified preparation of COMT. The reaction is linear up to 5 minutes.

forward experiment was designed in order to establish that the inhibition was not due to competition for the methyl group donor by the latter enzyme. This experiment is described below.

Effect of increasing the S-Ame concentration on the inhibition by DIHBA. For this experiment a COMT preparation purified about 30-fold (cf., section X) was employed. The inhibitor was added to a final concentration of 10^{-4} M and 10^{-3} M adrenaline was present. S-Ame was present at the following final concentrations: 10^{-4} M, 10^{-3} M, 3×10^{-3} M, 6×10^{-3} M and 10^{-2} M. The incubation time was 20 minutes. From figure 10 it is clear that a 100-fold increase in the concentration of the methyl donor does not affect the level of activity in the presence of DIHBA, i.e., the inhibition is not reversed by excess S-Ame. This failure to reverse the inhibition can be considered as conclusive evidence that the inhibition is not due to a limiting availability of the methyl group donor. It should also be noted that the inhibition is present even when a more highly purified enzyme preparation is employed.

Effect of 3,5-diiodo-salicylic acid (DISA). Iodophenol-O-methyltransferase has been reported to methylate DIHBA

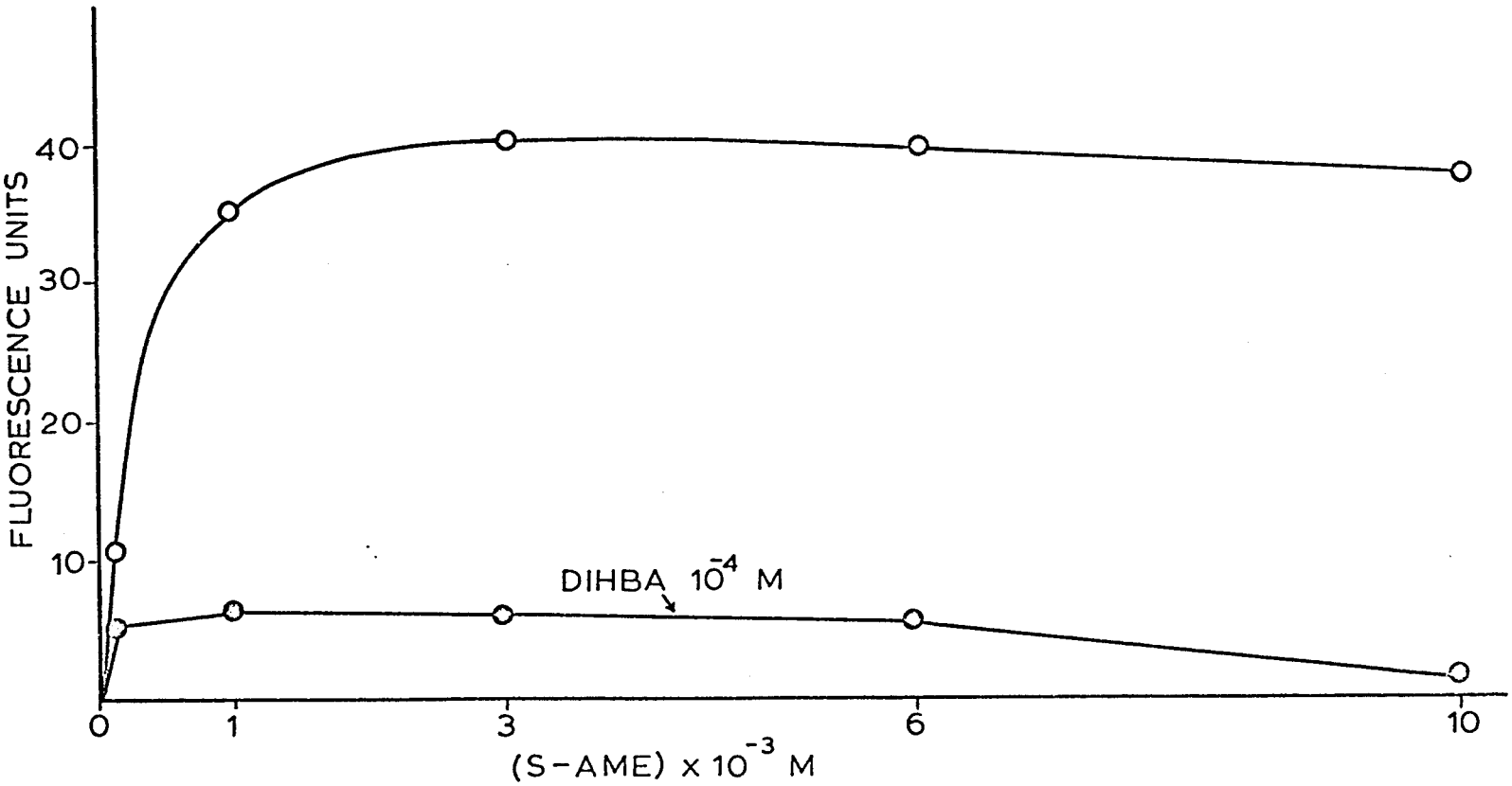


Fig.10. Lack of effect of excess amounts of S-Ame in the presence of 10^{-4}M DIHBA on the activity of COMT. Upper curve represents control activity.

and tetraiodothyroacetic acid (Tetrac) (TOMITA and CHA 1963). Both substances are characterized by the presence of two iodine atoms in positions ortho to the hydroxyl group (see addendum). It is not known if o-mono-iodophenols serve as substrates for this enzyme. DIBA, an ortho-mono-iodophenol at a concentration of 10^{-3} M is shown to inhibit COMT by 57.7% from the 5 minute level (fig. 11). It is very likely that the iodine atom at position 5 of the ring is not essential for the inhibitory action of DIBA. Strong evidence for this view comes from the inhibitory action of iodovanillin, a mono-iodophenol, which will be described later in this section.

Effect of 3,5-diiodo-4-hydroxypyridine (DIHP). This substance when present at 10^{-3} M concentration produces 20% inhibition - which is rather poor compared to the effects of the previously described inhibitors.

Effect of 3,5-diiodo-4-hydroxyphenylpyruvic acid (DIHPA). In figure 12 the effect of DIHPA is illustrated, when present at a final concentration of 10^{-3} M. The unusual feature in this plot is the complete titration of the enzyme by the end of 10 minutes of incubation. The activity of the enzyme is seen to remain constant throughout the additional 20 minutes of incubation. The new feature

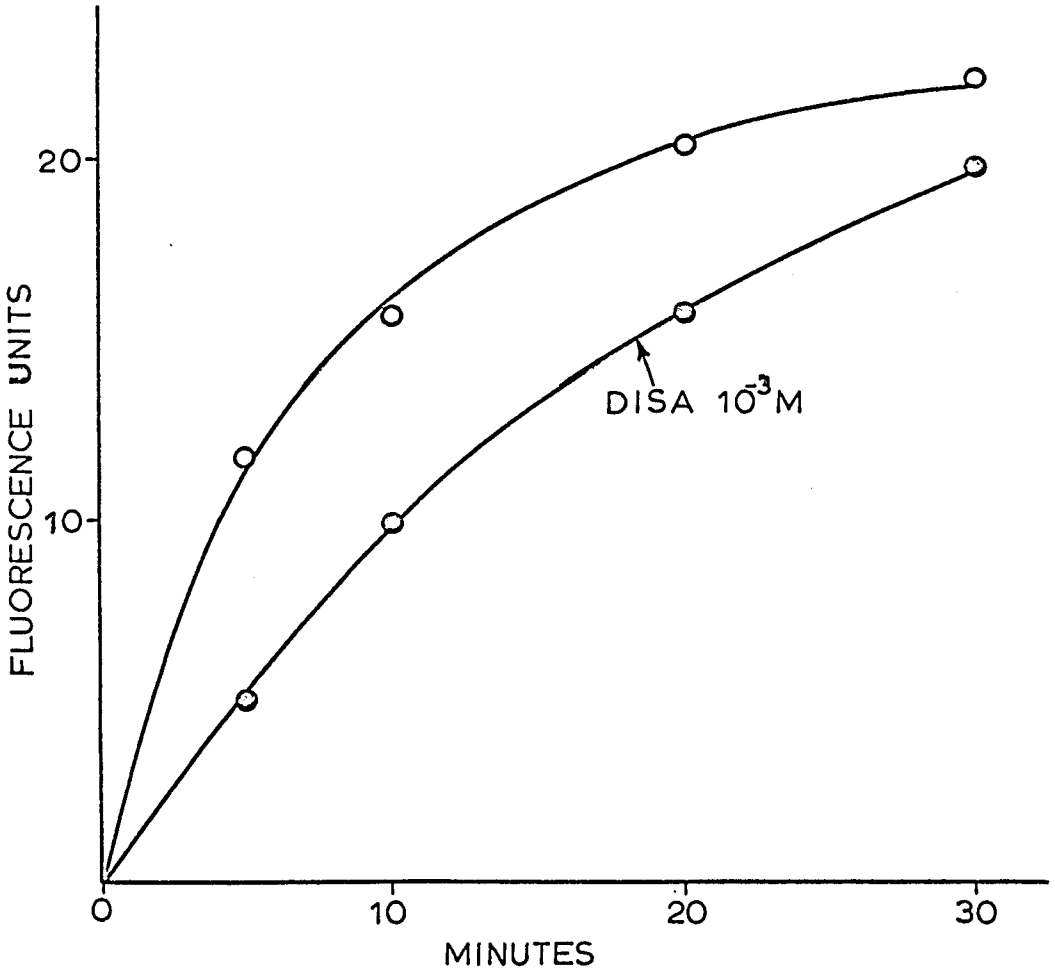


Fig.II. Rate of COMT activity in the presence of DISA. Upper curve indicates control activity.

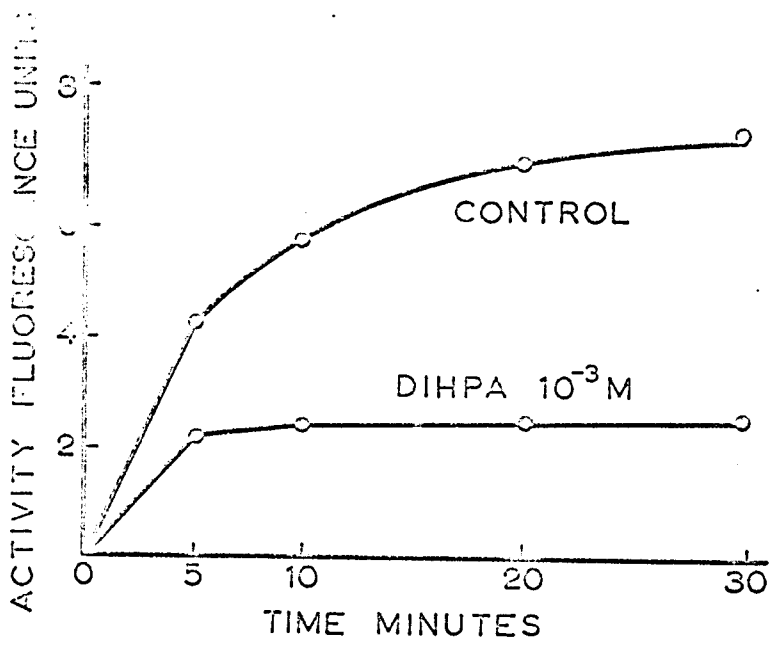


Fig.I2. Inhibition of catechol-O-methyl transferase (COMT) by DIHPA.

present in the molecule is the pyruvic acid chain. The possibility that the presence of the α-ketoacid side chain might in itself give rise to this type of inhibition is eliminated by the following two tests.

Lack of effect in the presence of phenylpyruvic acid and p-hydroxyphenylpyruvic acid. When these two compounds were present at 2×10^{-3} M concentrations no effect whatsoever was noted on the activity of the enzyme. This lack of effect demonstrates that the progressive inhibition exhibited by DIHPA is the overall effect of the presence of both the iodophenolic function and the pyruvic acid chain.

Lack of effect in the presence of 3,5-diiodotyrosine (DIT). Additions of DIT amounting to 10^{-3} M and 3×10^{-3} M final concentrations did not produce inhibition over the usual incubation time of 30 minutes. This absence of inhibitory capacity on the part of DIT was most puzzling. It is simply reduced to the observation that substitution of the pyruvic acid residue in the DIHPA by the alanine residue (by an imaginary transamination) resulting in the formation of DIT renders the latter completely devoid of inhibitory properties. This could only mean either of two things: (a) assuming that the alanine side chain

does not interfere in any way with the function of the diiodophenolic group, it would indicate that the latter is not capable in itself of binding the enzyme in such a fashion as to produce inhibition. This would mean that for inhibition two distinct points of attachment must be provided on the part of the inhibitor. One would be the iodophenolic function, the other the "supporting" function, such as the carboxyl group in DISA and DIHBA or the pyruvic acid residue in DIHPA. (b) On the other hand, one might assume that the influence of the alanine side chain is such as to completely abolish the inhibitory capacity, presumably solely borne by the iodophenol function. It is difficult to visualize such a total effect. Evidence that the iodophenol function cannot support inhibitory properties by itself is presented below.

Lack of effect of O-iodophenol. Ortho-iodophenol at a final concentration of 1.5×10^{-3} M produced no inhibition. This experiment then provides conclusive evidence that the iodophenol function alone cannot impart inhibitory properties to the benzene ring. The possibility that two iodine atoms rather than one in ortho position to the hydroxyl group might be absolutely required in the absence of any other functional group in the ring, could be

ideally tested if 2,6-diiodophenol were available. However, this substance was not available but the fact that DISA with only one iodine atom in ortho position to the hydroxyl group is an inhibitor, already indicated that two iodine atoms are not absolutely required. Further evidence for this is presented below.

Effect of 5-iodovanillin: (3-methoxy-4-hydroxy-5-iodobenzaldehyde). This compound became available to us at a late date and was tested with a 30-fold purified COMT. When it was added in a final concentration of 10^{-4} M it produced 37% inhibition. As a matter of fact this inhibitor is inferior only to DIHBA as will be shown in the next section. 5-iodovanillin is an ortho-monoiodophenol (see addendum) with an aldehyde function at para and a methoxy group at ortho positions relative to the hydroxyl group. It is equivalent with 3-iodo-4-hydroxybenzaldehyde since the methoxy group is inert as far as inhibitory capacity is concerned (cf., below). Vanillin can be visualized as the enzymatic end product of the catalytic action of COMT on protocatechuic aldehyde. The latter is in fact a substrate for the enzyme as any catechol is. It is remarkable that the introduction of an iodine atom converts the product of an enzymatic reaction into an inhibitor of the enzyme.

Therefore, here is clear demonstration that the monoiodophenolic function in conjunction with the "right" substituent in the ring is sufficient for the display of inhibitory properties. That the methoxy group is not significant in this respect is evidenced by the following experiment.

Lack of effect of 3,5-diiodo-4-methoxybenzoic acid (O-methyl-DIHBA). No effect was produced by the presence of 3×10^{-4} M O-methyl-DIHBA. This experiment supports the view that the methoxy group in 5-iodovanillin is inactive. On the other hand, the absence of inhibitory properties in the molecule of O-methyl-DIHBA demonstrates that a free hydroxyl is indispensable for the manifestation of such properties, even in the presence of two iodine atoms.

Lack of effect in the presence of meta-fluorotyrosine and ortho-fluorophenol. These two fluorophenols when present at 2.7×10^{-3} M and 10^{-3} M final concentration, respectively, do not affect the activity of the enzyme.

Summary and discussion. It was shown that over a wide range of S-Ame concentration covering the optimal concentration (fig. 10) the inhibition produced by DIHBA

remained practically unchanged. This indicates that the inhibition is not a function of S-Ame concentration and eliminates the possibility that it could be due to a parallel methylation of DIHBA catalyzed by another enzyme present as contamination, such as the iodophenol-O-methyltransferase.

Whether an active site for S-Ame exists on the enzyme surface is unknown (but cf. section VII). However, assuming that such a site exists and that the inhibition is due to the DIHBA (and its analogues) competing for and blocking this site, one would expect some reversal of inhibition by the excess S-Ame. No such reversal was noted and one is compelled to conclude that the inhibitor(s) do not bind at such a hypothetical site.

The experiments with o-iodophenol, DIT and the other substituted phenols provide conclusive evidence that the o-monoiodophenolic function per se cannot render the benzene ring an inhibitor of COMT. It appears then that the alanine side chain in DIT does not suppress any inhibitory capacity in o-iodophenol but, rather, is incapable of providing a second point of binding apparently required for inhibition. This is provided by the carboxyl groups in DIHBA and DISA, the carbonyl group in 5-iodovanillin and the pyruvic

acid residue in DIHPA. In 3,5-diiodo-4-hydroxypyridine (DIHP) it is apparently provided by the pyridine ring itself. It should be noted that DIHP is the weakest inhibitor of all. No direct comparison can be made between this molecule and the other inhibitors since the pyridine ring introduces a new element here. The inhibition by 5-iodovanillin shows that the presence of one iodine atom alone in ortho position relative to the hydroxyl group is sufficient to support inhibition. This does not eliminate the possibility that the presence of two iodine atoms might result in better inhibition as in DIHBA.

A free hydroxyl group is indispensable as the lack of effect of O-methyl-DIHBA has demonstrated.

S E C T I O N IV

TYPE OF INHIBITION AND INHIBITION CONSTANTS OF THE ISOPRENOL INHIBITORS

The double reciprocal plot of LINEWEAVER and BURK (1934) was employed in order to determine the type of inhibition and the inhibition constants (Ki) as well as the Michaelis constant (Km) for the substrate, adrenaline.

The equations are as follows (DIXON and WEBB 1955):

In the absence of inhibitor: $\frac{1}{v} = \frac{Km}{V} \cdot \frac{1}{S} + \frac{1}{V}$

In the presence of a competitive inhibitor: $\frac{1}{v} = \frac{Km}{V} \cdot \left(1 + \frac{I}{Ki}\right) \cdot \frac{1}{S} + \frac{1}{V}$

In the presence of a non-competitive inhibitor: $\frac{1}{v} = \frac{Km}{V} \cdot \left(1 + \frac{I}{Ki}\right) \cdot \frac{1}{S} + \frac{1}{V}\left(1 + \frac{I}{Ki}\right)$

where: V = maximum velocity

v = initial velocity

I = concentration of the inhibitor

S = concentration of the substrate

Km = Michaelis constant

Ki = inhibition constant (dissociation constant of the enzyme - inhibitor complex)

All three equations give straight lines when $\frac{1}{v}$ is plotted versus $\frac{1}{S}$. The slope in presence of either a competitive or a noncompetitive inhibitor is given by the expression

$$\text{slope} = \frac{Km}{V} \cdot \left(1 + \frac{I}{Ki} \right)$$

from which Ki can be calculated.

Conditions. Initial velocities are expressed as arbitrary fluorescent units produced in the first 5 minutes of incubation during which the reaction is linear with time. The Km for adrenaline has been reported to be equal to 1.2×10^{-4} M (AXELROD and TOUCHICK 1958) under similar conditions. This is indeed the value found in a large number of our experiments under the same conditions.

Measurements were taken at molar concentrations for the substrate, adrenaline, equal to 0.5×10^{-4} M, 1×10^{-4} M, 1.5×10^{-4} M and 3×10^{-4} M. This is a convenient range for the fluorometric method and covers the Km value for adrenaline for optimal O-Ame concentration which was found to be equal to 2.7×10^{-4} M (cf. section VII).

The measurements were therefore taken under conditions for first order kinetics (BRAY and WHITE 1957). It was shown in the previous section that varying the S-Ame concentration does not affect the inhibition. The standard concentration (1.5×10^{-4} M) for S-Ame was generally used. For DIHBA the S-Ame was present at 10^{-3} M concentration. The points in the figures represent mean values of duplicates. The duplicates were very close to each other and frequently identical. Since DIHPA produces a progressive type of inhibition no Ki is available for it.

DIHBA. Fig. 13 shows that DIHBA is a competitive inhibitor of COMT. The Ki = 1.3×10^{-5} M indicates that it is a strong inhibitor. Its inhibitory potency is of the same order of magnitude as that of pyrogallol (Ki = 0.8×10^{-5} M, CROUT 1961) and equal to that of 4-methyl-tropolone (cf. section VI, BELLEAU and BURBA 1963).

5-iodovanillin. From fig. 14 a Ki value of 1.6×10^{-4} M is calculated. The inhibitor is seen again to act in a competitive fashion. However it is less potent than DIHBA.

DISA. In fig. 15 the competitive type of inhibition by 3,5-diiodosalicylic acid is demonstrated. A Ki value of

3.4×10^{-4} M indicates that it is a less powerful inhibitor than 5-iodovanillin.

3,5-diiodo-4-hydroxypyridine (DIHP). From fig. 16 it is seen that this inhibitor acts in a noncompetitive fashion. A Ki of 1.1×10^{-3} M shows this inhibitor to be the poorest of all.

Discussion. Inhibition constants (Ki) are the dissociation constants of the enzyme-inhibitor complex. The reciprocal of the inhibition constant is the affinity of the enzyme for the inhibitor. Making use of the Ki values one can calculate that the affinity of COMT for DIHBA is twelve times as great as that for 5-iodovanillin. Since the two inhibitors differ in two respects, namely, in the number of iodine atoms next to the hydroxyl group and in the additional group, it is difficult to assign the difference in affinity exclusively to either of these structural differences. However, as will be shown in a later section, the 3,5,3',5'-tetraiododerivatives of the thyroformic, thyroacetic and thyropropionic acids have affinities 2.2-4.8 times as great as that of the 3,3'5-triiododerivatives. This indicates that the presence of two instead of one iodine atom next to the hydroxyl group makes for a better inhibition. It is reasonable

then to assume that the superior inhibitory properties of DIBBA as compared to those of 5-iodovanillin must be, partially at least, ascribed to the presence of two iodine atoms instead of one in positions ortho to the hydroxyl group. If this were so and if the presence of the methoxy group in iodovanillin is disregarded it would seem that the carboxyl group is more effective in supporting inhibition than the aldehyde group.

The affinity of COMT for 5-iodovanillin is in turn approximately twice as great as that for DIBA. This would seem to indicate, given also the possible inferior role of the aldehyde group just considered, that location of the supporting group (carboxyl) in position para to the hydroxyl is a more favorable condition for inhibition.

3,5-diiodo-4-hydroxypyridine (DIHP) displays 30 times less affinity for COMT than DIBA does. No valid comparisons can be made with the other inhibitors because of the presence of the pyridine ring. However, the indispensable character of the hydroxyl group was shown in the previous section. Hydroxypyridines do not possess normal phenolic properties and exist mainly in the pyridone forms. This may account for the poor inhibitory properties of DIHP. The presence of the non-competitive component cannot be related to any experimental

data obtained in this work. Table 5 summarizes the results of this section.

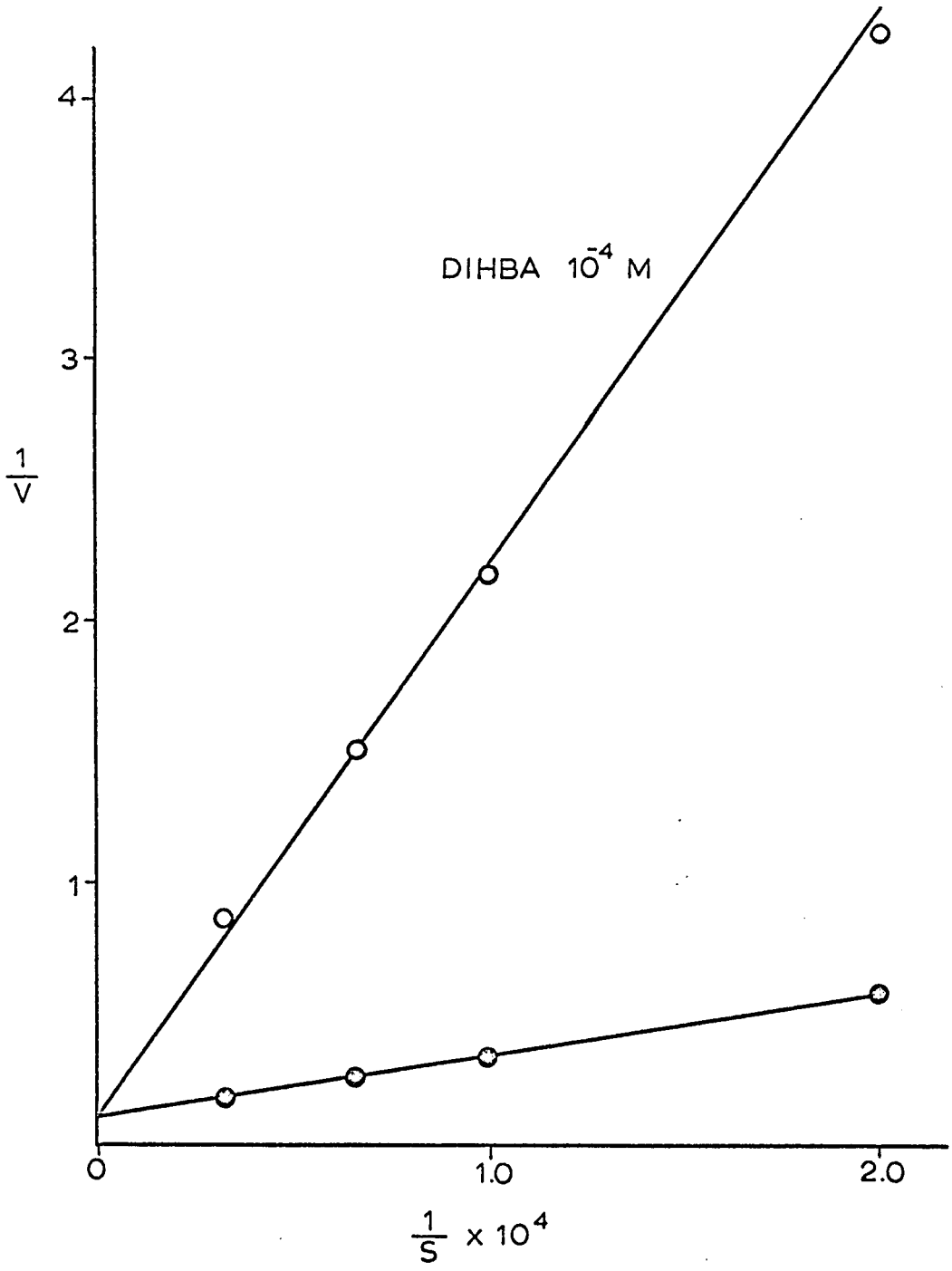


Fig. 13. Lineweaver and Burk plot in the absence and presence of 10^{-4}M DIHBA.

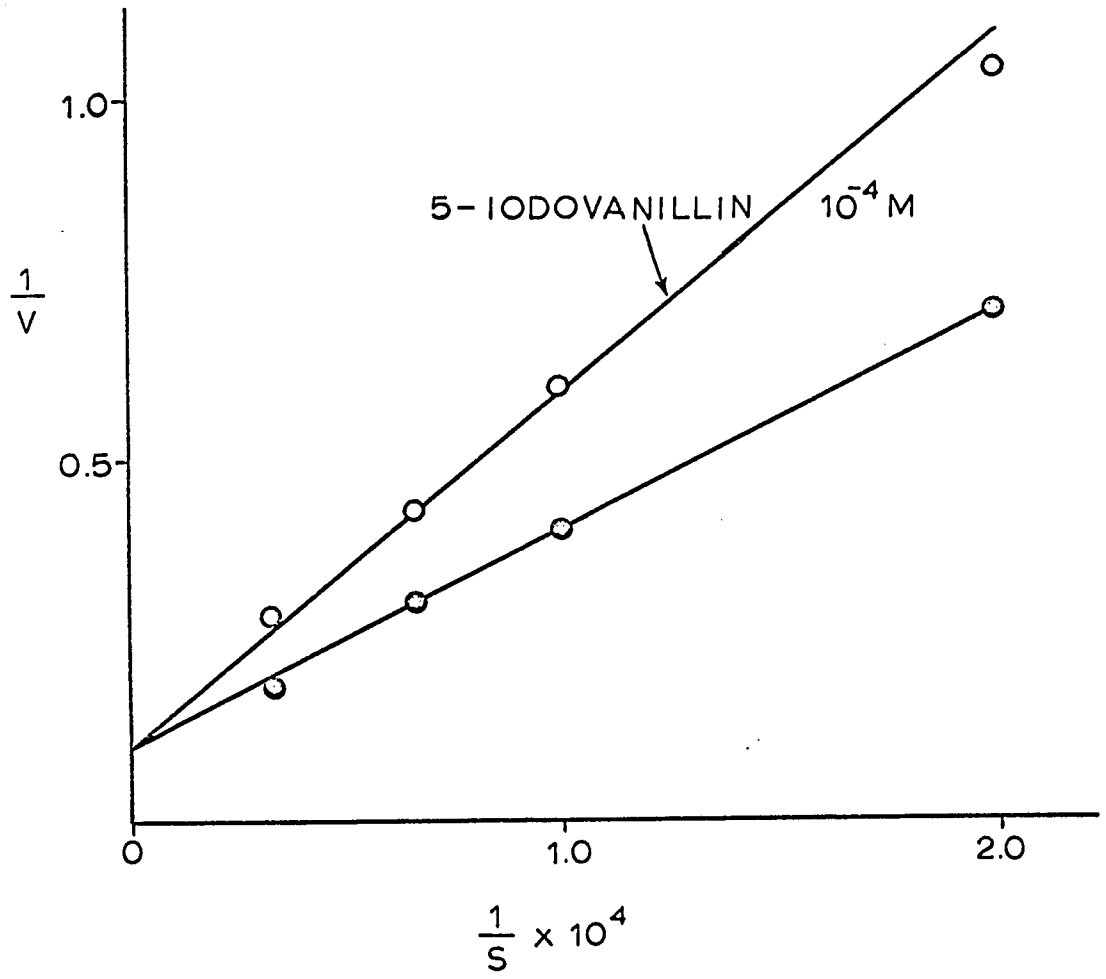


Fig.14. Lineweaver and Burk plot in the absence and presence of 10^{-4}M 5-iodovanillin.

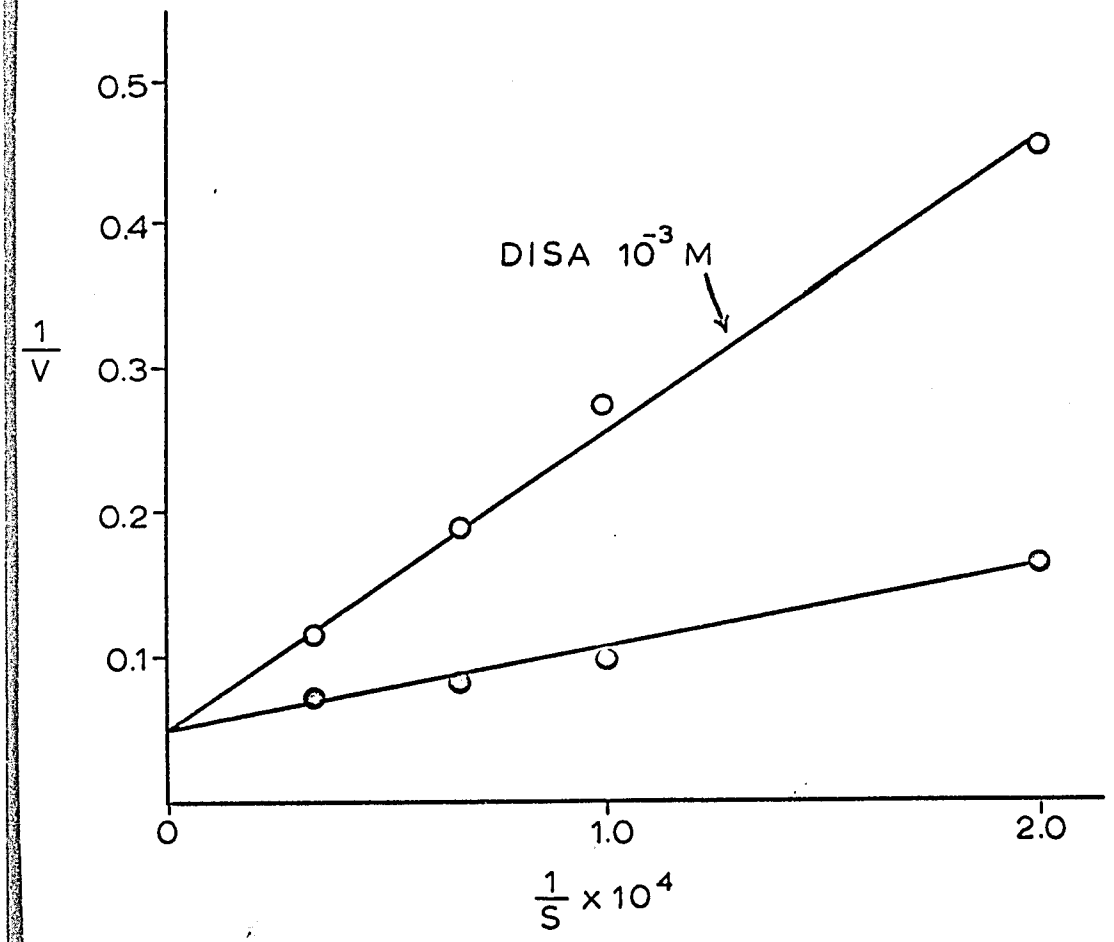


Fig.I5. Lineweaver and Burk plot in the absence and presence of IO^{-3}M DISA.

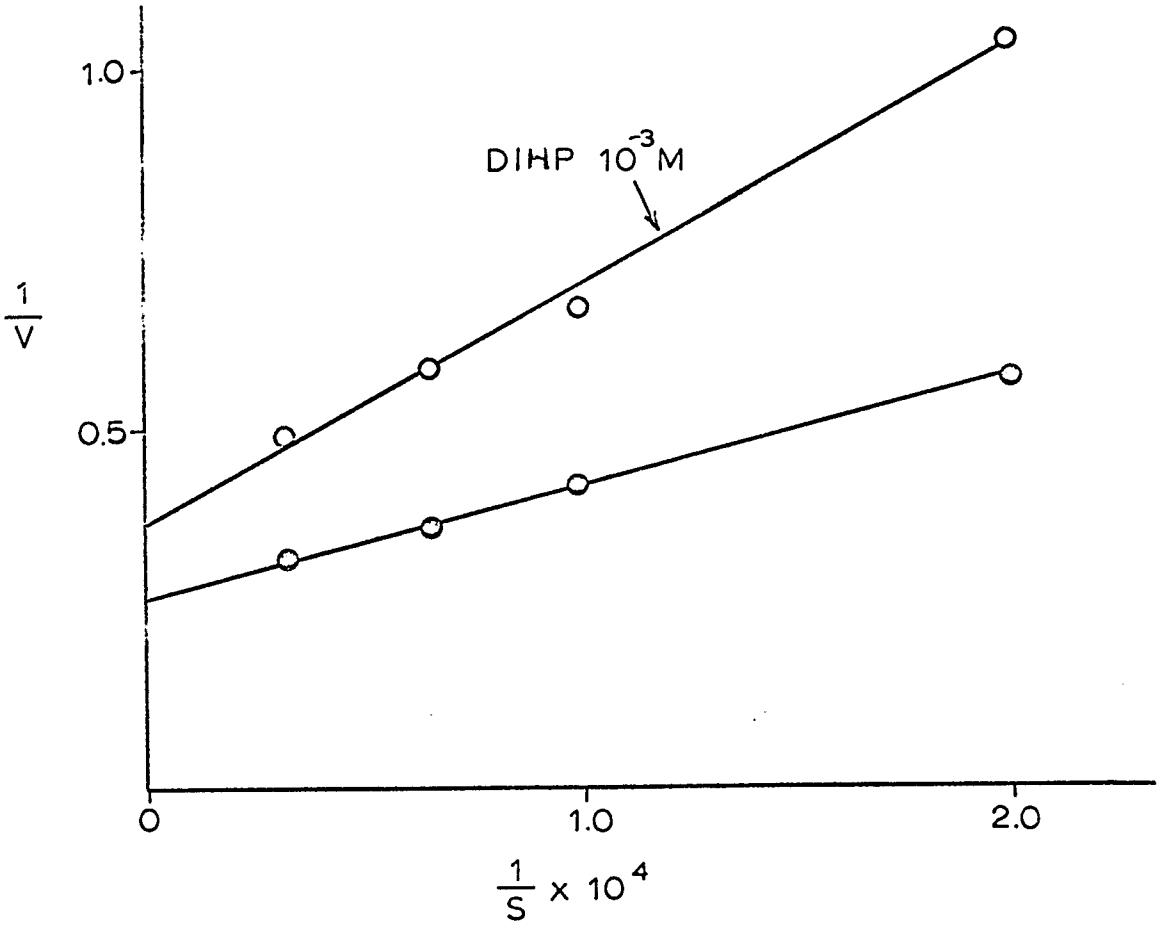


Fig.I6. Lineweaver and Burk plot in the absence and presence of 10^{-3}M DIHP.

Substances tested		Type of inhibition	Ki, M
Chemical name	Abbreviation		
3,5-diiodo-4-hydroxy-benzoic acid	DIHBA	Competitive	1.3×10^{-5}
3,5-diiodo-4-methoxy-benzoic acid	O-methyl-DIHBA	No effect	
3,5-diiodosalicylic acid	DISA	Competitive	3.4×10^{-4}
5-iodovanillin	-	Competitive	1.6×10^{-4}
3,5-diiodo-4-hydroxy-phenylpyruvic acid	DIHPA	Progressive	
Phenylpyruvic acid		No effect	
p-hydroxyphenyl pyruvic acid		"	
3,5-diiodotyrosine	DIT	"	
m-fluorotyrosine		"	
O-iodophenol		"	
O-fluorophenol		"	
3,5-diiodo-4-hydro-xypyridine	DIHP	Noncompeti-tive	1.1×10^{-3}

Table 5 - Summary of the effects of various halophenols on COMT.

S E C T I O N V

EXPERIMENTS WITH 3,5-DIIODO-4-HYDROXYPHENYLPYRUVIC ACID (DIHPA)

In section III it was shown that DIHPA titrates the enzyme quantitatively by the end of ten minutes of incubation. Before proceeding to further investigation the experiment (fig. 12) was repeated and confirmed. The same type of plot as in fig. 12 was obtained, indicating that most of the enzyme had been titrated by the end of 5 minutes and all of it by 10 minutes. This would mean that preincubation of the enzyme with DIHPA for 10 minutes in the presence of the methyl donor or the substrate should result in complete lack of activity after addition of the substrate or the methyl donor.

Preincubation in the absence of adrenaline. The complete system except substrate (adrenaline) was preincubated at 37^{0} with 10^{-3} M DIHPA for 10 minutes. Control tubes without the inhibitor were also included. The reaction was initiated by adding the substrate. The result is shown in fig. 17. The usual progress curve was obtained in the absence of inhibitor but no activity at all could be

demonstrated in its presence.

Preincubation in the presence of adrenaline. The complete system except S-Ame was preincubated as above with DIHPA and control tubes were included. The reaction was initiated by adding S-Ame. In fig. 18 it can be seen that the enzyme had been practically quantitatively titrated as in the previous experiment and negligible activity was noticeable.

Effect of increasing the S-Ame concentration. In this experiment the complete system except S-Ame was preincubated in the usual manner with 10^{-3} M DIHPA, for 10 minutes. The reaction was initiated by the addition of S-Ame in amounts calculated to bring its final concentrations to 1×10^{-4} M, 4×10^{-4} M, 7×10^{-4} M and 1×10^{-3} M. Control tubes (inhibitor absent) were included. The incubation was carried out for 30 minutes. In fig. 19 it can be seen that no reversal of inhibition is possible even at optimal concentrations for the methyl donor. It appears again that DIHPA does not compete for the (hypothetical) active site for S-Ame.

Effect of increasing the substrate concentration. The possibility of excess substrate reversing the inhibition

was tested in this experiment. The complete system except substrate was preincubated in the presence of 10^{-3} M DIMFA for 10 minutes. Control tubes (inhibitor absent) were included. The reaction was initiated by the addition of adrenaline in amounts calculated to produce concentrations of $3x10^{-4}$ M (the standard concentration used in the previous experiments), $1x10^{-3}$ M, $3x10^{-3}$ M, $7x10^{-3}$ M and $1x10^{-2}$ M. The incubation was continued for another 30 minutes. (beyond concentration of 10^{-2} M substrate inhibition becomes noticeable). Fig. 20 illustrates the results. For some reason a small residual activity (about 6% of the control) was present after the usual 10 minutes incubation. As can be seen a gradual reversal of inhibition occurs with increasing amounts of substrate. The optimal reversal (69% of control activity recovered) occurs with a $7x10^{-3}$ M concentration of substrate. Further increase in substrate concentration does not promote the reversal of inhibition to any significant extent.

The experiment suggests that, to a considerable extent, no reversible change is suffered by the enzyme due to the action of the inhibitor, since most of its activity is recovered by excess substrate.

Plot according to ACKERMAN and POTTER (1949). Regarding

inhibition the words "reversible" and "irreversible" are not clearly defined. ACKERMAN and POTTER (1949) regard an irreversible reaction as one in which "for all practical purposes the enzyme is effectively titrated or stoichiometrically combined with a definite amount of inhibitor". They, further, define as a "truly irreversible reaction" the one in which "the enzyme is converted to a form which cannot be converted back into active enzyme". DIXON and WEBB (1958) recognize these two alternative meanings assigned to irreversible inhibition. To them, an irreversible inhibition is characterized by a progressive increase with time and also inability to recover the activity by other means such as dialysis. It appears that DIHPA is an "irreversible" inhibitor in the first sense used by ACKERMAN and POTTER. DIHPA simply titrates the enzyme progressively with time, but most of the activity (70%) can be recovered by simply adding excess substrate. DIHPA then cannot be considered an "irreversible" inhibitor in the sense defined by DIXON and WEBB.

According to ACKERMAN and POTTER (1949) this type of inhibition can be recognized from the reversible type if the rate of the reaction is plotted versus amount of enzyme. The plot gives a straight line passing through the origin in the absence of the inhibitor. A

parallel line is obtained in the presence of the inhibitor, which intersects the abscissa. Such a plot was obtained in the absence and the presence of 10^{-3} M DIHPA (fig. 21). The enzyme solution contained 3 mg of enzyme per 0.1 ml. Incubation 5 minutes. This "formal" experiment established the type of inhibition.

Discussion. The experiments just described demonstrate that DIHPA is capable of quantitatively titrating COMT in the absence or the presence of the substrate or the methyl donor, with or without preincubation. The inhibition can be reversed up to 70 % by excess substrate but not S-Ame. This indicates that the inhibitor binds the enzyme at the active site involved in the activation of adrenaline. It is interesting to note that whereas the alanine side chain in DIT does not support inhibition the pyruvic acid chain converts the molecule into a titrating inhibitor. It may be significant in this respect the discovery that DIHPA is present in rat thyroid glands (HANDY and LISSITZKY (1962) and that DIT is enzymatically transaminated by rat kidney mitochondria (HAYANO et al. 1963a). An integrated discussion on these findings shall be reserved for the general discussion at the end of this Thesis.

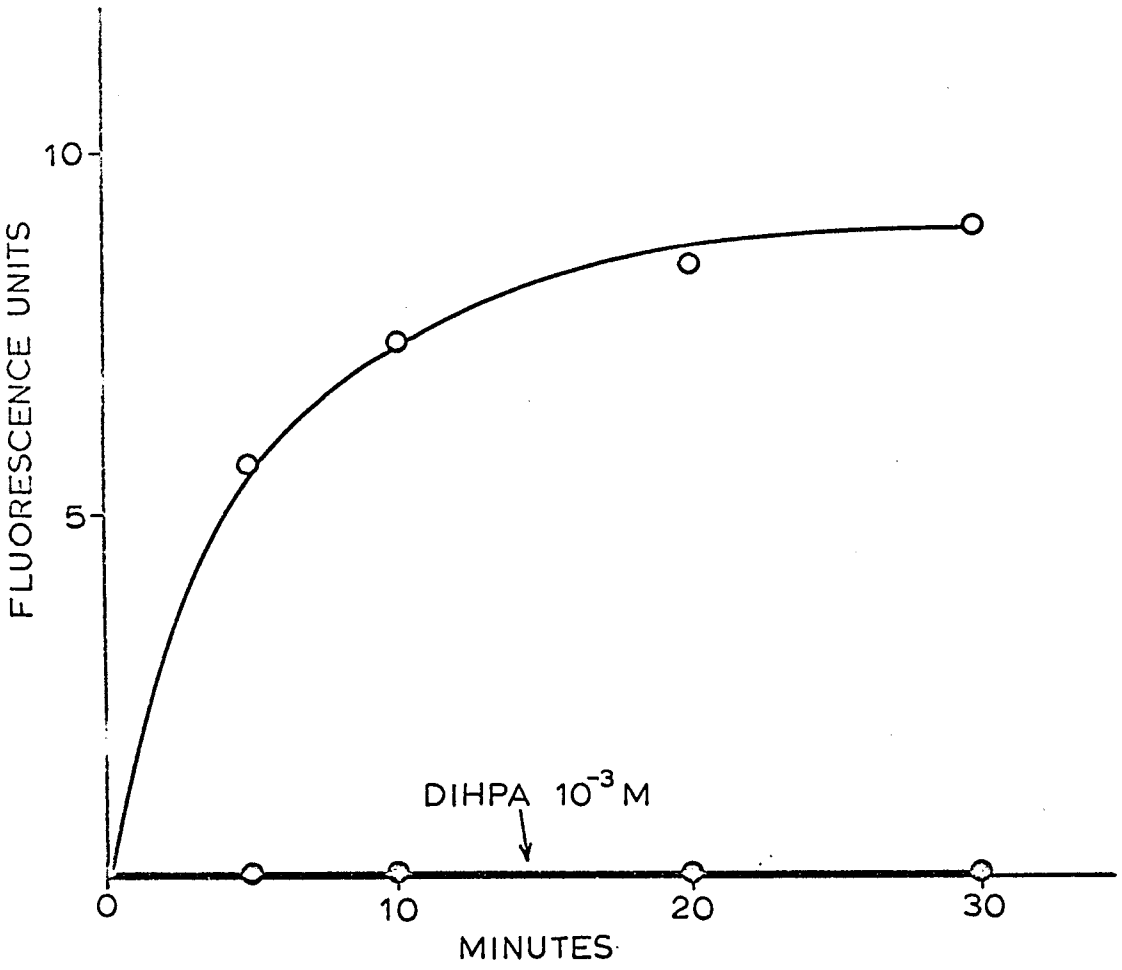


Fig.I7. Preincubation for IO minutes of COMT with DIHPA in the absence of adrenaline. The usual progress curve is obtained when the enzyme is preincubated in the absence of both adrenaline and DIHPA.

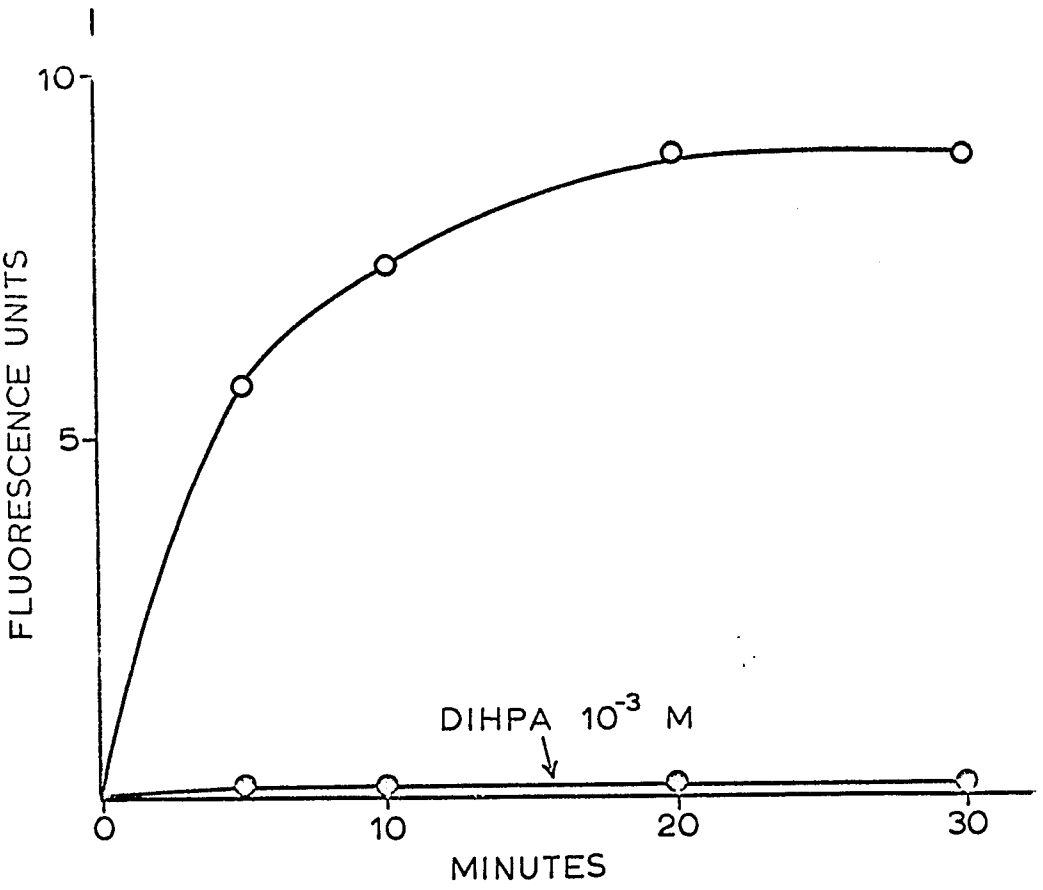


Fig.I8. Preincubation of COMT for IO minutes With DIHPA in the presence of adrenaline. The upper curve is the usual progress curve and was obtained after preicubation of COMT in the absence of both adrenaline and DIHPA.

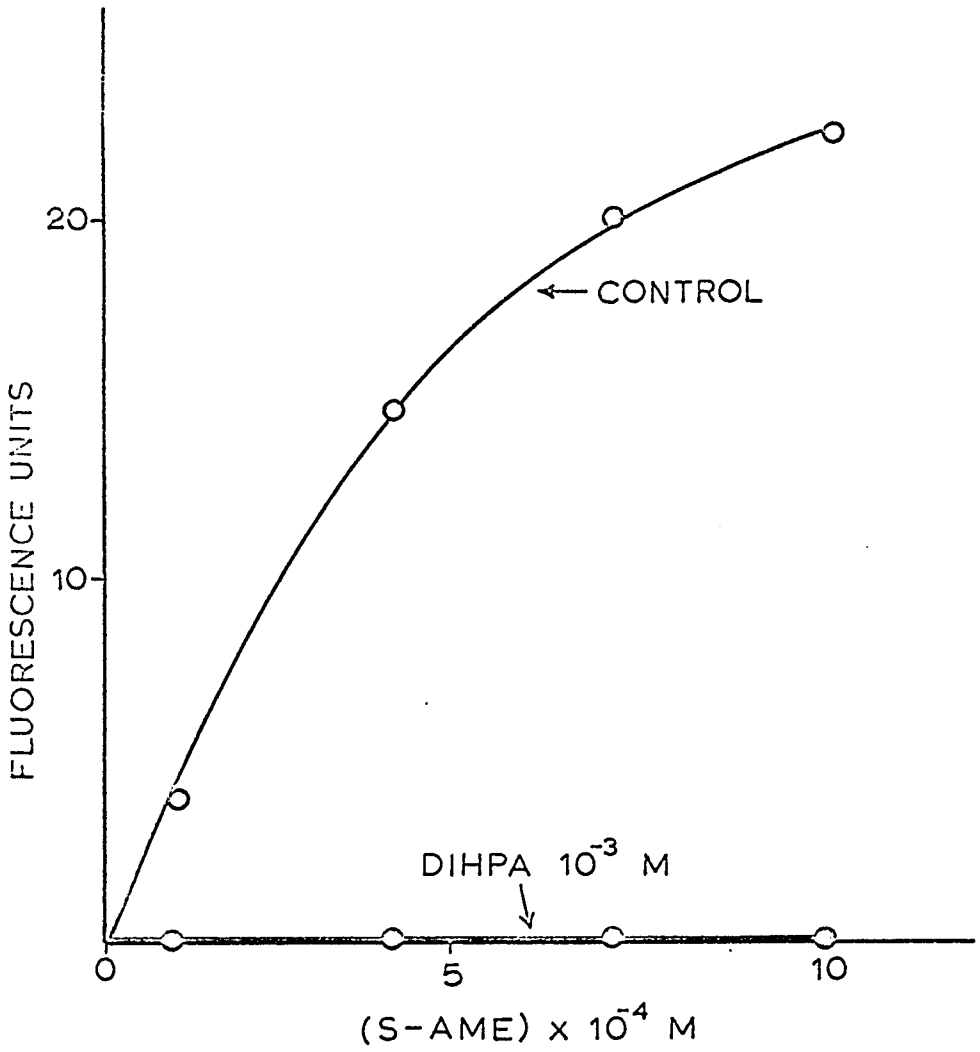


Fig.I9. Preicubation of COMT with 10^{-3}M DIHPA for 10 minutes. No reversal of inhibition was obtained by increasing amounts of S-Ame. Incubation 30 minutes.

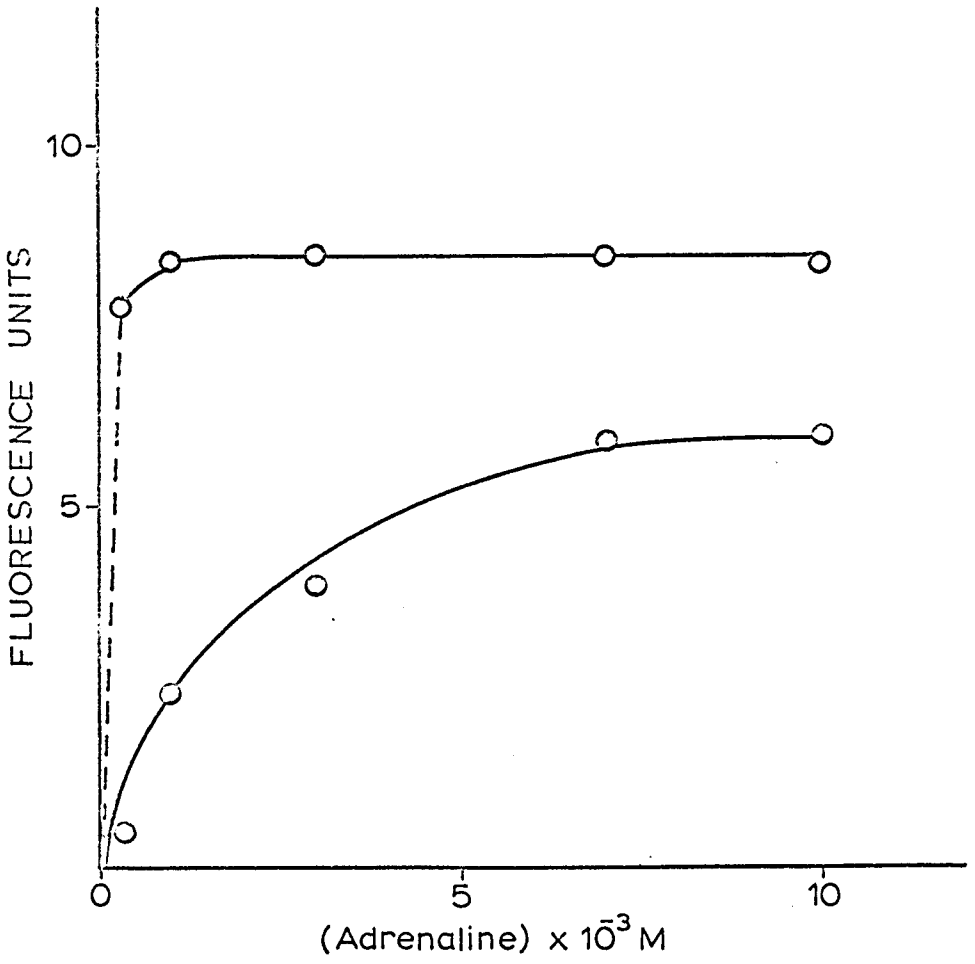


Fig.20. Reversal of DIHPA inhibition by excess adrenaline. Preincubation with 10^{-3}M DIHPA for 10 minutes. The reaction was initiated by the addition of adrenaline and the incubation was continued for another 30 minutes. Upper curve, activity in the absence of DIHPA. Lower curve, activity in the presence of DIHPA.

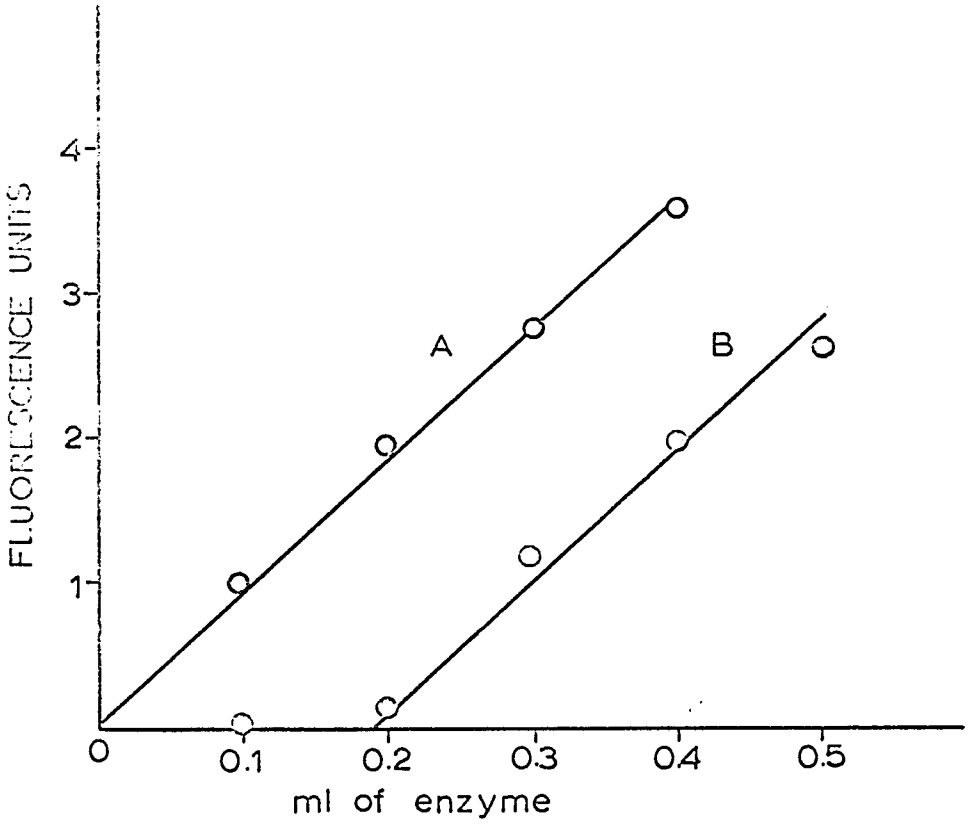


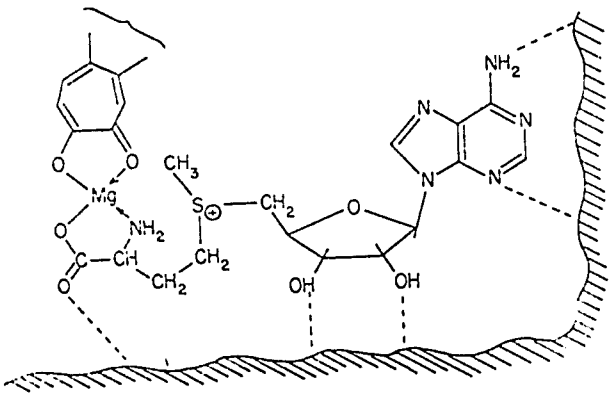
Fig.2I. Plot according to ACKERMANN and POTTER (1949). Line A in the absence of DIHPA. Curve B in the presence of 10^{-3}M DIHPA.

S E C T I O N VI

EXPERIMENTS IN VITRO WITH 4-METHYLTROPOLONE

While the present investigation was being carried out, interest arose in a new class of COMT inhibitors. BELLEAU and BURBA (1961) reported that tropolones and particularly 4-methyltropolone (see Addendum) are potent noncompetitive inhibitors of COMT in vitro. The latter claim regarding the type of inhibition was withdrawn later (BELLEAU and BURBA 1963) as a result of the work described in the present section.

Proposed mechanism of inhibition (BELLEAU and BURBA 1963). A mechanism of inhibition was proposed by BELLEAU and BURBA whereby tropolones are thought to act by virtue of their magnesium chelating properties. The assumed complex forms in such a way as to eliminate specifically magnesium and S-Ame. The binding of tropolone with the enzyme has no specific character since it presumably involves widely different side chains in the tropolone molecule.



Complex I

In the above complex it is obvious that the magnesium chelating properties of tropolones serve not the binding of the enzyme but the elimination of S-Ame. Since magnesium is present in the medium in large excess it is evident that the limiting factor could be either the S-Ame or the enzyme or both. MELLMAN and BURRA (1963) found that a five-fold increase in the magnesium concentration (already in excess in the medium) does not reverse the inhibition caused by 4-methyltropolone. This experiment is invoked as a proof of the existence and operation of the proposed complex in which, however, the active site of the enzyme is masked by the

tropolone in an unspecified way. It appears that the primary function of tropolones according to (I) is its ability to bind the enzyme at its active sites - but this is not related to its metal-chelating properties. It is difficult to visualize how an excess of magnesium would reverse or affect in any way the inhibition produced by such a complex in which, the actual process of enzyme-binding does not involve magnesium. As a matter of fact no complexation through magnesium between tropolone and S-Ame need be postulated if tropolone is also claimed to occupy and eliminate directly the active site in some other way not involving magnesium. If such a complex in its entirety were necessary for inhibition, lack of effect on addition of excess magnesium (which is always present in relative excess) cannot constitute the proof of its existence and operation.

The following experiments were performed with 4-methyltropolone and the usual partially purified enzyme preparation.

Time course. In fig. 22 the rate of the reaction is followed in the absence and presence of 10^{-3}M 4-methyltropolone. The tropolone was added dissolved in 0.1 ml of water.

Type of inhibition and inhibition constant. In fig. 23a it is shown that 4-methyltropolone is a competitive inhibitor of COMT. The Ki is calculated to be equal to 1.7×10^{-5} M.

In fig. 23b the inhibitor (7.5×10^{-5} M) was pre-incubated with the enzyme at 37° for 10 minutes and the reaction was initiated by addition of the substrate. The S-Ame was present at 7.5×10^{-4} M concentration. It is seen that the inhibition is again of the competitive type. The Ki is calculated to be equal to 1.5×10^{-5} M. The experiments demonstrate the competitive type of inhibition. The inhibition is not affected quantitatively or qualitatively by preincubation.

Effect of excess S-Ame on the inhibition. Fig. 24 illustrates this experiment. It is seen that the optimal S-Ame concentration is about 10^{-3} M. 4-methyltropolone was present at 10^{-4} M. Some reduction in the rate of the reaction at high concentrations may be due to impurities present in S-Ame. The main point here is (as in the inhibition with DUBA) that excess S-Ame does not reverse the inhibition. This does not favor the implication borne out by the type of complex proposed by BELLEAU and DUBA that elimination of S-Ame occurs.

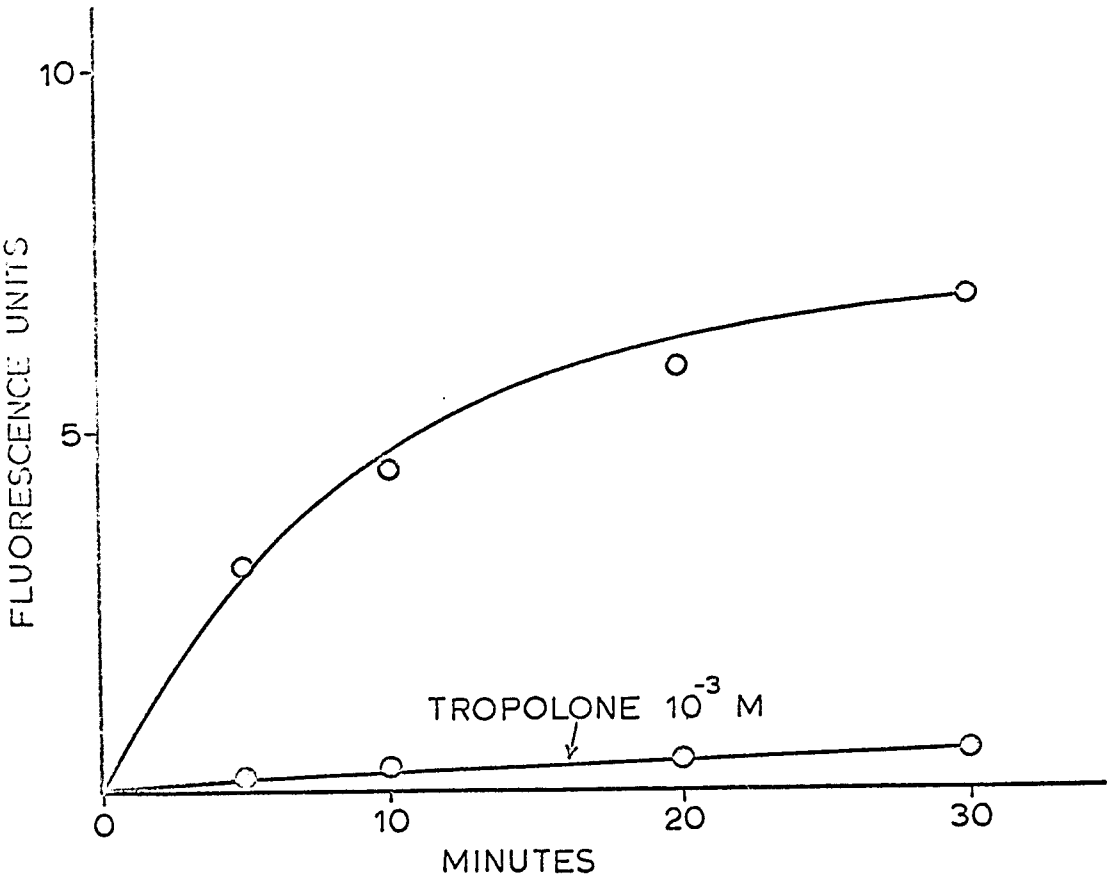
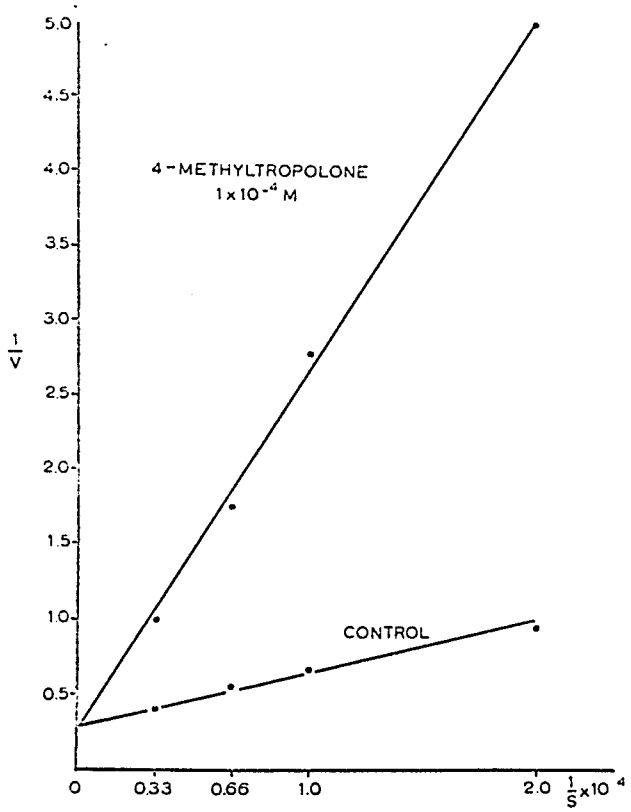


Fig.22. Inhibition of COMT in the presence of 10^{-3}M 4-methyl-tropolone. Upper curve, control.



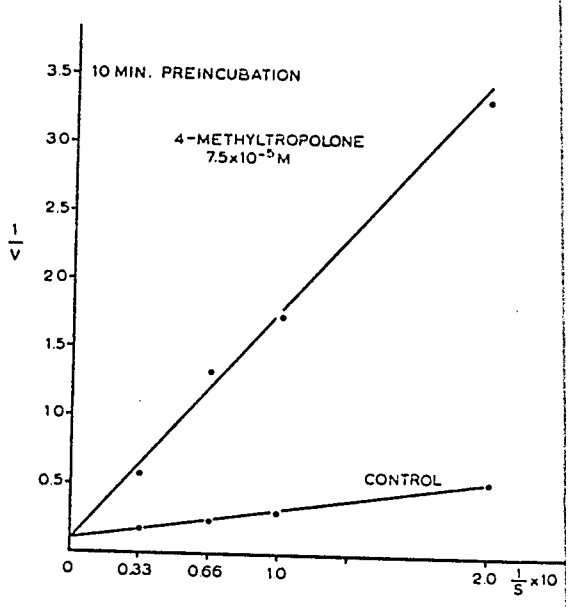


Fig.23a.(left). Lineweaver and Burk plot in the presence and absence of 4-methyl tropolone. No preincubation.

Fig.23b (right). Lineweaver and Burk plot as in 23a but after preincubation of the enzyme with tropolone for 10 minutes.

Effect of excess substrate on the inhibition. In this experiment the enzyme was preincubated with 4-methyltropolone present at 10^{-4} M concentration for 10 minutes. Increasing amounts of substrate were added and the incubation was continued for 5 minutes. The result is illustrated in fig. 25. It is seen that the inhibition is reversed from 80% to 46%. In another similar experiment in which the tropolone was not preincubated with the enzyme the inhibition was reversed from 75% to 23% under, otherwise, similar conditions. These experiments present evidence, in agreement with the other kinetic data indicating competitive inhibition, that tropolone competes for the active site with the substrate and that the enzyme-tropolone complex can be reversed *in* its greatest part by excess substrate.

Lack of effect of Kojic acid (5-hydroxy-2-hydroxymethyl-4-pyrone). This substance has a six-membered ring and a structure strikingly analogous to tropolones. Furthermore it shares the metal-chelating properties of the latter (BRANT and [illegible] 1954). More specifically, it is also a magnesium chelating agent (OKAC and KOLARIC 1960; MURAKAMI 1962). If tropolones indeed act by virtue of their magnesium chelating properties kojic acid should be expected to act as an inhibitor of COMT. This possib-

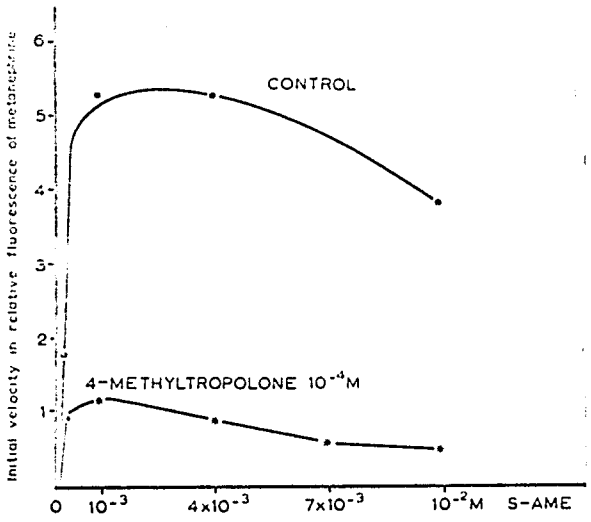


Fig.24

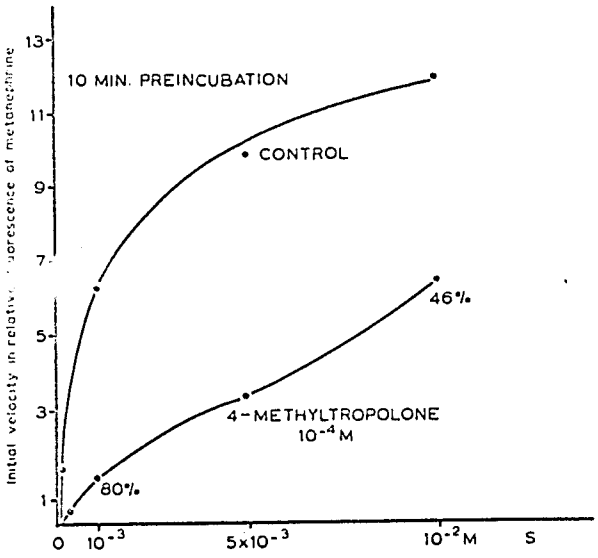


Fig. 25

Fig.25. Rate of metanephrine production with increasing adrenaline concentration. In the control curve the enzyme was preincubated for IO minutes before the addition of substrate. In the lower curve the enzyme was preincubated for IO minutes with the inhibitor. The final concentration of S-adenosyl methionine was $IO^{-3}M$.

Fig.24. Rate of metanephrine production with increasing concentration of S-adenosyl methionine. The concentration of adrenaline was $3XIO^{-4}M$.

ility was tested and in fig. 26 it is shown that kojic acid present at 10^{-4} M concentration does not exert the slightest effect on the activity of the enzyme.

The conditions for this experiment were:

Adrenaline bitartrate	3×10^{-4} M
Magnesium chloride	1×10^{-2} M
Phosphate buffer	5×10^{-2} M
3-AMC	1×10^{-4} M
Enzyme	10 mg

Discussion. 4-methyltropolone was shown in these experiments to be competitive inhibitor of DOPA. No convincing experimental evidence has been produced to support the claim that tropolones act by virtue of their magnesium chelating properties in the proposed complex (I). This claim is invalidated by the lack of inhibitory properties on the part of kojic acid. It appears that the tropolone inhibition is due to subtler and as yet unrecognized features in both the molecule of tropolones and in the enzyme. Our kinetic data indicate that 4-methyltropolone and adrenaline compete for the same active site on the enzyme.

The proposed mechanism of inhibition (MELLEAU and BURBA 1963) is based on the hypothetical complex proposed by SEMON et al. (1959), in which the place of

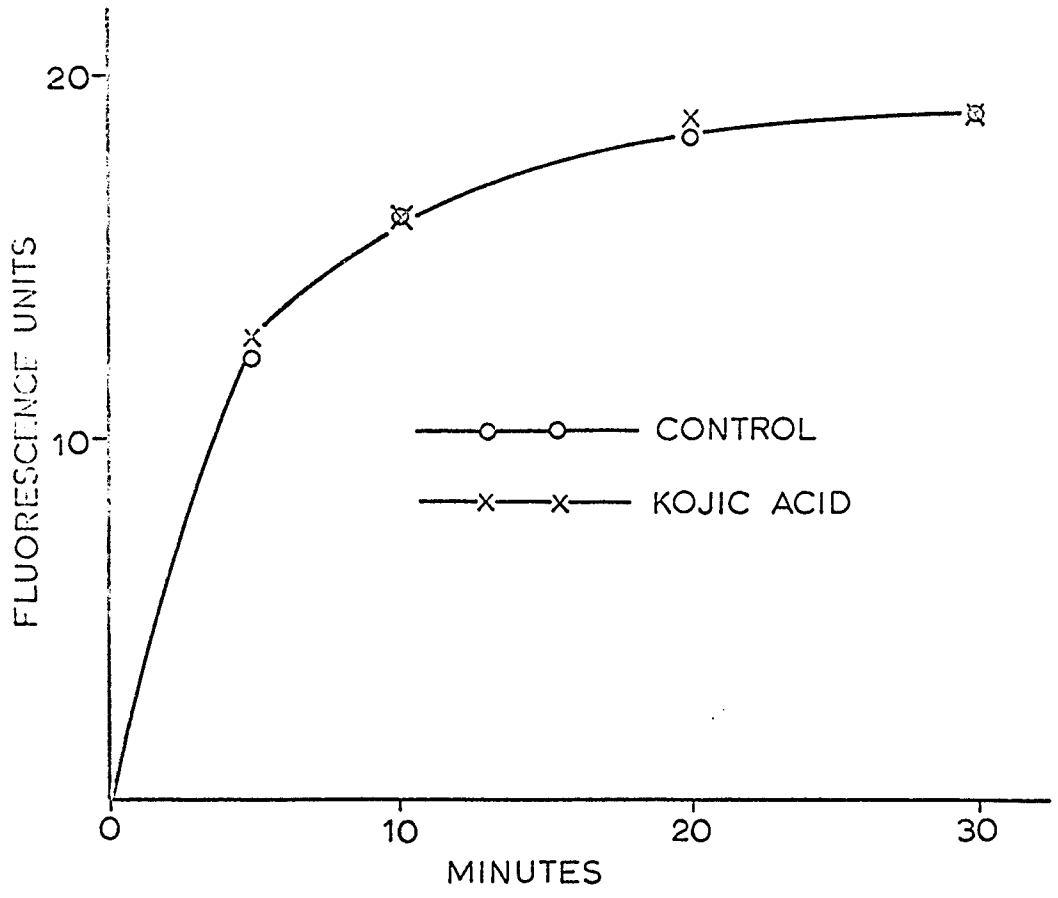


Fig.26. Progress curve demonstrating that kojic acid present at 10^{-4}M concetration does not inhibit COMT.

tropolone is occupied by the catechol in (I). Both groups of investigators refer to the same information source (MAIMAR 1956) to support the view that such a magnesium involving complex is possible. A closer examination of the relevant chapter in the aforementioned reference reveals that the metal chelating properties of catechol are mentioned with respect to copper only. Chelates with Fe^{+++} are also mentioned (p.231). Furthermore in p. 25 of the same reference the specific statement is made that catechol forms stable complexes with heavy metals. Obviously, magnesium is not a heavy metal. It becomes evident from the above that since magnesium chelates of catechols are not established, the magnesium complex proposed by SIMON et al. (who carefully state that it is hypothetical) cannot serve as a sound base to construct a reaction mechanism or a mechanism of inhibition.

S E C T I O N VII

CALCULATION OF Ks FOR ADRENALINE

In the experiments described in the preceding sections it was noted that the Km values for adrenaline varied with the amount of S-Ame present in the mixture.

In the Michaelis theory of enzyme action it is assumed that the equilibrium betweem enzyme E and substrate S is attained so rapidly thatthe enzyme-substrate complex ES remains in equilibrium with E and S while the complex breaks down to products P and enzyme:

$$E + S \rightleftharpoons ES \qquad (1)$$

$$ES(+\text{any second reactant}) \rightarrow P+E \qquad (2)$$

The dissociation constant Ks of ES into E and S is equal to the substrate concentration (S) at half maximum velocity V/2 where V = maximum velocity. The experimentally found value of (S) which gives the half maximum velocity is called the Michaelis constant Km. Km equals Ks if the assumption that equilibrium exists between ES and E + S, obtains during the reaction. Frequently this assumption is not satisfied. In such cases the BRIGGS -HALDANE theory postulates that the rate of formation and the

rate of breakdown of ES are essentially equal so that the concentration of ES can be considered constant over a short period of time:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (3)$$

where k_1, k_2 and k_3 are velocity constants.

In such cases the experimentally determined Km is not an equilibrium constant but it includes a kinetic element and is shown that (DIXON and WEBB 1958):

$$Km = \frac{k_2+k_3}{k_1} = Ks+\frac{k_3}{k_1} \qquad (4)$$

From the above relation it is concluded that Km=Ks when k_3 is very small compared to k_1 and the BRIGGS-HALDANE treatment is reduced to the MICHAELIS treatment. Otherwise Km is greater than Ks.

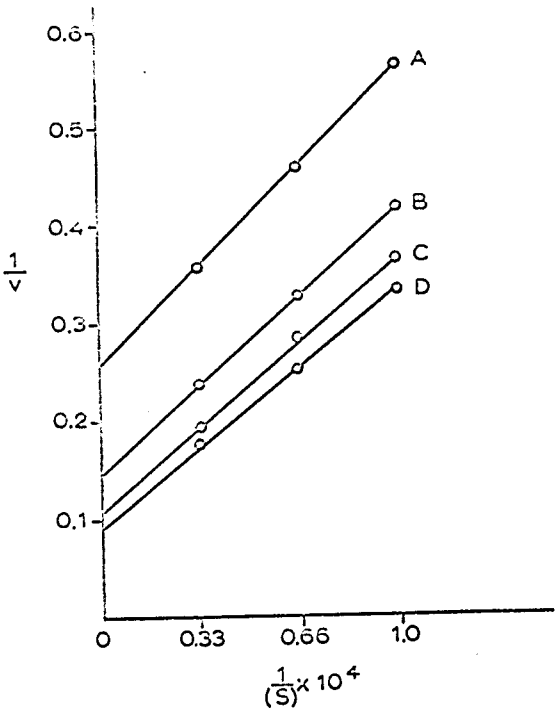
In reactions which involve two substrates A and B, where there is the possibility of varying k_3 by varying the second substrate B, this affords a method for determining the Ks for substrate A. SLATER and BONNER (1952) suggested that if Km is plotted against V a straight line is obtained which intersects the Km axis at a point representing the Ks. The equation is the following (DIXON and WEBB 1958, p. 104):

$$Km = Ks+\frac{V}{k_1 e} \qquad (5)$$

where e = enzyme concentration.

The variations of Km for adrenaline mentioned in the beginning are in agreement with the theory as outlined above, the two reactants being here adrenaline and S-Ame. It was decided to attempt the determination of Ks for adrenaline. A set of Km values and the corresponding maximum velocities were first required and to this end the experiment illustrated in fig. 27 was performed. The concentration of S-Ame ranged from 10^{-4} M to 10^{-3} M and as is seen in the figure a set of four nearly parallel lines was obtained. The Km values ranged from 1.25×10^{-4} M to 2.70×10^{-4} M (see detailed legend in fig. 27). This corresponds to slightly more than a two-fold increase in Km for a 10-fold increase in the S-Ame. The substrate (adrenaline) concentration was kept at 3×10^{-4} M. When these Km values were plotted against maximum velocities (calculated from fig. 27) the plot illustrated in fig. 28 was obtained. Extrapolation to zero maximum velocity gives Ks = 0.5×10^{-4} M.

CROUT (1961) has reported a Km value of 4×10^{-5} M for S-Ame. Preliminary experiments established that true initial velocities for low S-Ame concentrations could be obtained if the reaction was carried out for only one minute, when adrenaline was present at concentrations of 1×10^{-3} M and 5×10^{-3} M. Fig. 29 shows two double reciprocal



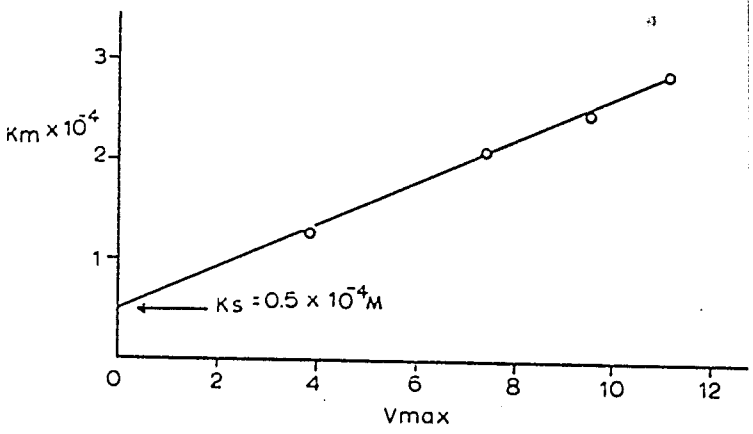


Fig.27(left). Lineweaver and Burk plots for adrenaline at four S-Ame concentrations, namely, $1X10^{-4}M$, $3X10^{-4}M$, $6X10^{-4}M$, and $1X10^{-3}M$ for lines A,B,C and D respectively. The respective Km values calculated from above plot are: $1.25X10^{-4}M$, $2.10X10^{-4}M$, $2.47X10^{-4}M$ and $2.70X10^{-4}M$.

Fig.28(right). Km values from fig. 27, plotted versus maximum velocity. The intersect at the vertical axis gives the substrate constant for adrenaline: $Ks=0.5X10^{-4}M$.

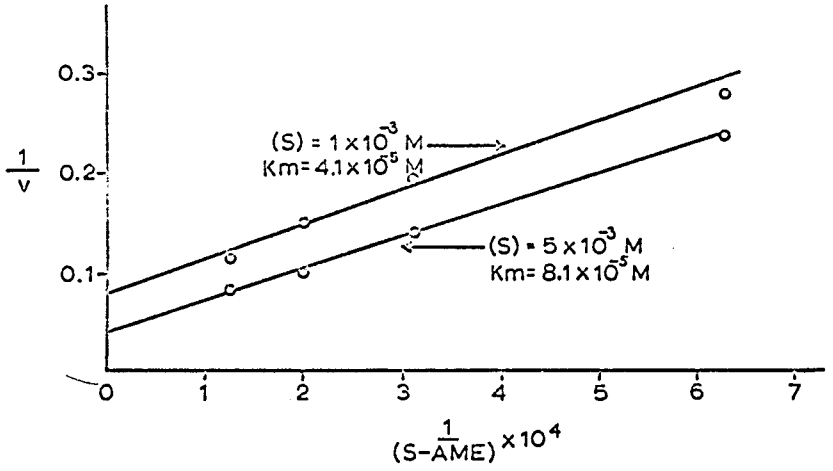


Fig.29. Lineweaver and Burk plots for S-adenosyl methionine at two adrenaline(S) concentrations, 1×10^{-3}M and 5×10^{-3}M. The respective Km values are found to be 4.1×10^{-5}M and 8.1×10^{-5}M. Velocity is expressed in fluorescence units produced in the first minute of incubation.

plots for S-Ame at the adrenaline concentrations just mentioned. The value $K_M = 4.1 \times 10^{-5}$ M is practically identical with that reported by CROUT (above).

Discussion. These experiments demonstrate that the Km values for adrenaline obtained under the usual experimental conditions are fairly representative of the enzyme-adrenaline affinity since their differences from the Ks are not very great.

It was shown in preceding sections that the inhibition caused by the two classes of true COMT inhibitors iodophenols and tropolones is competitive with respect to adrenaline and could be reversed by the latter. This indicates that an enzyme-adrenaline complex does form. The inhibition could not be reversed by excess S-Ame. This negative finding does not necessarily exclude the possibility of an enzyme-(S-Ame) complex. The hypothetical complex proposed by SENOH et al. (1959) favors the notion that no specific site on the enzyme is occupied by S-Ame. This complex is thought to form through chelation of magnesium resulting in a catechol-Mg-(S-Ame), complex. The complex is favored by some work on the metal ion requirements of COMT. However, it should be remembered that S-Ame serves as the methyl group donor in a great number of biological transmethylation reactions

besides the O-methylation of catechols. These include the methylation of nicotinamide (CANTONI 1951a), the methylation of guanidoacetate to creatine (CANTONI and VIGNOS 1954, 1955), the methylation of purines (REMY and SMITH 1959) and pyrimidines (REMY 1962), the formation of adrenaline from noradrenaline (KIRSHNER and GOODALL 1957), the formation of melatonin from acetylserotonin (LERNER et al. 1960; AXELROD and WEISSBACH 1960) and the formation of phasphatidyl choline (BREMER and GREENBERG 1961a). The methylation of 2,3-dimercaptoethanol (BAL) by S-Ame has also been demonstrated (BREMER and GREENBERG 1961b). To these the methylation of iodophenols should be added (TOMITA 1962; TOMITA and CHA 1963). Some of these (such as the last one) do not even require magnesium. This fact makes the hypothetical complex of SEKOH et al. (1959) somewhat questionable. On the other hand if a specific site for S-Ame is a prerequisite for methylation one wonders if such an identical site is present in all methylating enzymes. In conclusion, however, our data and the data of others are not sufficient to postulate mechanisms of action for COMT. Much higher purification and more detailed kinetic experiments are required.

S E C T I O N VIII

EXPERIMENTS WITH THYROID HORMONE ANALOGUES

Methods. The partially purified preparation of COMT described in section III was used in these experiments also, and the conditions were kept the same. The compounds were added in the usual fashion dissolved in 0.1 ml of 0.1N NaOH and the activity was followed with time up to 30 minutes. Solubility problems were encountered with tetraiodothyropropionic acid and triiodothyronine. When these derivatives were added in a final concentration of 10^{-3} M, they were only partially dissolved, sufficiently however to produce inhibition.

Addition of thyroxine had no effect. It was found that in order to get thyroxine in solution at 3×10^{-4} M concentration the pH of the mixture had to be raised to 10.4. At this pH the enzyme is inactivated.

Results. The percentage inhibition at the specified molar concentrations of the inhibitors is shown in table 6. The inhibition is that produced after 5 minutes of incubation. As is seen, thyroxine itself

does not act as inhibitor of COMT. The experiments were not designed to evaluate the relative inhibitory powers of the compounds but merely to test whether they could act as inhibitors or not. However, even at this early exploratory stage it is clear that the degree of inhibition is augmented with increasing number of iodine atoms present in the molecule, starting from the diiodo-derivatives. More informative in this respect and others are the experiments described in the next section.

Iododerivatives	Concentration Molar	Percent Inhibition
Thyronine	10^{-3}	nil
3,5-Diiodothyronine	2.9×10^{-3}	32
3,5-Diiodothyroformic acid	10^{-3}	36
3,5-Diiodothyroacetic acid	10^{-3}	21
3,5-Diiodothyropropionic acid	10^{-3}	30
3,3',5-Triiodothyronine	Unknown	39
3,3',5-Triiodothyroformic acid	10^{-3}	51
3,3',5-Triiodothyroacetic acid	1.1×10^{-3}	39
3,3',5-Triiodothyropropionic acid	10^{-3}	46
Thyroxine	Unknown	nil
3,5,3',5'-Tetraiodothyroformic acid	10^{-3}	76
3,5,3',5'-Tetraiodothyroacetic acid	10^{-3}	70
3,5,3',5'-Tetraiodothyropropionic acid	Unknown	33

Table 6 - Inhibition produced by thyroxine analogues on COMT after 5 minutes of incubation.

S E C T I O N IX

TYPE OF INHIBITION AND INHIBITION CONSTANTS OF THE THYRONINE ANALOGUES

The double reciprocal plot was again used as in section IV. The points are the mean values of duplicates. At least two and as many as five separate plots were constructed for each substance. The agreement was always very good. For instance, for 3,5-diiodothyronine five plots were constructed over a period of several months and the Ki values calculated from these plots were: 2.3×10^{-3} M, 2.0×10^{-3} M, 2.1×10^{-3} M, 2.0×10^{-3} M and 2.1×10^{-3} M. Average Ki=2.1×10^{-3} M. The Ki values reported here are those calculated from plots which were obtained under optimal S-Ane concentration (10^{-3} M). However, for some of them, such as for diiodothyroformic acid, triiodothyroformic acid and tetraiodothyroformic acid, plots were constructed from experiments in which S-Ane was present in suboptimal concentration (10^{-4} M). The Ki values calculated from these plots were found to be practically identical with those found under optimal S-Ane conditions. This is in agreement

with the previously reported finding that the inhibition is independent of the S-Ame concentration. Two types of inhibition were encountered in these experiments: the strictly noncompetitive type and the mixed type (DIXON and WEBB 1958). The results are presented in detail below.

3,5-diiodothyronine. In fig. 30 the double reciprocal plot is given in the absence and presence of 3,5-diiodothyronine. The Ki calculated from this particular plot is equal to 2.0×10^{-3} M. The inhibition is of the mixed type.

3,5-diiodothyroformic acid. Fig. 31 shows a double reciprocal plot in the absence and presence of this substance at 1.5×10^{-3} M concentration. The inhibition is of the strictly noncompetitive type. Ki = 1.3×10^{-3} M.

3,5-diiodothyroacetic acid. A plot similar to the one referred to above was obtained in the presence of this acetic acid derivative at 3×10^{-3} M concentration, indicating a strictly noncompetitive type of inhibition. Ki = 1.2×10^{-3} M.

3,5-diiodothyropropionic acid. This compound gave

initially a plot showing the presence of a competitive component to a small extent - i.e., as if the inhibition was of the mixed type. This was considered to be a rather suspect result since the two analogous compounds just described gave a noncompetitive type of inhibition. Contamination might be responsible for the effect. 100mg of the compound were dissolved with heating in 75ml of 30% ethanol.. The warm solution was filtered through a hard filter and allowed to cool at room temperature and further overnight in the cold room. The crystalline precipitate was filtered and washed with water and then dried over calcium chloride in a vacuum dessicator. When this purified material was tested at 3×10^{-3} M concentration, a perfect double reciprocal plot typical of noncompetitive inhibition, was obtained ($K_i = 1.3 \times 10^{-3}$ M.)

3,3',5-triiodothyroformic acid. A double reciprocal plot similar to that of figs. 30 and 32 was obtained with this derivative present at 10^{-3} M concentration. ($K_i = 7.5 \times 10^{-4}$ M).

3,3',5-triiodothyroacetic acid. The same as above type of inhibition was obtained in the presence of 1.1×10^{-3} M triiodothyroacetic acid. ($K_i = 11 \times 10^{-4}$ M).

3,3',5-triiodothyropropionic acid. The action of this acid at 10^{-3}M concentration gives a plot illustrated in fig. 32 (Ki = 8.5×10^{-4}M.

3,3',5-triiodothyronine. A mixed type of inhibition was obtained with this compound too, when present at 0.75×10^{-3}M concentration (Ki = 2.6×10^{-3}M).

3,3',5,5'-tetraiodo-thyroformic, thyroacetic and thyropropionic acids. Both thyroformic and thyroacetic derivatives were present at a concentration of 10^{-3}M while the concentration of the thyropropionic acid was 5×10^{-4}M. All three substances produced plots typical of a mixed type inhibition as in figs. 30and 32. Their Ki values in the above-mentioned order are: 1.9×10^{-4}M, 2.3×10^{-4}M and 3.8×10^{-4}M.

The results are summarized in Table 7.

Discussion. A definite and instructive pattern emerges from the experimental data just presented. Thyronine does not act on COMT. However, 3,5-diiodothyronine as well as the 3,5-diiododerivatives bearing the formic, acetic and propionic acid residues inhibit the enzyme. This can only mean that the introduction of the two iodine atoms at positions 3 and 5 impart to the molecule

inhibitory properties. The presence of the ether bridge oxygen in ortho position is most probably of significance here. Furthermore this arrangement produces a non-competitive inhibition, with one exception: 3,5-diiodothyronine exerts a mixed type of inhibition. With the data at hand this discrepancy cannot be rationalized. In section III it was concluded that the alanine side chain in 3,5-diiodotyrosine could not serve as a supporting function for the display of inhibitory properties. In the case of 3,5-diiodothyronine the alanine side chain seems to be able to modify the type of inhibition. Furthermore, it suppresses the inhibiting capacity of the molecule as is evidenced by its higher inhibition constant as compared to the inhibition constants of the other three diiododerivatives. This same effect is witnessed in the higher Ki value for triiodothyronine as compared to those of the other three triiododerivatives. It appears then, that whereas in the one-ring system (iodophenols)the alanine side chain does not function as far as inhibition is concerned, its presence in the thyronine molecule antagonizes the inhibitory properties established by the presence of the iodine atoms at positions 3 and 5. However, this is merely recognizing its effect and no explanation can be offered.

Addition of a third iodine atom at position

Compounds Tested	Type of Inhibition	Ki M
3,5-diiodothyronine	mixed type	2.1×10^{-3}
3,5-diiodothyroformic acid	noncompetitive	1.3×10^{-3}
3,5-diiodothyroacetic acid	"	1.3×10^{-3}
3,5-diiodothyropropionic acid	"	1.3×10^{-3}
3,3',5-triiodothyronine	mixed type	2.6×10^{-3}
3,3',5-triiodothyroformic acid	" "	7.5×10^{-4}
3,3',5-triiodothyroacetic acid	" "	11×10^{-4}
3,3',5-triiodothyropropionic acid	" "	8.5×10^{-4}
Thyroxine	no effect	-
3,5,3',5'-tetraiodothyroformic acid	mixed type	1.9×10^{-4}
3,5,3',5'-tetraiodothyroacetic acid	" "	2.3×10^{-4}
3,5,3',5'-tetraiodothyropropionic acid	" "	3.8×10^{-4}

Table 7 - Summary of the in vitro effects of the thyroxine analogues on COMT.

3' results in lower Ki values indicating higher affinities for the enzyme sites. A competitive component is now present as is seen in the double reciprocal plots given by all triiododerivatives. Both higher affinity and competitive component are introduced by the iodophenol function at positions 3' and 4' of the molecule. This is in agreement with the inhibition exhibited by the iodophenols. As supporting groups here seem to serve the formic, acetic and propionic acid side chains. From their Ki values it is clear that diiodothyronine and triiodothyronine are inhibitors of equal strength. This simply means that the alanine side chain does not support the 3',4'-iodophenol function in producing inhibition as the formic, acetic and propionic acid chains do in the corresponding triiododerivatives. This is in agreement with the lack of support noted previously in the molecule of 3,5-diiodotyrosine.

Introduction of a fourth iodine atom at position 5' results in even lower Ki values, i.e., even higher affinities and the inhibition is of the mixed type as in the triiododerivatives. This augmentation of inhibitory properties is in agreement with the superior inhibitory capacity of DIHDA with two iodine atoms in ortho positions to the hydroxyl, as compared to that of 5-iodovanillin with one only iodine atom.

A marginal difference exists in the inhibition constants of tetraiodothyroformic acid and tetraiodothyroacetic acid. However, the Ki value for tetraiodothyropropionic acid indicates a lower affinity of the enzyme for the derivative than for the other two, as a result of the length of the side chain.

A comparison of Ki values reveals that the affinity for the tetraiododerivatives is much lower than that for DIHBA (it is about equal to that for 5-iodovanillin and diiodosalicylic acid). This might mean that, besides unknown effects caused by the presence of the iodines at positions 3 and 5, the size of the molecule and the remoteness of the carboxylic functions in the thyronine derivatives result in a less efficient "fit" on the enzyme active sites. The same is true for the triiododerivatives as compared to 5-iodovanillin.

It was shown that the inhibition of DIHBA cannot be reversed by excess S-Ame and this was claimed to be conclusive evidence that the inhibition is not due to a complex between iodophenol methyltransferase* and S-Ame. Obviously this is sufficient argument speaking

*That such an enzyme exists has become known from two abstracts (TOMIDA 1962 and TOMIDA and Cha 1963). No detailed publication has appeared.

against such a possibility. However, more evidence is provided by the data with the thyroxine analogues. Should the inhibition be ascribed to such a complex one would expect competitive inhibition or a unique type of inhibition. In fact, competitive, noncompetitive, and mixed type of inhibition were established. This can only mean that the underlying mechanism is as variable as the types of inhibition. It should be emphasized at this point, that magnesium, which in any case is present in large excess, cannot become a limiting factor since it is not a requirement of iodophenol methyltransferase.

A comparison of the inhibition constants for the tetraiododerivatives (especially of those for tetraiodothyroformic and tetraiodothyroacetic acids) with the Ks for adrenaline reveals that eventhough the affinity of the enzyme is higher for adrenaline, it is however of the same order of magnitude as those for the inhibitors.

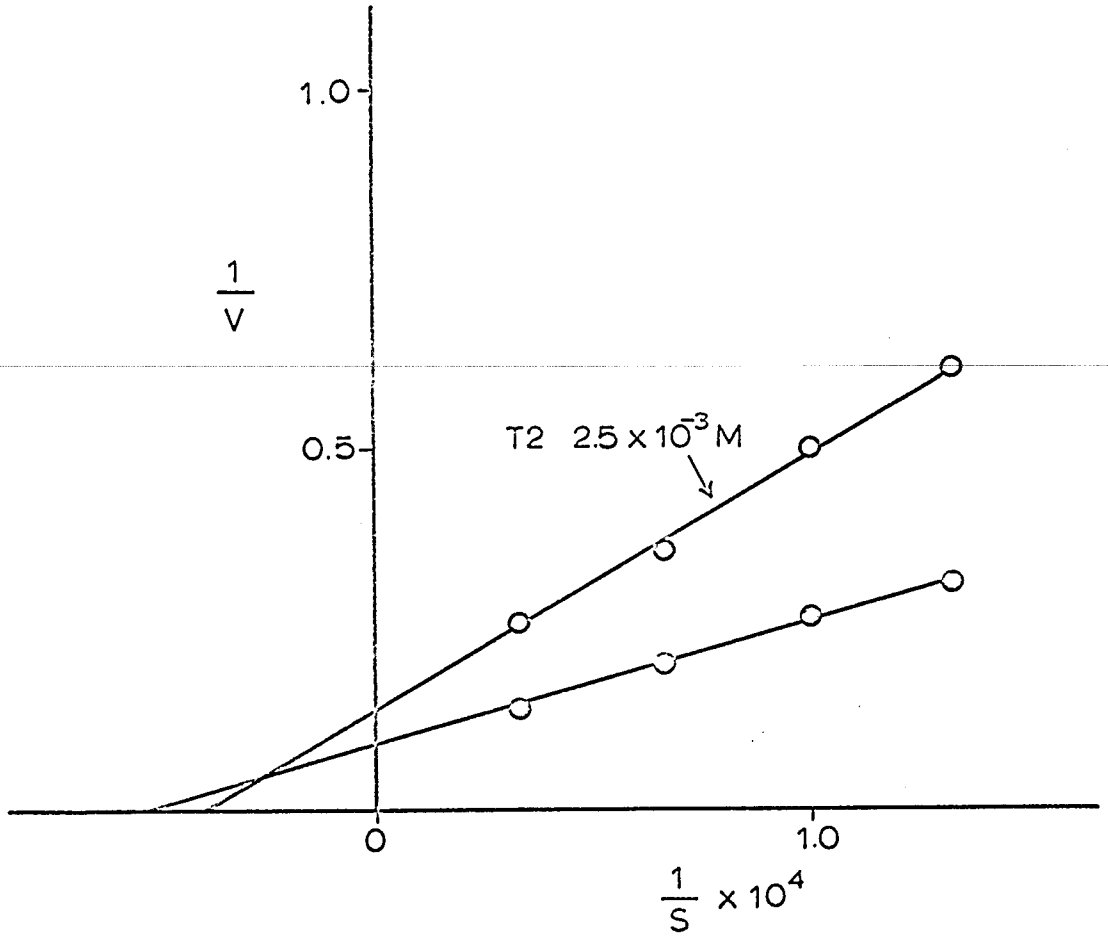


Fig.30. Lineweaver and Burk plot in the absence and presence of 3,5-diiodothyronine. The inhibition is of the mixed type.

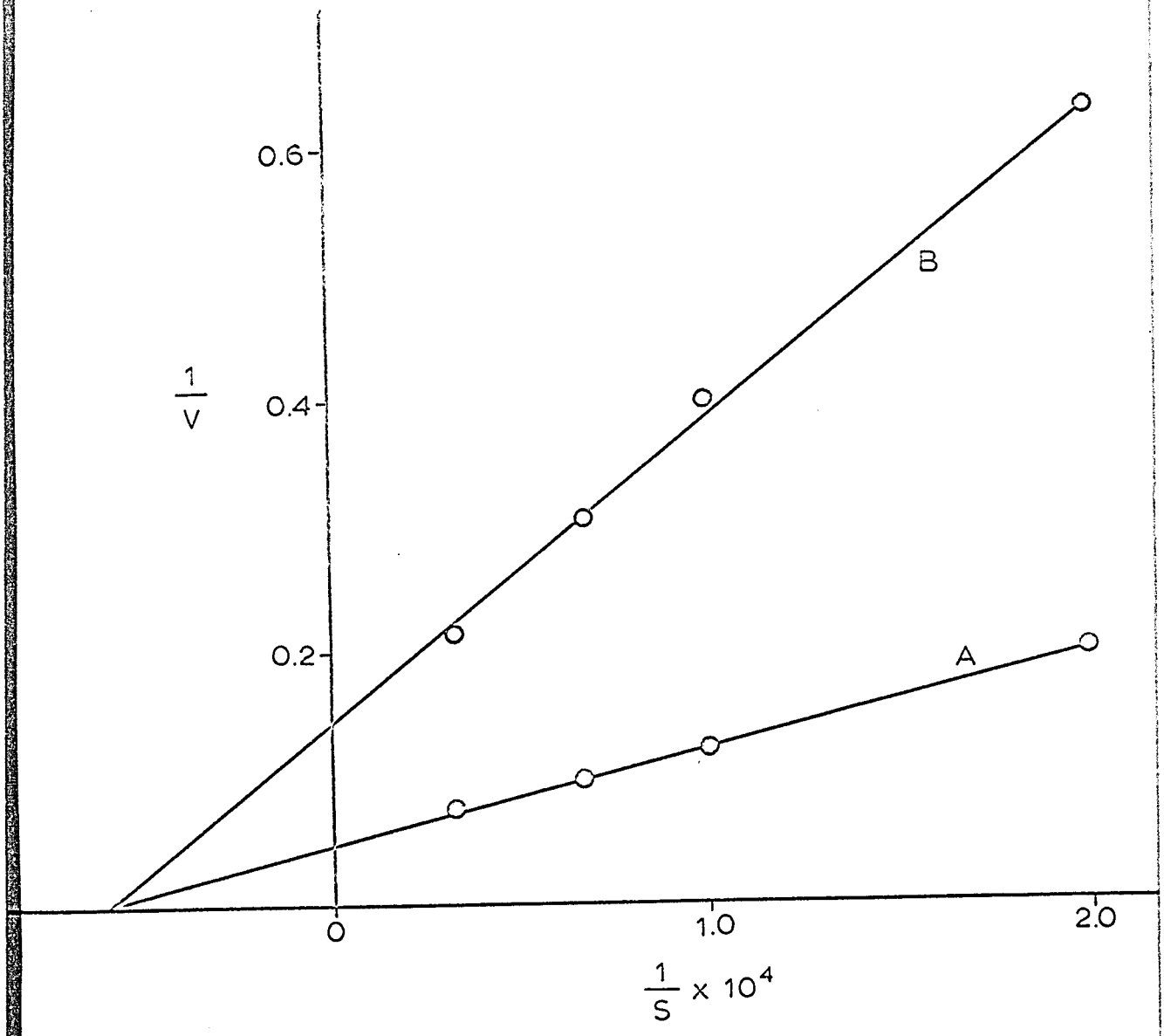


Fig.3I. Lineweaver and Burk plot in the absence(line A) and presence (line B) of 1.5×10^{-3}M 3,5-diiodothyroformic acid. The inhibition is strictly noncompetitive.

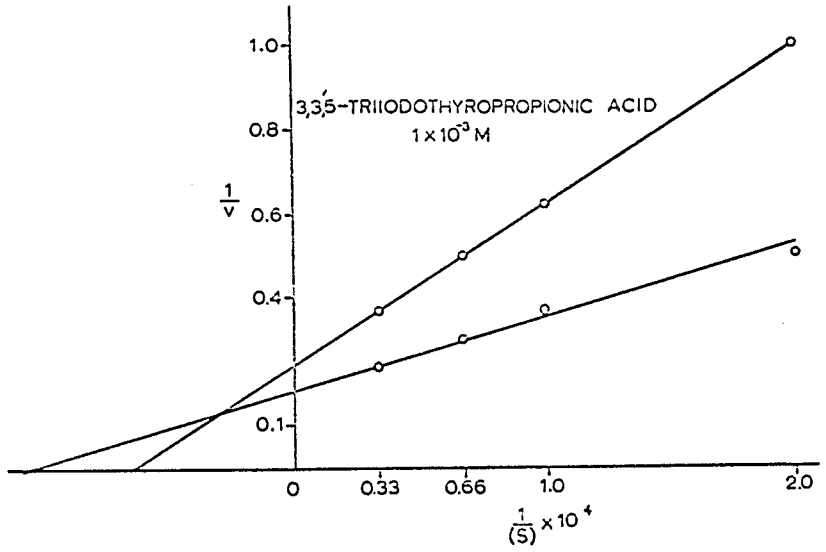


Fig.32. Lineweaver and Burk plot in the absence and presence of 3,3',5-triiodothyropropionic acid. Inhibition is of the mixed type.

S E C T I O N X

ATTEMPTED PURIFICATION OF COMT. - PURIFICATION WITH CALCIUM PHOSPHATE GEL

AXELROD and TOMCHICK (1958) have achieved a 30-fold purification of COMT by ammonium sulfate fractionation and calcium phosphate gel adsoption and selective elution. No further attempts were made to obtain higher purification (AXELROD 1962). However, only a highly purified enzyme would permit a deeper insight on specificity and reaction mechanism. The work described in the preceding sections led us to believe that such a purified enzyme was desirable and worthy of the time and effort usually invested in this type of work.

Although considerable time was devoted to this attempt, it is not considered that it has been exhaustive by any means. Since the attempt failed in its main objective only a sketchy account of it is given below. The purification by means of calcium phosphate gel is given in some detail since it deviates from the original method mentioned above.

Preliminary experiments consisted of the detailed study of ammonium sulfate fractionation. This confirmed that the bulk of enzymatic activity was collected in the protein fraction which precipitates on saturation of the supernatant fraction from 30 to 50% with ammonium sulfate. Gel filtration of this fraction with Sephadex G-25 did not result in protein resolution but it proved to be fast and effective in removing the ammonium sulfate from the protein component.

Sephadex G-50 was next used, of a particle size 100-200 mesh. The "fines", were removed with repeated washing with water. Considerable difficulties were encountered with this type of Sephadex. The rate of flow was low and columns higher than 30 cm were not practical. Application of pressure resulted in considerable contraction of the column and, consequently, this means of speeding up the rate of flow was abandoned. At any rate, the protein mixture of the 30-50% saturation fraction emerged from the column as a single peak on elution with water.

The rate of flow with columns made of Sephadex G-75 was even slower. No resolution was obtained with this type of gel either.

Calcium phosphate gel was used next for further purification and this preparation was passed through

DEAE - cellulose columns. The results were not promising and the effort was finally abandoned.

The net result of these experiments was the further purification of CONT with calcium phosphate gel according to the method of AXELROD and TOCHICK (1958). Since some modifications were introduced the method is described below in some detail.

Methods

Estimation of ammonium sulfate. Ammonium sulfate was estimated colorimetrically by the phenol-hypochlorite method as described by RUSSELL (1944). In alkaline solution, ammonia, phenol and hypochlorite produce an intense blue color which is believed to be indophenol or a related substance.

Reagents

Alkaline phenol reagent. 25% phenol in 2.7% NaOH.

Hypochlorite solution. This is prepared from calcium hypochlorite (bleaching powder) and sodium carbonate. The latter is added in excess so that the solution after removal of the calcium carbonate does not contain calcium ions.

Mangenous chloride 0.003M. (For maximum color development).

Procedure

1.5ml of the sample was placed in a test tube and one drop of mangenous chloride was added followed by one ml of cold phenol reagent and 0.5ml of hypochlorite solution. The mixture was placed for 5 minutes in a boiling water bath. After cooling the volume was made up to 6 ml with water and the color was read at 625mm in a BAUSCH and LOMB Spectronic 20 spectrophotometer.

Standard curve. A straight line was obtained on plotting optical density versus micrograms of ammonia nitrogen (from a standard solution of ammonium sulfate). The plot covered a range up to 6 micrograms and the optical density to 0.410.

Protein estimation. The FOLIN-LOWRY method was abandoned as time consuming. Moreover, phosphate buffer eluates from DEAE - cellulose columns gave a positive reaction even in the absence of protein. The UV absorption method of WARBURG and CHRISTIAN (1941) was employed as modified by LAYNE (1957). The optical density of the protein mixture was measured at 260mμ and 280 mμ in a BECKMAN DU spectrophotometer in square cuvettes of one cm light path.

The amount of protein in mg per ml was calculated by the following formula:

$$\text{Protein concentration (mg/ml)} = 1.55D_{280} - 0.76D_{260}$$

Preparation of phosphate gel. The gel was prepared as described by KEILIN and HARTREE (1938a) and quoted by COLOWICK (1955). 150 ml of a calcium chloride solution (67.8 gm of anhydrous calcium chloride per liter) was diluted to about 1600 ml with water. 150 ml of a sodium triphosphate solution (152 gm of $Na_3PO_4 \cdot 12H_2O$ per liter) was then added with stirring. The acid was brought to pH 7.4 with 1N acetic acid and the precipitate was divided into two equal parts. Each part was washed four times with 10 liters of water each time. Tap water is suggested in the method. However, use of tap water resulted in a brown-colored gel due to impurities contained in the water. Use of distilled water throughout the procedure corrected this anomaly. The precipitate was centrifuged and resuspended in water. Solids were determined by heating a small volume of the gel suspension in a watch-glass at 100° overnight. The gel used in the following experiments contained 25 mg of solids per ml.

Purification procedure

Preliminary experiments. As was mentioned previously

the protein fraction precipitating on 30-50% saturation with ammonium sulfate was found to give the highest specific activity. Prior to ammonium sulfate fractionation adjustment of the supernatant to pH 5.0 did not increase significantly the specific activity in terms of protein, however it removed a great deal of insoluble material.

The use of the conventional dialysis technique was replaced by the use of the gel filtration technique. Sephadex G-25 was found very satisfactory in this respect. A particle size of 100-200 mesh was employed. The gel was suspended in tap water in a volumetric cylinder and the "fines" were removed by repeated decantation and resuspension. The column was prepared according to the method of FLODIN (1961). The gel suspension in a 2 liter separatory funnel and under constant agitation with the aid of a glass-rod agitator driven by a small motor, was allowed to enter dropwise into a column 1.9 cm in diameter. The top of the column and the tip of the separatory funnel were connected with the aid of a perforated rubber stopper. A column 40 cm high was prepared in 5-6 hours. A circular filter paper was placed on top of the column which was further allowed to settle under overnight percolation with distilled water. All operations were carried out in the cold room. The column could be used

for a large number of times over a period of several weeks if care were taken to avoid its drying up.

When the protein-ammonium sulfate mixture was placed on such a column and elution with distilled water followed, two distinct symmetrical peaks were obtained. The fastest moving peak was the protein. It was rather closely followed by the ammonium sulfate peak. With the aid of an automatic fraction collector the protein was collected completely free of the salt. The entire procedure could be accomplished in 1½ hour. The use of Sephadex was a departure from the method of AXELROD and TOMCHICK.

The protein fraction freed of ammonium sulfate was then absorbed on calcium phosphate gel at pH 5.0. It was established that 95-98% of the protein was adsorbed with a gel: protein ratio of 2:1. It was further established that maximum specific activity was obtained by selectively eluting with sodium phosphate buffer 0.02M, pH 6.4. This is also a departure from the method of AXELROD and TOMCHICK in which pH 6.9 was employed. The discrepancy may be due to the fact that distilled water was used for the preparation of our calcium phosphate gel. It is here confirmed that enzyme purification methods cannot be followed blindly.

Procedure for enzyme purification. 68 gm of rat liver was homogenized with 4 volumes of isotonic KCl. The homogenate was centrifuged at 40,000 xg for one hour. The clear supernatant was adjusted to pH 5.0 with 1N acetic acid. The precipitate was removed by centrifugation and to 200 ml of the supernatant 35.2 gm of solid ammonium sulfate was added slowly under constant stirring with a magnetic stirrer (0-30 saturation). The precipitate obtained after centrifugation was discarded and to 208 ml of the supernatant 26.4 gm of ammonium sulfate was added as above (30-50 saturation). The new supernatant obtained after centrifugation was discarded and the precipitated protein was dissolved in 10 ml of distilled water and placed on a Sephadex G-25 column. The column was eluted with distilled water and the protein fractions were collected in an automatic fraction collector free of ammonium sulfate. The fractions were pooled. To 17.5 ml of the pool (43.75 mg protein per ml) 20 ml of water and 37 ml of 0.02M acetate buffer pH 5.0 were added. To this 62 ml of calcium phosphate gel (solids 25 mg per ml) was added and mixed thoroughly. The mixture was allowed to stand for 15 minutes with occasional stirring and then centrifuged. The supernatant was discarded and the gel was eluted twice with 10 ml portions of 0.02M phosphate buffer pH 6.4. The experiment is summarized in table 8. A

34-fold purification was obtained. The yield was poor as indicated in the table but it was later found that instead of two even twelve elutions from the phosphate gel could be performed. All these elutions were of comparable yield and specific activity. The combined eluates would raise the yield by a factor of 5 to 6. A difference to be mentioned is that AKILOV and TORCHICK achieved an almost 7-fold purification up to the ammonium sulfate stage and an additional 5-fold purification with the calcium phosphate gel, whereas we have not been able to achieve even a five-fold purification with ammonium sulfate. However, the difference is compensated by a superior (8-fold) purification with calcium phosphate gel alone.

Procedure	Volume ml	Activity units/ml	Total units	Protein mg/ml	Purity units/mg	Yield %	Purification
Supernatant	202	40.1	8,100	34.3	1.17	100	
pH 5.0 ppt.	200	40.0	8,000	31.0	1.29	98	
30-50% saturation passage through Sephadex G-25 and dilution to 10.3 mg protein per ml	74.5	50.4	3,755	10.3	4.90	46	4.2
Adsorption to and elution from gel	20	13.5	270	0.34	39.70	3.3	34.0

Table 8 - Purification of COMT. The units are micrograms of metanephrine produced in the first five minutes of incubation. S-Ado was present at 5×10^{-4} M and adrenaline at 10^{-3} M in the assay method.

GENERAL SUMMARY AND DISCUSSION

Ortho-iodophenol does not inhibit COMT in vitro. The presence of a carboxyl group in position para (as in DIHPA) or ortho (as in DIHA) to the hydroxyl group converts iodophenols into inhibitors of COMT. The presence of an aldehyde group (as in 5-iodovanillin) or of the pyruvic acid residue (as in DIHPA) also confers inhibitory properties to the ortho-iodophenol. These substituted iodophenols act as competitive inhibitors of the enzyme. DIHPA is a titrating ("irreversible") inhibitor of COMT in the sense that no equilibrium is established and the enzyme is quantitatively titrated and made entirely unavailable to the substrate. For this reason no inhibition constant could be established for this inhibitor. The inhibitor seems to compete for the active site normally occupied by the substrate (catechol) since when adrenaline is added in excess the inhibition is reversed by 70%. The presence of two iodine atoms is more effective than the presence of one as estimated from the superior inhibitory properties of DIHBA compared to those of 5-iodovanillin and those

of the tetraiododerivatives of the thyroxine analogues as compared to those of the triiododerivatives. The alanine side chain does not support inhibition in iodophenols as is shown by the inertness of 3,5-diiodotyrosine (DIT).

Thyroxine itself could not be demonstrated to inhibit because of its extreme insolubility. Since it carries the alanine side chain it is not expected to be a potential inhibitor of much importance in vivo. As a matter of fact it cannot be a better inhibitor than diiodo or triiodothyronine. We have seen that the two latter substances are the poorest inhibitors described in this work. Thyroxine, then, in the light of these results cannot be considered a potentially effective inhibitor in vivo, and the same is of course true for triiodothyronine. The iodo- and especially the tetraiododerivatives of the thyroformic, thyroacetic and thyropropionin acids were shown to inhibit, with a noncompetitive component introduced by iodine in positions 3 and 5. The affinity of the enzyme for the tetraiododerivatives is shown to be of comparable magnitude to that for the normal substrate. It is inferior however to that for DIBA. Tetraiodothyropyruvic acid was not available for testing but one might expect it to act as a titrating inhibitor, much like DIBPA.

No O-methylation of DIHBA could be demonstrated. Iodophenol-O-methyltransferase has been shown (TOMITA and CHA 1963) to be an enzyme distinct from COMT, slowly acting on DIHBA and tetraiodothyroacetic acid (tetrac) to give the O-methylated derivatives. Its presence in vivo is supported by the finding of small amounts of O-methyl-thyroxine in the urine of rats injected with physiological doses of thyroxine-I^{131} (ROCHE et al. 1961). The enzyme requires methionine and ATP or S-AMe as the methyl group donor. It does not require magnesium as COMT does. The distinctness of iodophenol-O-methyltransferase from COMT and the demonstration that excess of S-AMe does not reverse the inhibition demonstrate that the latter is due to a direct action on COMT and not to O-methylation of the inhibitor or removal of the methyl group donor by the first enzymatic system.

No confirmed mechanism for the O-methylation of catechols exists. The role of magnesium is conjectural. AXELROD (1960) suggests that magnesium serves to attach the two hydroxyl groups of the catechol to the SH groups of the enzyme. SH groups are thus assigned the role of essential groups of the active site. The magnesium chelating properties of tropolones would serve in explaining the inhibition in such a complex but the failure of kojic acid, a magnesium chelating agent also, to inhibit,

suggests that more subtle and unrecognized factors are at play here.

SENOH et al. (1959) attributed a similarly hypothetical role to magnesium, Magnesium is assumed to enter a complex formation between catechol and S-Ado. Whatever the role of magnesium, there seems to be no compelling reason to assume that chelation is the only condition for inhibition. Iodophenols are not known to form metal chelates yet they inhibit the enzyme. The progressive inhibition produced by EDTA is interesting but it does not provide any clue for further understanding. In the final analysis it must be admitted that no mechanism seems to be obvious enough to account for the inhibitory effects discovered in this work. Unless the enzyme is highly purified such questions as specificity, reaction mechanism and mechanism of inhibition cannot be answered with any degree of reliability.

To what extent the lower activity of COMT in vivo in hyperthyroid animals can be ascribed to a direct inhibition of the enzyme is difficult to assess. Our in vitro experiments clearly established that iodinated thyronines are poor inhibitors of COMT. For reasons already discussed in detail in section IX, thyroxine itself is expected to be an equally poor inhibitor as triiodothyronine. However, a number of analogues have

been shown to be good inhibitors of the enzyme, especially the tetraiododerivatives. The thyroacetic acids have been found in the kidneys and liver of mice which were injected with large doses of radioactive iodide and are believed to be the true metabolic products of the endogenous hormones and not merely the result of unphysiological doses of thyroxine or triiodothyronine (PITT-RIVERS and TATA 1959). Tetraiodothyroacetic acid (tetrac) has been identified in the plasma of hepatectomized dogs after injection of thyroxine (GLOCK et al. 1957). Thyropyruvic acids have not been detected but as pointed out by PITT-RIVERS and TATA (1959) they may be intermediates in the metabolic pathway of thyroid hormones to thyroacetic acids. The oxidative deamination of thyroxine and triiodothyronine to the corresponding acetic acid derivatives has been described. TOMITA and LARDY (1960) demonstrated that an extract of rat kidney mitochondria converts thyroxine and triiodothyronine to the corresponding acetic acid analogues. YAMAMOTO (1959) obtained evidence for the presence of a thyroxine-α-ketoglutarate transaminase in rat kidney homogenate which converted thyroxine to tetraiodothyroacetic acid. These experiments were continued and verified for various rat tissues (YAMAMOTO et al. 1960a; 1960b) and extended to include triiodothyronine which was shown to be converted

to 3,3',5-triiodothyroacetic acid by rat kidney mitochondria (YAMAMOTO et al. 1960c). NAKANO and DANOWSKI (1962) described the conversion of triiodothyronine to triiodothyroacetic acid by an extract prepared by repeated freezing and thawing of rat kidney mitochondria. More recently NAKANO et al. (1963) demonstrated the presence in extracts of sonically treated rat kidney mitochondria of two enzymes catalyzing the deamination of thyroxine to mainly tetraiodothyroacetic acid. One of these enzymes is an oxidase, the other is a transaminase requiring ketoglutarate and pyridoxal phosphate for activity. It appears then quite possible that direct inhibition of COMT in vivo can occur, if not by thyroxine itself, by its metabolic products such as tetraiodothyroacetic acid. PITT-RIVERS and TATA (1959) emphasize that from the quantititative point of view the oxidative deamination of the thyroid hormones is not as important as deiodination and phenolic conjugation in vivo. This would mean that tetrac under physiological or even hyperthyroid conditions would be present in much too low concentration to produce inhibition of COMT. However, tetraiodothyropyruvic acid could conceivably act as an irreversible inhibitor. It is unfortunate that this compound was not available to us to be tested. The experiments of THIBAULT (1948a) may indicate

precisely that. She found that one hour of preincubation with thyroxine was needed in order to demonstrate an augmentation of the inhibitory effect of adrenaline on rabbit intestine. She ascribed this lag period (SMITHARD 1950) to a requirement for "active thyroxine" which could be obtained by preincubation of thyroxine with intestinal tissue (cf. section IV). It is not inconceivable that this "active thyroxine" might be a thyroxine analogue such as tetrac or tetraiodothyropyruvic acid. This mechanism could possibly explain the potentiating effects of thyroxine in vitro. In vivo the situation becomes more complicated. It is not known whether the lower levels of COMT in the hyperthyroid state are due to direct inhibition, reduced protein synthesis or both. Problems such as membrane permeability should also be kept in mind. Our finding that one injection of thyroxine does not result in lower COMT activity is in keeping with the data and conclusions derived from the experiments in vitro establishing that iodothyronines (alanine side chain present) are poor inhibitors of the enzyme. A second injection of thyroxine resulted in significant but not drastic diminution in activity. This time interval may be required for the accumulation of metabolites in amounts sufficient to inhibit the enzyme to a significant extent. It must be emphasized at this point that the

relatively high inhibition constants established for the thyroxine analogues as compared to the amounts of circulating thyroxine do not necessarily speak against such an inhibition. In normal serum the concentration of thyroxine is about 10^{-7}M. (PITT-RIVERS and TATA 1959). However the intracellular concentration of thyroxine is not known and the evidence (reviewed by PITT-RIVERS and TATA 1959) is that it increases considerably in the hyperthyroid state. The best reason however for believing that such direct inhibition is possible, is derived from the inhibition characteristics of 3,5-diiodo-4-hydroxy-phenylpyruvic acid (DIHPA) and those anticipated, by analogy, of the tetraiodothyropyruvic acid. DIHPA has been shown to be a titrating inhibitor of COMT in vitro. The degree of inhibition does not depend on an instantaneously achieved equilibrium whose constant, among others, would determine its effectiveness in vivo. The inhibition is progressive and results ultimately in the quantitative inhibition of the enzyme as long as sufficient amounts of the inhibitor are present. NAKANO et al. (1963a) demonstrated the presence in rat kidney mitochondria of a transaminase converting 3,5-diiodotyrosine to DIHPA. NAKNY and LISSITZKY (1962) demonstrated the presence of 3-iodo-4-hydroxyphenylpyruvic acid and DIHPA in rat thyroid

glands. DIPPA is then a physiologically occuring substance resulting from the transamination of diiodotyrosine. The latter is known to be an intermediate in thyroxine biosynthesis and it has also been claimed to be a metabolite of thyroxine by many investigators. A shade of doubt is cast on the latter claim by PITT-RIVERS and TATA (1959) on the basis that chromatographic identification of diiodotyrosine is not unambiguous.

WURTMAN et al. (1963) confirm the results of D'IORIO and LEDUC (1960) that large doses of thyroxine injected into rats cause a significant decrease in COMT activity. They claim however that part, at least, of the hyperresponsiveness to adrenaline in the hyperthyroid state is due to a decreased capacity of the heart to inactivate catecholamines by binding.

In conclusion, we do not consider that our results provide the entire answer as to the mechanism underlying the potentiation of the actions of adrenaline by thyroxine. However, by tackling the problem at the enzyme level we have shown that direct inhibition of COMT by physiologically occuring analogues of thyroxine is possible and may account, at least partially, for the observed potentiation phenomena.

A summary of the original contributions of this work follows.

SUMMARY OF ORIGINAL CONTRIBUTIONS

1- In the one-ring system (iodophenols) we discovered a number of new inhibitors of COMT. These inhibitors are substituted ortho mono- or diiodophenols. The additional substituent required for inhibition could be a carboxyl or aldehyde group or the pyruvic acid residue. The pyridine ring carrying the iodophenol function can also be an inhibitor as is the case with 3,5-diiodo-4-hydroxypyridine. (DIHP)

2- The kinetics of the inhibition was studied and the inhibition constants were calculated. All inhibitors except two were shown to be of the competitive type. DIHP, the poorest inhibitor of all is of the noncompetitive type. DIHPA was shown to be a titrating inhibitor.

3- DIHPA was studied in some detail. Its inhibition could be reversed by excess substrate (adrenaline) but not S-Ame. This indicated that the inhibition was not due to denaturation of the enzyme but to competition for the site normally involved in the

activation of adrenaline. Analysis according to ACKERMAN and POTTER (1949) showed that DIHPA is irreversible or pseudoirreversible inhibitor.

4- We have shown that the alanine side chain does not support inhibition of iodophenols in the one-ring system.

5- In the two-ring system we discovered that a large number of thyroxine analogues act as inhibitors of COMT.

6- The kinetics of inhibition of this class of inhibitors was studied and their inhibition constants were derived. The 3,5-diiododerivatives, except diiodothyronine, were shown to be noncompetitive inhibitors. In these analogues the presence of the alanine side chain suppressed this noncompetitive inhibition introducing a competitive component. In the tri - and tetraiododerivatives, the proximity of the hydroxyl group and the iodine atom(s) introduced a competitive component in agreement with the experiments with the one-ring system. The strength of inhibition was augmented with increasing number of iodine atoms as is indicated by the inhibition constants.

7- It was shown that in the two-ring system the alanine side chain did not support inhibition in conjunction

with the 3',4'-iodophenolic function. This is in agreement with the results obtained with the one-ring system. The important conclusion was drawn that thyroxine itself, since it carries the alanine side chain, cannot be a potentially valuable inhibitor in vivo.

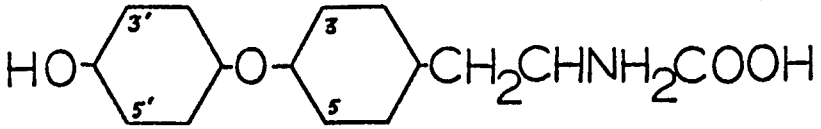
8- It was shown that the Michaelis constant for adrenaline is a function of the concentration of S-Ame. It was further possible to derive the substrate constant Ks for adrenaline by a graphical method. This is equal to 0.5×10^{-4} M.

9- The kinetic study of the inhibition of COMT by 4-methyltropolone as given by BEILEAU and BURBA (1961) was corrected. The inhibition was shown to be strictly competitive with or without preincubation and not noncompetitive as was claimed. The inhibition constant for 4-methyltropolone was derived and was shown not to be significantly affected by preincubation.

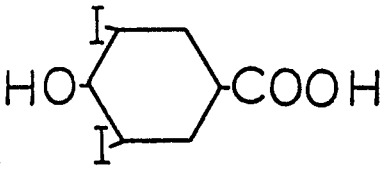
10- The validity of the proposed mechanism for the inhibition of COMT by tropolones (BEILEAU and BURBA 1963) was questioned. Kojic acid, a magnesium chelating agent was shown not to act as inhibitor of COMT. This finding challenges the claim that tropolones act by virtue of their magnesium chelating properties.

ADDENDUM

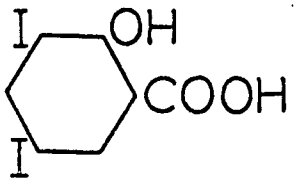
Key structures of substances used in the experiments



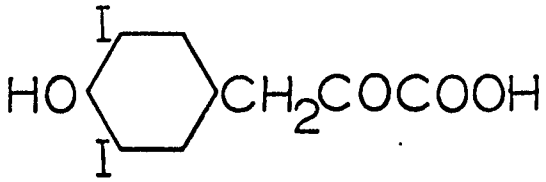
Thyronine



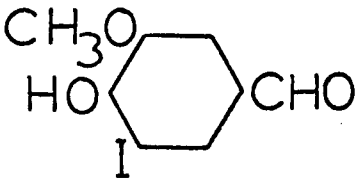
3,5-diiodo-4-hydroxybenzoic acid (DIHBA)



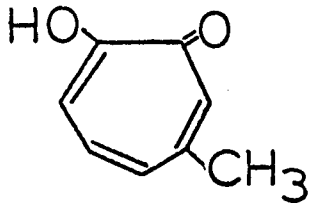
3,5-diiodosalicylic acid (DISA)



3,5-diiodo-4-hydroxyphenylpyruvic acid (DIHPA)



5-iodovanillin



4-methyltropolone

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