THE EFFECT OF CHOLINE 2:6 XYLYL ETHER BROMIDE AND RELATED COMPOUNDS ON DOPAMINE-β-OXIDASE

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ABSTRACT

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The Effect of Choline 2:6-xylyl ether bromide (T.M.10) and related Compounds on Dopamine-β-Oxidase

The effects of T.M.10 and of β-Methyl T.M.10 on dopamine-β-oxidase were investigated. Both bovine adrenal medulla and human pheochromocytoma were used as a source of the enzyme on the assumption that the properties of dopamine-β-oxidase of these tissues are representative of those of chromaffin tissue in general.

Both whole homogenates and isolated chromaffin granules prepared from these two tissues were incubated with C^{14}-dopamine. The catechol amines were subsequently separated by ion exchange chromatography and identified by paper chromatography and autoradiography. Their concentrations were determined spectrophotometrically and their radioactivity also measured. The effects of T.M.10 and of β-Methyl T.M.10 on the conversion of C^{14}-dopamine to C^{14}-noradrenaline by the whole homogenates and by the isolated chromaffin granules were determined by comparing the specific radioactivity of the isolated noradrenaline from the sample to which T.M.10 had been added with that of a control sample incubated without T.M.10. Both stimulation and depression of the conversion of C^{14}-dopamine to noradrenaline were observed with whole homogenates of bovine adrenal medulla depending on the concentration of T.M.10. Only inhibition was observed with homogenates of pheochromocytoma or with isolated chromaffin granules.

Because T.M.10 is known to inhibit amine oxidase, a few experiments were carried out in which Marsilid was added to the incubation medium.

The conversion of C^{14}-dopamine to noradrenaline was stimulated in the presence of concentrations of Marsilid which would be expected to inhibit amine oxidase in bovine adrenal medulla.

The experimental evidence suggests that both T.M.10 and β -Methyl T.M.10 are inhibitors of the two enzymes, dopamine-β -oxidase and amine oxidase, and that the sensitivity of amine oxidase to T.M.10 and its β -Methyl analogue is greater than that of dopamine-β -oxidase.

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CHAPTER I

INTRODUCTION

During the past five years there has been considerable interest in a number of compounds which interfere with the release of noradrenaline from sympathetic nervous tissue. Two such compounds are: T.M.10 (choline 2:6 xylyl ether bromide) and Bretylium (N-o-bromobenzyl-N-ethyl:N:N dimethylammonium bromide). These compounds have been or are being used for the treatment of hypertension.

The first of these compounds to be studied was T.M.10. It was synthesized by Hey in 1952. The initial biological studies were those of Hey and Willey (1954), who examined T.M.10 and other related nuclear substituted choline phenyl ethers for nicotine-like activity. They observed that a first intravenous injection of T.M.10 (choline 2:6 xylyl ether bromide) and related compounds into cats produced a brief, rapidly diminishing rise in blood pressure, which they found to be a characteristic property of T.M.10 and of its analogues. However, subsequent injections of these compounds did not produce any further rise in blood pressure even when given following a lapse of several hours, but they did prevent responses to postganglionic sympathetic nerve stimulation. It was also shown (Hey and Willey, 1954; Exley, 1957) that except for an initial and transient effect, doses of T.M.10 which effectively blocked the responses to postganglionic sympathetic nerve stimulation did not modify the effects of injected adrenaline. This sympathetic "inhibitory" activity of T.M.10 and related compounds

suggested that these choline xylyl ethers possessed local anaesthetic properties.

Edge, Mason and Wyllie (1957) confirmed the suggestion of Hey and Willey (1954) that T.M.10 produced long lasting local anaesthesia. They also showed that choline 2:6 xylyl ether bromide and its related compounds had nicotine-like, muscarinic, weak anti-adrenaline and vasodilator properties; all of these compounds caused some neuromuscular blocking as well.

In 1956, Exley studied the blocking action of T.M.10 on adrenergic nerves. His preliminary work showed that doses of T.M.10 that effectively blocked adrenergic nerve activity indicated, for example, by the relaxation of the nictitating membrane of cats did not suppress the postganglionic action potentials. Further work by Exley (1957) extended the observations of Hey and Willey (1954) to other nerves and effector organs. A comparison of the effects of T.M.10, even in rather large doses, on the parasympathetic and sympathetic nerves supplying such tissues as the heart and submaxillary gland of cats showed that T.M.10 given systemically in tolerated doses which effectively block the responses to sympathetic nerve stimulation, does not block the effects of cholinergic nerves. Other experiments showed that T.M.10 prevented the secretion of noradrenaline from the spleen in response to splenic nerve stimulation, but in acute experiments, did not affect the release of pressor amines from the adrenal medulla, stimulated either chemically with tetramethylammonium or through the splanchnic nerves. Whereas related choline phenyl ethers exhibited similar local anaesthetic properties, Exley (1957) found that they did not produce any

adrenergic nerve blocking such as produced by T.M.10. Exley's studies led him to conclude that T.M.10 acted near or at nerve terminals, exerting its effect through interference with either the release or the synthesis of noradrenaline.

In 1956 Bain and Fielden, working on the hypothesis that the inhibitory action of T.M.10 on sympathetic nervous activity was due to its interference with the synthesis of the adrenergic transmitter, studied the effects of various, supposed precursors of noradrenaline on the sympathetic blocking activity of T.M.10. Using isolated, innervated rabbit intestine in vitro (Finkelman, 1930) they discovered that dopamine (3,4-dihydroxyphenylethylamine) could reverse the blocking action of T.M.10, but that the effects of T.M.10 recurred if the dopamine were removed by washing. Tyrosine, phenylalanine and dopa (3,4-dihydroxy-phenylalanine) failed to reverse the blocking action of T.M.10. They also looked for an inhibition of dopa decarboxylase by T.M.10 and therefore a block in the biosynthesis of dopamine. Experiments with dopa decarboxylase from guinea pig kidney did not show any inhibition of the synthesis of dopamine. Bain and Fielden (1956) therefore suggested that either the dopa decarboxylase of adrenergic nerve differed from that of guinea pig kidney or that T.M.10 acted in some other manner. Later work (Bain and Fielden 1957) supported the latter possibility. Bain and Fielden found that the formation of noradrenaline from dopamine by human pheochromocytoma tissue was inhibited by T.M.10, presumably through the inhibition of "dopamine dehydrogenase" (dopamine-β-oxidase).

In 1956 Brown and Hey made a study of the relationship between amine oxidase inhibitory activity and the chemical structure of substituted

choline phenyl ethers including T.M.10. They found that these compounds cause competitive inhibition of amine oxidase.

A histochemical study of the effects of T.M.10 upon the amine content of the adrenal medulla of rats was made by Coupland and Exley in 1957. They claimed that daily subcutaneous injections of T.M.10 acted upon the normal adrenal medulla and upon adrenal medulla grafts to produce a progressive depletion of the catechol amine content to an extent of fifty per cent in a period of two weeks. The pressor amine content of the medulla returned to normal in the same period of time following the cessation of T.M.10 administration. The results produced by Coupland and Exley agree very favourably with the estimated half turn over time of seven to nine days for the pressor amines of rat medulla calculated by Udenfriend et al (1953). The close correlation of their results with the data produced by Udenfriend and co-workers drew Coupland and Exley to the conclusion that T.M.10 exerts its action through interference with the biosynthesis of the pressor amines.

In 1958, Coupland compared the effects of a single injection of T.M.10 on the catechol amine content of normal rat adrenal medulla and of autografts of chromaffin cells in the anterior chamber of the eye. He showed that T.M.10 failed to produce a measurable reduction in the catechol amine content of the adrenal medulla or of the implanted chromaffin cells. He therefore discounted the suggestion that T.M.10 functioned as a direct nicotine-like stimulant. He claimed this result supported the hypothesis that T.M.10 reduced sympathetic nerve activity by inhibiting the synthesis of the pressor amines.

Nasmyth and Andrews (1959) studied the antagonism of cocaine to

the action of T.M.10 at sympathetic nerve endings. They demonstrated that an injection of cocaine prior to the injection of T.M.10 prevented the blocking action of the choline xylyl ether and that, following the attainment of a block with T.M.10, an injection of cocaine partially restored the secretion of noradrenaline at the sympathetic nerve endings on nerve stimulation and the responses of the organs they innervate. The degree of the antagonism seemed to be dependent upon the relative amounts of the two substances present. They also confirmed the observations of Hey and Willey (1954) that T.M.10 blocks the response of the nictitating membrane to sympathetic stimulation and the results of Exley (1957) demonstrating that choline 2:6 xylyl ether bromide prevented the secretion of noradrenaline at splenic nerve endings.

A series of new anti adrenergic compounds was described by Boura, Copp and Green in 1959. The compounds in this series are all benzyl quaternary ammonium compounds. One of the most active compounds of the series is Bretylium and it has been studied to a greater extent by Boura et al (1959, 1959a, 1959b) than any of the other compounds in the series.

Following the subcutaneous injection of C^{14} labelled Bretylium into cats, the radioactivity was found to be concentrated chiefly in the adrenergic nerves, sympathetic ganglia and tissues with a rich sympathetic innervation (Boura et al 1959a). Studies (Boura and Green 1959b) with various isolated preparations such as rabbit ileum (Finkelman, 1930) demonstrated that, like T.M.10, Bretylium blocked the response to adrenergic nerve stimulation. The sensitivity of these preparations to exogenous adrenaline and noradrenaline was greatly enhanced following the injection of Bretylium. In the intact animal there was no inhibition of the actions of circulating or injected adrenaline or noradrenaline. Boura and Green

(1959b) also demonstrated that the concentration of Bretylium which produced blocking of adrenergic neurones had no effect on the secretion of the catechol amines from the adrenal medulla in response to splanchnic nerve or chemical (dimethylphenylpiperazinium iodide) stimulation. They concluded that Bretylium acts by impairing the conduction of impulses in adrenergic nerves and also suggest that the selective accumulation of Bretylium (Boura et al 1959a) in peripheral sympathetic nerves provides additional supporting evidence that the blocking action of Bretylium is restricted to the peripheral sympathetic nerves. There is no direct evidence to suggest that Bretylium inhibits the synthesis of noradrenaline. However, since it has been shown (Boura et al 1959a) that Bretylium does not cause depletion of the pressor amines from the adrenal medulla of neither rats nor cats, and assuming that the properties of adrenal medulla tissue are representative of those of chromaffin tissue in general, it seems unlikely that Bretylium should have a different effect on adrenergic nerves.

More recently Bain (1960) has repeated his claim that the antiadrenergic action of T.M.10 is due to its inhibition of dopamine-β-oxidase. He again reports experiments in which T.M.10 incubated with human pheochromocytoma tissue inhibited the conversion of dopamine to noradrenaline. He states that "To account for the antiadrenergic activity of T.M.10 in terms of an inhibition of noradrenaline formation from dopamine, we have only to suppose - and there is a good deal of presumptive evidence for this - that release of the transmitter on nerve stimulation occurs only when the "pool" of preformed amine can be maintained constant, or nearly so, by the synthesis of fresh transmitter

from dopamine. When synthesis of new material is prevented, liberation of noradrenaline on nerve stimulation does not occur. Even the propagation of the impulse in adrenergic nerves may depend in some way on the sudden formation of noradrenaline from dopamine - there is certainly plenty of dopamine there (Shumann, 1960) - so that axonal block, if it does occur in the terminal ramifications of adrenergic nerves under the influence of T.M.10, may result from interference with this conversion. In any event, interference with noradrenaline synthesis could account for the way in which T.M.10 acts wherever the transmitter may be formed or released - whether that be in the nerves themselves, from structures in intimate association with the nerves, or from more peripheral structures. Nor must it be forgotten that it can account too for the fact that dopamine may prevent, delay, or abolish the block produced by T.M.10. It looks, indeed, as though T.M.10 and, presumably, related compounds may prove to be true adrenergic blocking drugs because they are competitive antagonists of dopamine dehydrogenase."

Although Bain's concept of the release of noradrenaline on nerve stimulation may not be correct, the evidence described to this point demonstrates that T.M.10 does act to prevent the release of noradrenaline in response to sympathetic nerve stimulation. One possibility is that T.M.10 might act by inhibiting the synthesis of noradrenaline in adrenergic nerves.

Biosynthesis of Noradrenaline

The experiments of Bain and Fielden (1957, 1960), Coupland and Exley (1957) and Coupland (1958) suggested that T.M.10 might be an inhibitor of the synthesis of noradrenaline in chromaffin tissue.

Noradrenaline is known to be synthesized from l-dopa (Demis, Blaschko and Welch, 1956; Hagen and Welch, 1957). Dopamine is an intermediate in

this conversion (Hagen, 1955). Although the conversion of dopa to dopamine is probably quantitative, only a small part of the dopamine is converted to noradrenaline (Hagen and Welch, 1957), the greater part of the dopamine being deaminated by amine oxidase to form dihydroxyphenylacetic acid. These reactions are shown on the opposite page. As it has proved difficult up to this time to demonstrate any considerable net synthesis of noradrenaline from dopamine in a purified enzyme system and as the cofactors required for this synthesis have not yet been definitely established, most of the studies carried out so far have involved the measurement of the incorporation of small amounts of radioactive (C^{14} labelled) precursors into noradrenaline.

The Problem

The experiments described in this thesis were carried out in order to determine whether T.M.10 can inhibit dopamine-β-oxidase of Chromaffin tissue.

The two tissues used in these experiments as a source of dopamine-β-oxidase were bovine adrenal medulla and human pheochromocytoma.

In view of the known, very low activity of dopamine-β-oxidase (Hagen and Welch, 1956; Kirshner, 1959) it would be difficult to evaluate the activity of this enzyme by measuring a net conversion of dopamine to noradrenaline. For this reason, the activity of the enzyme has been measured by studying the conversion of C^{14} labelled dopamine to C^{14} labelled noradrenaline by homogenates of bovine adrenal medulla and of human pheochromocytoma in vitro. The effect of T.M.10 added to the incubation mixture on this conversion has been investigated.

CHAPTER II

TECHNIQUES

I Preparation of Tissue Fractions (all manipulations were carried out at 2°C)

1. Separation and isolation of the tissue fractions: In each preparation of medulla tissue, 40 or more grams of adrenal medulla were dissected out from bovine adrenal glands obtained from a local slaughter house. The medulla tissue was finely minced with a scalpel and then homogenized in 0.4 M sucrose solution (containing 0.04 M phosphate buffer pH 7.4 and 0.01 M sodium versenate) using an all glass homogenizer to make a 1:3 or 1:4 homogenate. This constituted the homogenate used in most of the experiments. It was general practice to add versenate to the isotonic sucrose solutions used because in its presence, better separations of the subcellular components (mitochondria and chromaffin granules) were obtained.

The homogenate was centrifuged at 900 X g for thirty minutes to yield a supernatant and a sediment. The sediment (cell nuclei and unbroken cells) was resuspended in 0.4 M sucrose solution and recentrifuged at 900 X g for thirty minutes in order to minimize the loss of large granules sedimented with the nuclei in the first centrifugation. The sediment was discarded.

The nuclei free supernatants were pooled and centrifuged at 11,000 X g for thirty minutes to bring down the large granules (mitochondria and chromaffin granules) leaving a first high speed supernatant. The large granules were resuspended in a minimum volume of 0.4 M sucrose. This

suspension is referred to as the large granule fraction.

The first high speed supernatant was centrifuged at 140,000 X g for sixty minutes to bring down the microsomes, leaving a final high speed supernatant which was used for the preparation of dopa decarboxylase.

2. Separation of Chromaffin Granules from Mitochondria: Three mls of the large granule fraction were carefully layered above 2.0 mls of 1.8 M sucrose in plastic centrifuge tubes (Fig. 3a) which were then centrifuged in a horizontal field at 140,000 X g for sixty minutes, in the Spinco SWL 39 swing out head. Three different fractions distributed along the length of the tube (Fig. 3b) were produced. The upper layer was clear and apparently lacked particulate material. The middle layer was of a greyish color and consisted of several bands of material with a reddish-brown layer at the junction with the bottom layer, which was creamy yellow and homogeneous in appearance. Some sediment adhered to the bottom of the tube. The tubes were cut so as to isolate the three fractions. The first cut was made at the bottom of the clear solution. The second cut was made just below the narrow reddish-brown pigmented band. All of the material collected between the first and second cuts is henceforth referred to as the mitochondrial fraction. All of the material remaining in the centrifuge tube following the second cut constituted the chromaffin granule fraction. Both the mitochondrial and chromaffin granule fractions were made up to a volume of 10 mls with 0.4 M sucrose and stored in a deep freeze until used.

3. Preparation of Dopa Decarboxylase: The following procedure was carried out primarily to obtain a more concentrated form of the enzyme

for manometric studies and not essentially as a purification step although undoubtedly, some degree of purification was achieved. The 100,000 X g supernatant obtained from bovine adrenal medulla homogenate as described above was used as the starting material. The enzyme was concentrated by precipitation from the high speed supernatant with ammonium sulphate at 2° C.

First, saturated ammonium sulphate was added to given an ammonium sulphate concentration of 10%. The precipitate formed was spun down by centrifugation. The supernatant was transferred to a separate container and more saturated ammonium sulphate was added to it to bring the ammonium sulphate concentration to 20%. The resulting precipitate was collected by centrifugation; the supernatant was transferred to a clean flask and more saturated ammonium sulphate added to bring the ammonium sulphate concentration to 30%. Again the precipitate was collected by centrifugation. The precipitates thus obtained by the addition of saturated ammonium sulphate to give saturations of 40%, 45%, 50% and 55% ammonium sulphate respectively were likewise sedimented by centrifugation. Each of the protein fractions isolated by the "salting out" action of saturated ammonium sulphate was resuspended in 10 mls of 0.01 M sodium phosphate buffer pH 6.5 containing 0.1 M mercaptoethanol. No attempts were made to remove any ammonium sulphate that may have been present in the precipitates. The dopa decarboxylase activity of each protein fraction was determined by measuring the rate of production of CO_2 at 37° C. with dopa as substrate, using the Warburg Apparatus. The protein fraction precipitated by 45% saturation with ammonium sulphate was found to contain the greatest dopa decarboxylase activity (Table I) and was subsequently used in the experiments reported.

II Ion Exchange Chromatography

1. Preparation of the resin: Dowex-50 resin was washed several times with water to remove the fine particles. The resin was then cycled through the acid form using concentrated hydrochloric acid. The acid form of the resin was washed several times with water and then converted to its sodium salt by suspension in 40% sodium hydroxide. The sodium form of the resin was washed several times with distilled water and re-converted to its acid form with concentrated hydrochloric acid. The acid form of the resin was washed several times with water and stored in water until used.

The carboxylic resin XE-64, was prepared in a very similar manner with the following modifications: after the resin had been successively cycled through its sodium and acid forms, it was again converted to its sodium form and then equilibrated with 0.2 M sodium phosphate buffer pH 6.8 (Experiments 1 to 4, 10 and 11). In experiments 12 and 13, concentrated ammonium hydroxide was used instead of 40% sodium hydroxide; the ammonium salt of the resin was equilibrated with 0.2 M ammonium acetate solution pH 6.8. The equilibrated XE-64 resin was stored in either 0.2 M sodium phosphate or 0.2 M ammonium acetate solution pH 6.8 until used.

2. Preparation of the columns: In each experiment, the columns of resin were packed to the specified heights under a pressure of 15 lbs. per square inch. Dowex-50 was used in a 68.0 X 1.0 cm column; XE-64 was used in a 25.5 X 1.0 cm column (sodium form) or in a 32.5 X 1.0 cm column (ammonium form).

3. Ion exchange chromatography of noradrenaline and adrenaline:

(a) Use of XE-64: The method used is a modification of the procedure described by Kirshner and Goodall (1957).

Preliminary experiments indicated that the best separation of the catechol amines was obtained by elution from a 25.5 X 1.0 cm column with 0.05 M sodium phosphate (containing 0.001 M $Na_2S_2O_4$) pH 4.3 (Fig. 4). The three catechol amines were completely eluted from the column by 1.5 litres of the sodium phosphate solution. The reservoir containing the elution solution was placed six feet above the top of the column.

It was also demonstrated that by either decreasing the length of the column or by increasing the concentration of the sodium phosphate solution, the degree of resolution of the catechol amines was greatly reduced.

One disadvantage of the above system is that sodium phosphate crystals remain on the planchets when samples of the eluates are evaporated to dryness in preparation for counting radioactivity. Therefore, if highly accurate counts were desired, each sample would have to be desalted before its radioactivity was measured. Also, because the separation is carried out at almost neutral pH, in spite of the presence of the reducing agent, some oxidation of the catechol amines occurred. Another factor rendering the carboxylic resin system unsatisfactory is that dopamine closely follows noradrenaline off the column. This creates the possibility of the isolated noradrenaline being contaminated with radioactive dopamine.

A satisfactory separation was also obtained (Experiments 12 and 13) when the catechol amines were eluted from a 32.5 X 1.0 cm column of

XE-64 with either 0.1 M or 0.2 M ammonium acetate.

Sodium dithionite, a reducing agent, was added to both the sodium phosphate and the ammonium acetate elution systems to prevent the oxidation of the catechol amines on the column. The highest concentration of dithionite which could be added without increasing the optical density of the eluting solution at 280 mμ was determined. This optimum concentration of dithionite was found by measuring the optical density at 280 mμ of a series of samples prepared by adding increasing amounts of sodium dithionite to 5 mls of 0.2 M sodium phosphate pH 6.8. The highest concentration of sodium dithionite that did not absorb any more light at 280 mμ than the solution without dithionite was 0.001 M dithionite (Table II). All concentrations of dithionite greater than 0.002 M produced opaque solutions when they were added to the sodium phosphate buffer.

(b) Use of Dowex-50: The sulphonic ion exchange resin, Dowex-50, had previously been used for the column chromatography of the catechol amines with hydrochloric acid as the eluting agent (Ellman, 1956), but no details of the method were given. Carlsson (1959) also used a column of Dowex-50, in the sodium form, to effect a good separation of noradrenaline and dopamine, but did not separate noradrenaline and adrenaline. He also used hydrochloric acid as the eluting agent. Thus it was necessary to determine the conditions for the separation of noradrenaline and adrenaline with this resin. Accordingly, a brief study was undertaken. The effects of column length and of hydrochloric acid in the eluent on the degree of separation of the catechol amines were investigated.

1. Separation of noradrenaline and adrenaline from dopamine: It was found that noradrenaline could be completely separated from dopamine by suing a 20.0 X 1.0 cm column of Dowex-50 and eluting the amines with a constantly increasing concentration gradient produced by passing 4 N hydrochloric acid into 0.4 N hydrochloric acid in the reservoir during the elution. A good separation of adrenaline from dopamine was also effected by the same system, but a mixture of noradrenaline and adrenaline could not be resolved.

2. Separation of noradrenaline and adrenaline: With a 20.0 X 1.0 cm column, a concentration of hydrochloric acid of less than 0.1 N was necessary to obtain a good separation between noradrenaline and adrenaline (Fig. 5 and 6). By increasing the column size (44.0 X 1.5 cms) a good separation was obtained with 0.15 N hydrochloric acid. However, 3.5 litres of the eluting solution were required for the complete elution of the amines, making the separation a very slow procedure. By further increasing the concentration of the hydrochloric acid, the required volume of eluting solution could be decreased, but there was also a proportionate decrease in the degree of resolution of noradrenaline and adrenaline (Fig. 7). Although 0.3 N hydrochloric acid was found to give a good separation of noradrenaline and adrenaline on a 55.0 X 1.5 cm column of Dowex-50 with a sufficiently small volume of eluate (1800 mls) (Fig. 8) to be practical for routine work, to ensure that a clean separation would be obtained between noradrenaline and adrenaline, the column length of Dowex-50 was increased. Accordingly, in most of the experiments (5 to 9, 14 to 16) the noradrenaline and adrenaline were separated on a 68.0 X 1.0 cm column of

Dowex-50 in the hydrogen form using 0.3 N hydrochloric acid for elution (Fig. 9). Although the noradrenaline and adrenaline were eluted from the column by 3 litres of the 0.3 N acid a further 1.5 to 2 litres were necessary to elute the dopamine from the column unless an increasing gradient elution was then used. It was found that the dopamine could be quickly eluted from the column following the complete elution of noradrenaline and adrenaline by gradually passing 3 N hydrochloric acid into the reservoir containing 0.3 N hydrochloric acid so that a constantly increasing hydrochloric acid concentration gradient was produced.

Because noradrenaline did not appear in the eluate until 1.4 to 1.5 litres had been collected, the practice was adopted of starting the collection of the 10 ml fractions only after 1 to 1.4 litres of eluate had passed through the column.

Some of the more important advantages of this system over the carboxylic resin system are:

(1) Noradrenaline is followed from the column by adrenaline.

(2) A considerable volume of eluent passes through the column after the elution of noradrenaline and adrenaline before the dopamine is eluted. Thus it is possible to obtain a "clean" separation of the radioactive noradrenaline from any radioactive dopamine that might remain after the incubation.

(3) The column is operated at a low pH and therefore oxidation of the catechol amines on the column is prevented.

(4) Due to the high volatility of the hydrochloric acid effluent, no material other than the eluted catechol amines remains on the planchets when samples are evaporated to dryness.

During the development of the Dowex-50 ion exchange chromatography procedure, it was discovered that a certain commercial brand of adrenaline which was being used as a reference amine consistently produced a "toothed" adrenaline peak (Fig. 5). Material from both adrenaline peaks were identified as adrenaline by paper chromatography. Another commercial brand of adrenaline used did not produce the "toothed" effect. It was concluded that the commercial adrenaline used contained traces of d-adrenaline. In other ion exchange separations of the catechol amines (Fig. 7, 8 and 14) certain irregular "humps" occurred on the graphed chromatograms. The sources of the amines in these latter instances were bovine adrenal medulla and human pheochromocytoma tissue respectively and no carrier catechol amines had been added. Because these humps were not consistently found in different chromatograms from the same tissue (Fig. 11 to 18), these irregularities are probably due to changes in the flow rate of the eluting solution through the column.

III Paper Chromatography

In principle, the procedure adopted for the paper chromatography of the catechol amines was essentially that described by Vogt (1952). The isolated catechol amines were spotted on 20.0 X 20.0 cm Whatman #1 filter paper with small, platinum wire loops. Standard noradrenaline and adrenaline were also spotted on the sheets as reference amines. The "spotted" sheets were dried in a stream of warm air supplied by a hair drier and then set up for ascending chromatography in a glass, Universal Chromatography Tank, the interior of which was first saturated with the developing solvent. The tank was not filled with any gas such as SO_2, CO_2, or NO_2 (Crawford and Outschoorn, 1950; Vogt,

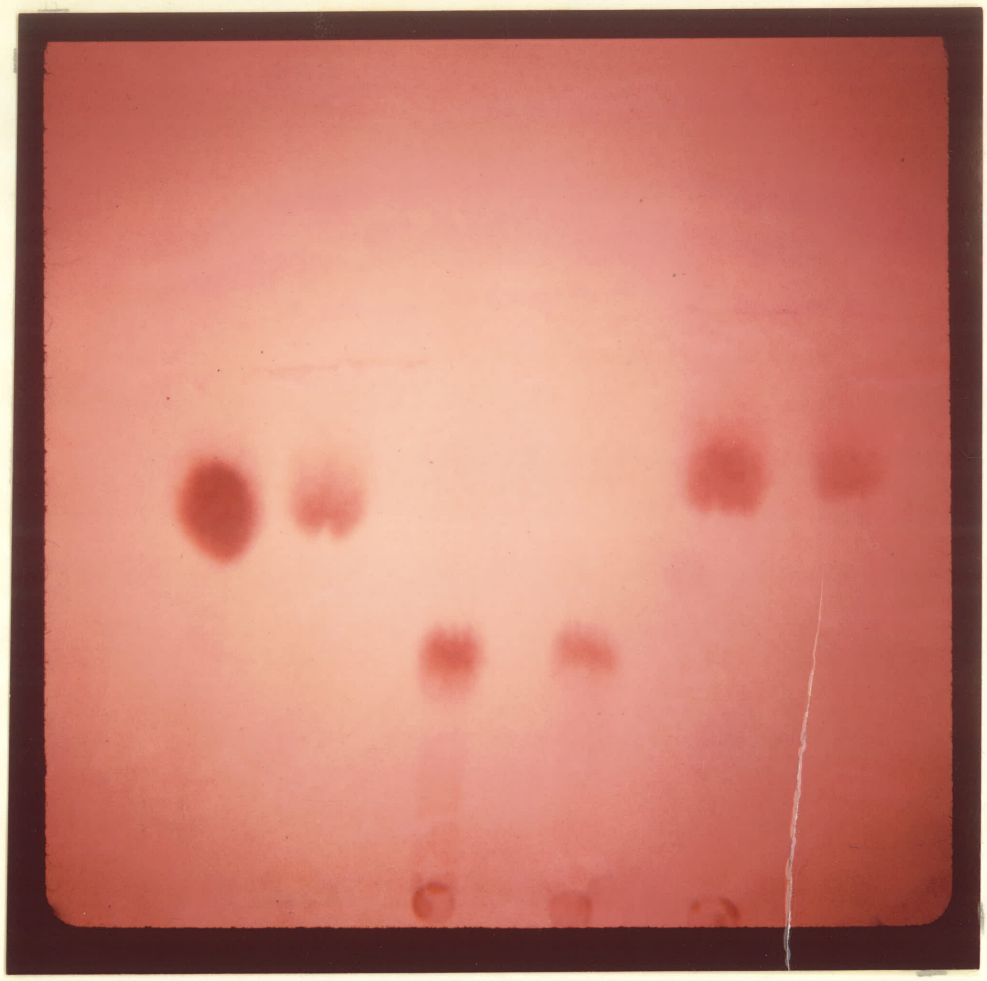


Fig. 10A Photograph of a typical paper chromatogram of the catechol amines separated by the ion exchange chromatography system shown in Fig. 9. The above chromatogram was obtained using phenol-HCl as solvent and was developed for 16 hours at room temperature. The amines were located with ninhydrin and the spots stabilized with acidified cupric nitrate and ammonia. The difference in the color of the dopamine and adrenaline is unfortunately not clearly shown on the photograph.

A. The third compound eluted off the Dowex-50 column.
B. Standard dopamine.
C. First compound eluted off the Dowex-50 column.
D. Standard noradrenaline.
E. Second compound eluted off the Dowex-50 column.
F. Standard adrenaline.

1952). The solvent used to develop the chromatograms was a mixture of phenol with 0.1 N hydrochloric acid (15% (V/W) hydrochloric acid in phenol). The chromatography was carried out at room temperature for sixteen hours and a good separation of the catechol amines was obtained (Fig. 10). The developed chromatograms were dried in a stream of air for several hours (overnight) to remove as much as possible of the phenol from the paper. The dried chromatograms were sprayed with 0.2% ninhydrin in acetone, air dried and then heated for three to five minutes in an oven at 100° C. The positions of the located amines were marked with a pencil. In order to stabilize the colors produced, the dried chromatograms were then sprayed with a ninhydrin fixer solution (80 parts acidified cupric nitrate solution and 20 parts freon) and while still wet, sprayed with an ammonia solution (85 parts ammonia and 15 parts freon). The chromatograms were then air dried.

CHAPTER III

Effect of T.M.10 on the conversion of Dopamine to Noradrenaline

I Experiments in which Homogenates of Bovine Adrenal Medulla were used

In these experiments (1 to 11), dopamine-β-C^{14} was incubated with homogenates of bovine adrenal medulla and the catechol amines subsequently separated by ion exchange chromatography. The radioactivity of the isolated noradrenaline served as an indication of the conversion of dopamine to noradrenaline. The effect of various concentrations of T.M.10 and β-Methyl T.M.10 on this conversion was determined by comparing the specific activity of the noradrenaline isolated from samples to which T.M.10 or β-Methyl T.M.10 had been added prior to incubation with that of the noradrenaline isolated from the control sample which had been incubated without any T.M.10 or β-Methyl T.M.10.

1. Incubation procedure: Each flask contained the following ingredients in a volume of 4 mls:

3 mls adrenal medulla (1:3 or 1:4) homogenate.
1 ml 0.2 M potassium phosphate buffer pH 7.4.
0.04 ml dopamine-β-C^{14} (120 µg dopamine containing 0.008 µc).
1.5 mgs ATP
1.5 mgs fumaric acid.

It has been shown (Levin et al, 1960) that the conversion of dopamine to noradrenaline is greatly enhanced by the addition of ATP and fumarate to the incubation medium.

Uniformity of composition, especially of specific activity, of both the test and control was ensured by first putting all of the ingredients of the control and test incubation media, except the possible "inhibitors" being examined, into a single container. After

thorough mixing, equal volumes of the incubation mixture were transferred to each of two or more small erlenmeyer flasks, one of which served as the control sample while T.M.10 or β -Methyl T.M.10 was added to the others. The following amounts of T.M.10 (milligrams per millilitre of incubation mixture) were used: 5.0, 11.4, 12.5, 22.8 and 50.0 mgs per ml. The concentrations (milligrams per millilitre of incubation mixture) of β -Methyl T.M.10 used were: 0.25, 0.50, 1.25, 2.5, 6.25, 12.5, 25.0 and 50.0 mgs per ml.

The flasks containing the test and the control samples were incubated in a Dubnoff Metabolic Shaking Incubator at 37° C. for five hours. After three hours incubation, 5 mg ascorbic acid in 0.5 ml 0.2 M potassium phosphate buffer pH 7.4 was added to each flask to prevent oxidation of the catechol amines. 2.0 mls of large granules (Experiments 2, 3, and 4) or 2.0 mls of mitochondria (Experiments 5 to 9) were also added to each flask at this time to supply carrier catechol amines and amine oxidase, the latter to destroy any dopamine remaining. Since, with the carboxylic resin system, dopamine follows noradrenaline off the column, the addition of excess amine oxidase was considered necessary to prevent contamination of the noradrenaline with radioactive dopamine.

The enzyme reaction was stopped after five hours by the addition of 6.5 mls of 0.8 N perchloric acid to each flask. The precipitated protein was removed by centrifugation. A few mgs of ascorbic acid were added to each flask to prevent oxidation of the catechol amines during neutralization and the supernatants neutralized to pH 6.8 with 40% potassium hydroxide. The supernatants were then decanted into separate flasks leaving behind the insoluble potassium perchlorate.

In some experiments (1 to 4) 9 µMoles of carrier noradrenaline were

also added to each flask at the end of the five hours incubation period in order to obtain better defined peaks during the ion exchange chromatography.

2. Preparation of extracts for ion exchange chromatography: Each of the neutralized, protein free supernatants was evaporated under reduced pressure to a volume of 1 to 3 mls. The resulting small volumes of solution were partially desalted by the addition of 1 to 1.5 mls of 95% ethanol to each sample and the ethanol treated samples cooled in a deep freeze for fifteen minutes. The desalted samples were then filtered onto separate ion exchange columns.

3. Ion exchange chromatography: Two types of resin were used, a carboxylic resin, Amberlite IRC-50 (XE-64) and a sulphonic acid resin, Dowex-50. The technical details of the procedures are discussed in Chapter II.

In experiments 1 to 4, 12 and 13, the catechol amines were separated on the carboxylic cation exchange resin, XE-64, by a modification of the procedure described by Kirshner (1957). The sodium form of the resin was used in a 25.5 X 1.0 cm column. The partially desalted extracts were applied to the columns and the columns then washed with a few mls of distilled water. The amines were eluted with 0.05 M sodium phosphate containing 0.001 M sodium dithionite. Five ml fractions of the effluent from the columns were collected with a fraction collector.

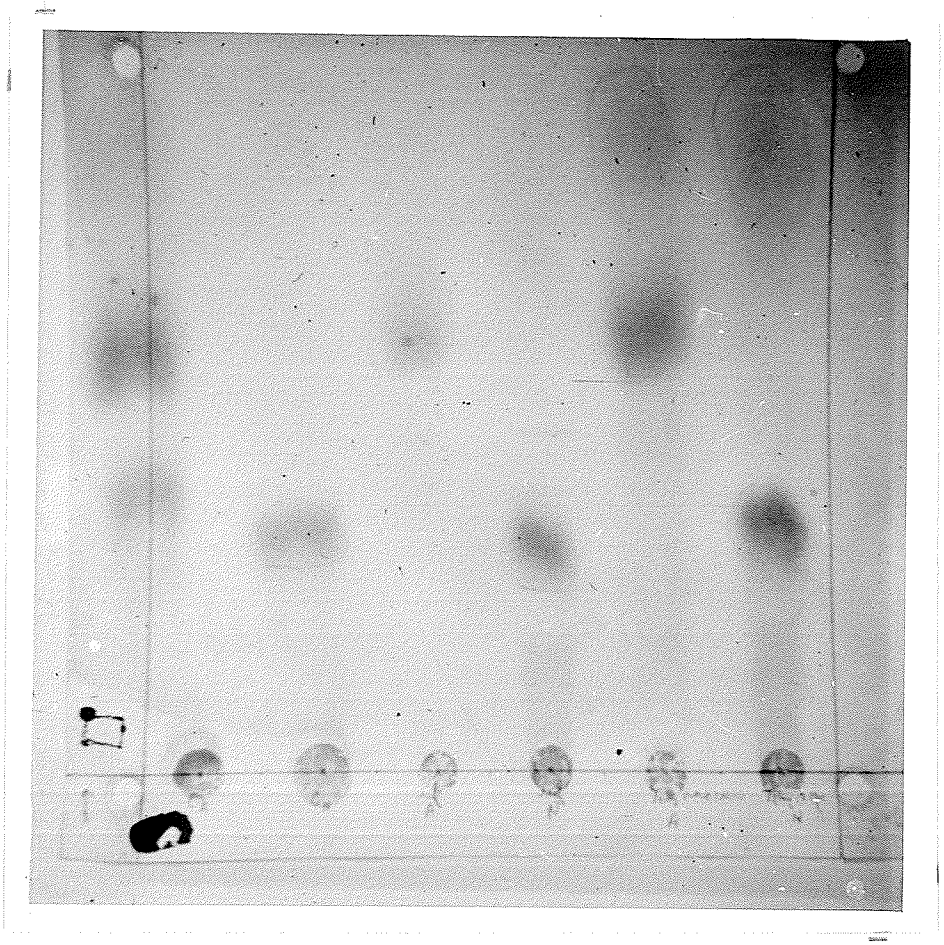
In the remainder of the experiments (5 to 9), Dowex-50, a sulphonic acid ion exchange resin, was used. The Dowex-50 resin was used in the hydrogen form in a 68.0 X 1.0 cm column. The partially desalted extracts were filtered onto the columns and washed into the resin with a few mls of distilled water. Elution was carried out with 0.3 N hydrochloric acid.

The effluent from the columns was collected in 10 ml fractions.

In all of the experiments, the adrenaline and noradrenaline content of each fraction was determined by measuring the optical density of the fractions at 280 mμ in a Beckman D.U. Spectrophotometer.

4. Measurement of radioactivity: One ml aliquots from those fractions containing adrenaline and noradrenaline were plated onto stainless steel planchets and evaporated to dryness under an infrared lamp. The radioactivity on each planchet was counted at infinite thinness in a gas flow counter fitted with a micromil window. This procedure was justified because, in some experiments in which one and two ml aliquots were plated out on planchets, it was found that doubling the volume produced twice the number of counts per minute obtained with one ml aliquots. In experiments 8 and 9, the radioactivity on the planchets was counted with a low background, anticoincidence gas flow counter equipped with an automatic sample changer.

When the optical density of each of the fractions (measured at 280 mμ) was graphed against tube number, the peaks due to the separated adrenaline and noradrenaline stood out clearly. The radioactivity of samples around each peak was plotted as counts per minute per ml of sample on the same graph. Thus it was possible to measure the specific activity of each sample. Specific activity was expressed as counts per minute per ml per unit change in optical density (measured at 280 mμ), or counts per minute per micromole of noradrenaline.

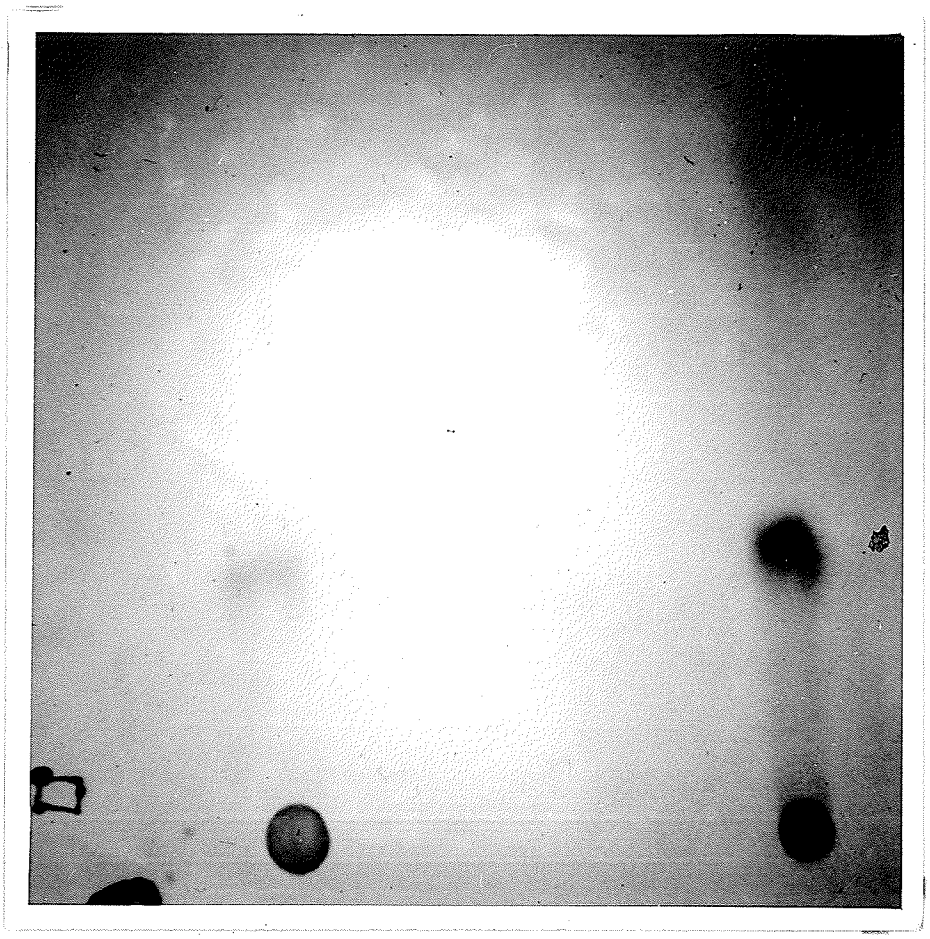


A B C D E F

Fig. 10B Picture of a typical, developed paper chromatogram of the noradrenaline and adrenaline separated by ion exchange chromatography (Fig. 9). This particular chromatogram is from experiment 9. The developed chromatogram was marked (lower left corner) with radioactive ink and then set up for autoradiography. The autoradiograph is shown in Fig. 10C.

A. The separated adrenaline from the control sample.
B. The separated noradrenaline from the control sample.
C. Standard adrenaline.
D. Standard noradrenaline.
E. The separated adrenaline from the test sample containing 0.25 mg /3 -Methyl T.M.10/ml.
F. The separated noradrenaline from the test sample containing 0.25 mg /3 -Methyl T.M.10/ml.

The extra spots at the top of strips E and F are believed to be melanins produced by oxidation of the catechol amines, possibly while the large volumes containing the amines were being evaporated down to a volume sufficiently small for paper chromatography. The lower spot in strip A is unidentified, but it might possibly be noradrenaline (unlabelled).



B F

Fig. 10C Picture of a typical, developed autoradiograph obtained by sealing a developed chromatogram (Fig. 10B) marked with radioactive ink in a manila folder with Kodak Medical X-ray film and leaving it in total darkness for one week. The developed autoradiograph was superimposed on the paper chromatogram so that the spots due to the radioactive ink (lower left corner) on the paper chromatogram coincided with those on the autoradiograph. Any other spots on the autoradiograph were due to radioactivity in the chromatographed samples.

B. The separated noradrenaline from the control sample of experiment 9.
F. The separated noradrenaline from the test sample of experiment 9 containing 0.25 mg /3-Methyl T.M.10/ml.

In most experiments, after the measurement of optical density and radioactivity, samples from the upper portion of each peak were pooled. The optical density of each pooled volume was determined at 280 mμ, its volume measured and a 1.0 ml aliquot plated out on a planchet to measure its radioactivity. Each pooled volume was then reduced in vacuo to a small volume, a portion of which was used for paper chromatography.

5. Confirmation of identity of isolated noradrenaline and of association of radioactivity with noradrenaline by paper chromatography and autoradiography: Chromatography was carried out as described in Chapter II. In experiments 5 to 9, a portion of the small volumes obtained above were spotted along the base line on 20.0 X 20.0 cm Whatman #1 chromatography paper using small, platinum wire loops. Standard solutions of adrenaline and noradrenaline were also spotted on the base line of each sheet. After drying, ascending chromatography was carried out using the phenol-hydrochloric acid solvent. The developed chromatograms were dried and the amines located with ninhydrin. The coloured spots were stabilized with cupric nitrate and ammonia solutions.

Autoradiographs of the chromatograms were then made. First, two radioactive ink marks were made along two edges of each chromatogram. Each chromatogram was then placed in contact with one sheet of Kodak Medical (no screen) X-ray film, sealed in a manila folder and kept for one week in darkness. The film was then developed, fixed and dried. It was then possible to superimpose the dark spots on the film due to the radioactive ink on the ink marks of the chromatogram.

The location of any other radioactivity on the chromatogram was then indicated by dark spots on the film.

II Additional Experiments in which Amine Oxidase was either inhibited or eliminated from the Incubation Mixture

In experiments 1 to 9 a complete homogenate of bovine adrenal medulla was used as a source of the enzyme dopamine-β-oxidase. As this preparation contains an active amine oxidase (Langemann, 1951), and as T.M.10 is an amine oxidase inhibitor (Brown and Hey, 1956), experiments were also carried out in which the amine oxidase was either inactivated with Marsilid (Zeller and Barsky, 1952) or eliminated from the incubation medium by using isolated chromaffin granules as the source of the enzyme. The dopamine-β-oxidase of adrenal medulla is localized in the chromaffin granules (Kirshner, 1959) which lack an amine oxidase (Blaschko, Hagen and Hagen, 1957). The procedure for separation of chromaffin granules from mitochondria is described in Chapter II.

1. Incubation procedure: Except for the addition of Marsilid (0.45 and 1.7 mg Marsilid per millilitre of incubation mixture) to the test incubation media in experiments 10 and 11, the incubation and isolation procedures were identical to that previously described (page 18).

In the experiments in which chromaffin granules were used (experiments 12 and 13), the incubation procedure differed from that previously described in that the mixture in each flask contained:

0.5 ml of chromaffin granules

0.5 ml potassium phosphate buffer pH 7.4

2.5 mg ascorbic acid

0.01 ml dopamine-β-C^{14} (15 μg dopamine containing 0.001 μc)

Neither crude homogenate nor fumaric acid was added. No carrier was added at the end of the incubation. The incubation period was only 2 1/4 hours.

2. Ion exchange chromatography and measurement of radioactivity: For the chromatographic separation of adrenaline and noradrenaline, the carboxylic cation exchange resin, XE-64 was used. In experiments 10 and 11, the sodium form of the resin was used as already described for experiments 1-4. In experiments 12 and 13, the resin was used in the ammonium form and in a 32.5 × 1.0 cm column. The amines were eluted with 0.1 M (exp. 12) or 0.2 M (exp. 13) ammonium acetate containing 0.001 M sodium dithionite. Five ml fractions of the effluent from the columns were collected with a fraction collector. One quarter ml of each sample was plated onto planchets. The adrenaline and the noradrenaline content and the radioactivity of the fractions were determined as described for experiments 1-9.

III Effect of T.M.10 on Dopa Decarboxylase Activity

Although the statement had been made that T.M.10 was not an inhibitor of dopa decarboxylase (Bain and Fielden, 1956), no experimental evidence was presented to support this claim. For this reason, the effect of T.M.10 on dopa decarboxylase was investigated (Experiments 17 and 18).

1. Procedure: Five Warburg flasks, each containing the reactants as listed in Table III (opposite page) were labelled Blank (used to obtain a measure of CO_2 output in the absence of substrate), Control, Test A (10 mg T.M.10), Test B (25 mg T.M.10) and Test C (50 mg T.M.10). The manometers and their corresponding flasks were gassed with nitrogen and then incubated at 37° C. After 5 minutes equilibration, the substrate, dopa, was added from the side arm. The manometers were then read at 3 minute intervals over a period of 15 minutes. Graphs (Figs. 19 and 20) illustrating the effects of T.M.10 on dopa decarboxylase were plotted by graphing the volume of CO_2 produced (microlitres) against time (minutes).

IV Experiments in which Human Pheochromocytoma Tissue was used

Fortunately, some human pheochromocytoma tissue became available and thus made it possible to extend the study with T.M.10 to this tissue. It was of interest to investigate the effect of T.M.10 in this tissue because:

(a) It was human tissue.

(b) It was the tissue used by Bain and Fielden in their original experiments.

In experiments 14, 15 and 16, the effect of T.M.10 on the conversion of C^{14}-dopamine to noradrenaline by both whole homogenates and isolated chromaffin granules of the pheochromocytoma was investigated.

1. An experiment in which a homogenate of human pheochromocytoma was used: In experiment 14, a 1:3 homogenate of the pheochromocytoma tissue was used as the source of dopamine-β-oxidase. The effect of various concentrations of T.M.10 (5.0, 10.0 and 20.0 milligrams of T.M.10 per millilitre of incubation mixture) was investigated. With the exception that 6 µMoles each of carrier noradrenaline and adrenaline instead of mitochondria were added to each flask at the end of the incubation period, the incubation and isolation procedures were identical to those already described in this chapter for experiments 5 to 9.

2. Experiments with human pheochromocytoma tissue in which amine oxidase was either inhibited or eliminated from the incubation mixture: Except for the addition of 6.0 mgs of Marsilid (1.5 milligrams per millilitre of incubation medium) to the test incubation medium in experiment 15, the incubation and isolation procedures followed were identical with those used in experiment 14.

In experiment 16, isolated chromaffin granules (free from mitochondria) were used as the source of dopamine-β-oxidase. The incubation procedure differed from that previously described for bovine tissue in that the mixture in each flask contained:

1 ml chromaffin granules

1 ml 0.2 M potassium phosphate buffer pH 7.4

0.01 ml dopamine-β-C^{14} (71 µg dopamine containing 0.001 µc)

1.5 mg fumaric acid

1.5 mg ATP

The incubation period was 4 hours. Six µMoles each of carrier noradrenaline and adrenaline were added to each flask at the end of the incubation. The procedures for the isolation and measurements of the catechol amines were identical to those already described for experiments 5 to 9.

CHAPTER IV

RESULTS

I Experiments in which Bovine Adrenal Medulla was used as the Source of Dopamine-β-Oxidase

1. Effect of T.M.10 on the conversion of C^{14}-dopamine to noradrenaline by homogenates of adrenal medulla: The results of individual experiments (1 to 4, 5 and 8) are shown in Tables IV and V according to the system used to isolate the amines. All concentrations of T.M.10 greater than 5 mgs per ml, but less than 50 mgs per ml were found to activate the conversion of the added C^{14}-dopamine to noradrenaline. A concentration of 50 mgs of T.M.10 per ml caused some inhibition (21.8%) of this conversion. Five mgs per ml did not result in any appreciable change in the conversion.

2. Effect of T.M.10 on the conversion of C^{14}-dopamine to noradrenaline by isolated chromaffin granules (free from mitochondria): The results of these experiments (12 and 13) are listed in Table VI. Two concentrations of T.M.10 were investigated (10 and 20 mg T.M.10 per ml of incubation mixture). The concentration of 10 mgs per ml had no appreciable effect on the conversion of C^{14}-dopamine to noradrenaline. However, a 10.9% inhibition of the conversion was caused by 20 mgs T.M.10 per ml.

3. Effect of Marsilid alone on the conversion of C^{14}-dopamine to noradrenaline by homogenates of adrenal medulla: The effect of Marsilid on

the conversion of C^{14}-dopamine to noradrenaline is summarized in Table VII. In both experiments (10 and 11) carried out, Marsilid alone activated the enzymic conversion of C^{14}-dopamine to noradrenaline. By increasing the concentration of Marsilid from 0.45 mgs per ml to 1.7 mgs per ml, there was a corresponding increase in the degree of activation (from 72 to 235%).

II Experiments in which Human Pheochromocytoma was used as the Source of Dopamine-β-Oxidase

1. Effect of T.M.10 on the conversion of C^{14}-dopamine to noradrenaline by a homogenate of human pheochromocytoma: All concentrations of T.M.10 investigated (Table VIII A) produced some inhibition. The degree of inhibition increased with increasing concentrations of T.M.10. (Fig. 11 to 14)

2. Effect of T.M.10 on the conversion of C^{14}-dopamine to noradrenaline by isolated chromaffin granules (free from mitochondria): The only concentration of T.M.10 used was 10 mgs per ml. It caused a 13.8% inhibition in the conversion (Table VIII B). (Fig. 15 and 16)

3. Effect of Marsilid alone on the conversion of C^{14}-dopamine to noradrenaline by a homogenate of human pheochromocytoma: The only concentration of Marsilid used, 1.5 mg per ml, produced 12.1% activation (Table IX). (Fig. 17 and 18)

III Effect of β-Methyl T.M.10 on the Conversion of C^{14}-Dopamine to Noradrenaline by Homogenates of Bovine Adrenal Medulla

The effect of β-methyl T.M.10 on this conversion was studied in

experiments 7 to 9. Various concentrations of β -Methyl T.M.10 from 0.25 to 50.0 mg per ml of incubation mixture were investigated. The results of these experiments are listed in Table X. Concentrations of β -Methyl T.M.10 from 1.25 to 12.5 mg per ml all resulted in approximately the same degree of activation (39 to 62%). At lower concentrations (0.25 and 0.50 mg per ml) the degree of activation was decreased considerably. Higher concentrations of the compound (25 and 50 mg per ml) produced inhibition. The degree of inhibition increased with increasing concentration of the "inhibitor".

IV Effect of T.M.10 on Dopa Decarboxylase Activity

The effect of T.M.10 (4.5 to 22.7 mgs/ml) on dopa decarboxylase activity was investigated in experiments 17 and 18 (Figs. 19 and 20). In one experiment some inhibition of dopa decarboxylase (prepared from bovine adrenal medulla) was found (Table XI).

V Summary of Experimental Results

Concentrations of T.M.10 varying from 5 to 23 mgs per ml stimulated the conversion of C^{14}-dopamine to noradrenaline by homogenates of bovine adrenal medulla. Higher concentrations of T.M.10 produced inhibition. The conversion of C^{14}-dopamine to noradrenaline by homogenates of human pheochromocytoma was inhibited to varying degrees by all the concentrations of T.M.10 studied. The degree of inhibition increased with increasing concentration of T.M.10. The conversion of C^{14}-dopamine to noradrenaline by chromaffin granules (free from mito-

chondria) isolated from both bovine adrenal medulla and human pheochromocytoma was slightly inhibited by 20 mgs per ml and 10 mgs per ml of T.M.10 respectively.

Marsilid alone, stimulated the conversion of C^{14}-dopamine to noradrenaline by homogenates of both bovine adrenal medulla and human pheochromocytoma.

All concentrations of β-methyl T.M.10 less than 25 mgs per ml activated the conversion of C^{14}-dopamine to noradrenaline by homogenates of bovine adrenal medulla. Higher concentrations produced inhibition.

Concentrations of T.M.10 from 4.5 to 22.7 mgs/ml produced some inhibition of dopa decarboxylase activity.

A summary of the results of all the experiments described above is given in Table XII.

CHAPTER V

DISCUSSION OF RESULTS

The experiments described in this thesis were carried out to investigate the effects of T.M.10 and of β-Methyl T.M.10 on dopamine-β-oxidase. Both bovine adrenal medulla and human pheochromocytoma tissue were used as a source of the enzyme on the assumption that the properties of dopamine-β-oxidase of the adrenal medulla and pheochromocytoma tissues are representative of those of chromaffin tissue in general.

In experiments 1 to 4, 5 and 8, whole homogenates of bovine adrenal medulla were used as the source of the enzyme. The effect of T.M.10 on the conversion of C^{14}-dopamine to noradrenaline by whole homogenates of bovine adrenal medulla was shown to vary with the concentration of T.M.10 (Tables IV and V).

No significant inhibition was observed in the presence of the lowest concentration of T.M.10 used (5 mg/ml). However, 21.8% inhibition was observed in the presence of the highest concentration of T.M.10 used (50 mg/ml). Intermediate concentrations of T.M.10 stimulated the conversion of C^{14}-dopamine to noradrenaline. Varying degrees of activation were observed in different experiments with identical concentrations of T.M.10. Thus a T.M.10 concentration of 11.4 mg/ml caused between 84.5% to 540% activation. This variation is probably due to the different sensitivities of the different tissue preparations to T.M.10 and might be explained in terms of a difference in the sensitivity to T.M.10 of the

dopamine-β-oxidase and amine oxidase from different animals. The bovine tissue used in these experiments came from different animals and as was previously mentioned, fresh homogenates were used in almost each new experiment. This argument is strengthened by a comparison of the experiments carried out with human pheochromocytoma tissue (Fig. 11 to 18). All of these latter experiments were carried out with tissue from the same source. Accordingly, the relationship between the effects produced by different concentrations of T.M.10 is very close. For example, Fig. 11 to 14 illustrate the increasing degree of inhibition obtained by increasing the concentration of T.M.10.

With homogenates of human pheochromocytoma all concentrations of T.M.10 studied produced inhibition. The only difference between the procedure in these experiments and those with the bovine adrenal medulla homogenates was the source of the tissue.

The completely opposite effect of T.M.10 with homogenates of human pheochromocytoma tissue from that observed with the intermediate concentrations of T.M.10 on homogenates of bovine adrenal medulla might be explained in at least two ways:

(1) there is a species difference in dopamine-β-oxidase or

(2) T.M.10 is not a specific inhibitor of dopamine-β-oxidase but also affects other enzymes in the homogenate such as amine oxidase.

The first possibility, namely a species difference, cannot be excluded on the basis of experiments reported in this thesis. Coupland and Exley (1958) discovered that T.M.10 acts to deplete the adrenal medulla of rats of catechol amines, and attributed this effect to an inhibition of dopamine-β-oxidase. If this is the mode of action of T.M.10, then

it is possible that human and rat and presumably also cat dopamine-β-oxidase differs from bovine dopamine-β-oxidase.

The alternative explanation is that T.M.10 acts on other enzymes in the whole homogenate. An inhibition of amine oxidase by T.M.10 has been reported (Brown and Hey, 1956). It is also known that homogenates of adrenal medulla contain an active amine oxidase (Langemann, 1951) and that when dopamine is incubated with bovine adrenal medulla homogenates much of it is destroyed by amine oxidase (Hagen and Welch, 1956). It has been observed that concentrations of iproniazid as high as 1.3×10^{-3} M are necessary to produce 50% inhibition of bovine adrenal medulla amine oxidase. Iproniazid at a concentration of 5.6×10^{-3} M produced complete inhibition of the enzyme (Hagen, P. and Black, W.; 1961). In experiment 11 described in this thesis, it was demonstrated that iproniazid at a concentration of 6.1×10^{-3} M stimulated the conversion of C^{14}-dopamine to noradrenaline to an extent of 225%. At a concentration of 1.6×10^{-3} M iproniazid produced 72% stimulation.

It has also been observed that a concentration of T.M.10 of 1.1×10^{-2} M likewise can inhibit bovine adrenal medulla amine oxidase and to an extent of 50%. A 1.1×10^{-1} M concentration of T.M.10 produced complete inhibition of the enzyme (Hagen, P. and Black, W.; 1961). In comparing these data to the results of experiments reported in this thesis (Table XII), one notes that the concentration of T.M.10 which produced 50% inhibition of amine oxidase had no significant effect on the conversion of C^{14}-dopamine to noradrenaline by a homogenate of bovine adrenal medulla. Also, 1.75×10^{-1} M T.M.10, which is sufficient to produce 100% inhibition of bovine adrenal medulla, produced depression of the conversion of C^{14}-dopamine to noradrenaline by a homogenate of bovine adrenal medulla to an extent

of 21%. All other concentrations of T.M.10 studied, which are intermediate to the above two concentrations, stimulated the conversion of C^{14}-dopamine to noradrenaline. Thus, during the prolonged (5 hours) incubation period used in these experiments, it is probable that the dopamine was protected by the amine-oxidase-inhibiting action of T.M.10 and was therefore present in higher concentrations for conversion to noradrenaline throughout the whole incubation period. This hypothesis is supported by experiments in which Marsilid (1-isonicotinyl-2-isopropylhydrazine), a well known inhibitor of amine oxidase (Zeller and Barsky, 1952), was incubated with homogenates of bovine adrenal medulla and of human pheochromocytoma. A marked stimulation of the conversion of C^{14}-dopamine to noradrenaline was observed with bovine adrenal medulla homogenates and a small, yet possibly significant (12%), stimulation with a homogenate of human pheochromocytoma tissue.

This hypothesis is further supported by experiments in which amine oxidase was omitted from the incubation mixture by the use of chromaffin granules free from mitochondria as a source of the enzyme. Whereas dopamine-β-oxidase is known to be localized in the chromaffin granules (Kirshner, 1959), the amine oxidase is found in the mitochondria (Blaschko, Hagen, and Welch, 1955). Whether the chromaffin granules were derived from bovine adrenal medulla or from human pheochromocytoma tissue, T.M.10 either had no significant effect at a low concentration (10 mg/ml in the case of bovine chromaffin granules) or caused inhibition (20 mg/ml and 10 mg/ml with bovine and human chromaffin granules respectively).

Thus a probable explanation of the experimental results is that both amine oxidase and dopamine-β-oxidase are inhibited by T.M.10 but

that amine oxidase is more readily inhibited. In homogenates of bovine adrenal medulla the lower doses of T.M.10 (10 to 20 mg/ml) could inhibit the amine oxidase to a greater degree than they inhibit dopamine-β-oxidase. Therefore, the C^{14}-dopamine is preserved and is available for conversion to noradrenaline throughout the whole of the incubation period, so that there is an increased conversion of the dopamine to noradrenaline. Therefore, with these concentrations of T.M.10, the stimulation of the conversion of C^{14}-dopamine to noradrenaline is not due to an activation of the dopamine-β-oxidase, but rather to an inhibition of the amine oxidase. On the other hand, higher concentrations of T.M.10 inhibit not only the amine oxidase but also the dopamine-β-oxidase so that the conversion of C^{14}-dopamine to noradrenaline would be reduced.

There must be some other explanation for the failure of T.M.10 to stimulate noradrenaline formation in homogenates of human pheochromocytoma at concentrations which stimulate the conversion of C^{14}-dopamine to noradrenaline in homogenates of bovine adrenal medulla, even though some inhibition was observed with both human and bovine chromaffin granules. It is possible that the amine oxidase activity of human pheochromocytoma is lower than that of bovine adrenal medulla or more particularly that the ratio of amine oxidase to dopamine-β-oxidase activity is higher in bovine adrenal medulla. In view of the fact that only a limited amount of human pheochromocytoma was available for the study, it was not possible to examine an adequate dosage range of T.M.10 to determine whether or not a qualitative difference existed between the sensitivities to T.M.10 of dopamine-β-oxidase and monoamine oxidase of bovine adrenal medulla and of human pheochromocytoma. Another possible explanation for the inhibition of the conversion of dopamine to noradrenaline

by human pheochromocytoma tissue by the same concentrations of T.M.10 as produced stimulation of the conversion in the bovine tissue might be that the dopamine-β-oxidase and monoamine oxidase of human pheochromocytoma are both sensitive to lower concentrations of T.M.10 than are the same enzymes found in ox adrenal medulla. An action of T.M.10 on both enzymes, dopamine-β-oxidase and amine oxidase, is not altogether surprising in view of the fact that dopamine is an excellent substrate for both enzymes. Thus similarities in the structure of both enzymes might be expected and it would seem probable that T.M.10 and β-Methyl T.M.10 can find attachment to either of the two enzymes, but have a slightly higher affinity for amine oxidase than for the dopamine-β-oxidase.

Except for the fact that different bovine tissue preparations were used as the source of dopa decarboxylase in experiments 19 and 20, no explanation can be offered for the absence of an inhibitory effect by T.M.10 on dopa decarboxylase in experiment 19. Although the slight inhibition of dopa decarboxylase by high concentrations of T.M.10 is at variance with Bain's (1960) claim that T.M.10 does not inhibit dopa decarboxylase, it seems unlikely that the high concentrations necessary to produce this inhibition can be obtained in sympathetic nerve fibres in vivo. It is also unlikely that the concentrations of T.M.10 and of β-Methyl T.M.10 necessary to inhibit dopamine-β-oxidase can ever be obtained in sympathetic nerve fibres in vivo. Therefore some other explanation is required for the pharmacological action of T.M.10. However, if, like Darenthin (Boura et al, 1959a), T.M.10 and β-Methyl T.M.10 can be selectively concentrated in sympathetic nervous tissue perhaps sufficiently high concentrations of these agents might occur to

produce inhibition of the dopamine-β-oxidase *in situ*. Such a possibility, however, seems unlikely.

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