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ACTION OF TM 10 (CHOLINE 2:6-XYLYL ETHER BROMIDE) AND ITS β-METHYL DERIVATIVE ON THE CONVERSION OF DOPAMINE TO NORADRENALINE BY CHROMAFFIN TISSUE

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Abstract—A study has been made of the effects of TM 10 and of β-methyl TM 10 on the conversion of ^{14}C-dopamine to noradrenaline by homogenates of ox adrenal medulla and of human pheochromocytoma tissue and by isolated chromaffin granules. TM 10, at concentrations between $1{\cdot}75 \times 10^{-2}$ M, and β-methyl TM 10, at concentrations of $4{\cdot}1 \times 10^{-3}$ to $2{\cdot}1 \times 10^{-2}$ M, stimulated the conversion of ^{14}C-dopamine to noradrenaline by homogenates of ox adrenal medulla. A TM 10 concentration of $1{\cdot}7 \times 10^{-1}$ M and a β-methyl TM 10 concentration of $8{\cdot}2 \times 10^{-2}$ and $1{\cdot}6 \times 10^{-1}$ M inhibited the conversion of ^{14}C-dopamine to noradrenaline in homogenates of ox adrenal medulla. The concentration of TM 10 which stimulated the conversion of dopamine to noradrenaline caused almost complete inhibition of the amine oxidase of adrenal medulla. Likewise, iproniazid (Marsilid) stimulated the conversion of ^{14}C-dopamine to noradrenaline by homogenates of ox adrenal medulla at concentrations which inhibited the amine oxidase of adrenal medulla.

As very high concentrations of TM 10 and β-methyl TM 10 are required to inhibit dopamine β-oxidase *in vitro*, it is unlikely that this can be the mechanism of the inhibition of sympathetic nervous activity produced by these drugs *in vivo*.

Details are given of a procedure for the separation of adrenaline, noradrenaline and dopamine by ion-exchange chromatography on Dowex-50.

DURING the past few years there has been considerable interest in a new group of antihypertensive compounds, which include TM 10 (choline 2:6-xylyl ether bromide), its β-methyl derivative and Bretylium (N-*o*-bromobenzyl-N-ethyl, N:N-dimethyl ammonium bromide). The first of these to be studied was TM 10, which was found to interfere with the secretion of noradrenaline at adrenergic nerve endings in response to sympathetic nerve stimulation, without abolishing conduction in the nerve.[1] Bain and Fielden[2] and Coupland and Exley[3] suggested that the action of TM 10 might be due to its ability to prevent the biosynthesis of noradrenaline in sympathetic nerves, and suggested that it probably inhibited dopamine β-oxidase. They subsequently demonstrated, with one concentration of TM 10, an inhibition of the conversion of ^{14}C-dopamine to noradrenaline in a homogenate of human pheochromocytoma.[4]

This paper reports a study of the effects of different concentrations of TM 10 on the conversion of ^{14}C-dopamine to noradrenaline by homogenates of ox adrenal medulla and of human pheochromocytoma tissue and by isolated chromaffin granules of ox adrenal medulla and of human pheochromocytoma tissue. Chromaffin granules

have been used in some of these experiments because they are the intracellular site of dopamine β-oxidase, the enzyme which effects the conversion of dopamine to noradrenaline. Both bovine adrenal medulla and human pheochromocytoma were used as sources of the dopamine β-oxidase on the assumption that the properties of the enzyme of these tissues are representative of those of chromaffin tissue in general.

METHODS

Dopamine-β-^{14}C was incubated with homogenates of ox adrenal medulla or of human pheochromocytoma tissue or with isolated chromaffin granules. The adrenaline and noradrenaline were subsequently separated by ion-exchange chromatography. The radioactivity of the isolated noradrenaline served as an indication of the conversion of dopamine to noradrenaline. The effects of various concentrations of TM 10 or of β-methyl TM 10 on this conversion were determined by comparing the specific activity of the noradrenaline, isolated from the samples to which TM 10 had been added prior to incubation, with that of the noradrenaline isolated from control samples, which had been incubated without any TM 10 or β-methyl TM 10.

Preparation of homogenate

Adrenal glands were removed from cattle as soon as possible after killing and placed on ice for transport to the cold room. All subsequent manipulations were carried out at 2 °C. The medulla tissue was finely minced and then homogenized in a sucrose solution (0·4 M) containing phosphate buffer (pH 7·4; 0·04 M) and sodium versenate (0·01 M), to make a 1 in 3 or 1 in 4 homogenate. This was "the homogenate" used in most experiments. It was general practice to include versenate in the isotonic sucrose solutions used for the homogenization, because in its presence better separations of mitochondria and chromaffin granules could be obtained subsequently, in those experiments in which they were required.

Preparation of chromaffin granules

The homogenate was centrifuged for 30 min at 900 **g** to remove nuclei and unbroken cells. Centrifugation of the nuclei-free supernatant fraction for 30 min at 11,000 **g** brought down the "large granules". The "large granules" were resuspended in a small volume of sucrose solution (0·4 M), layered over 3 ml of a more concentrated solution (1·8 M) of sucrose in a plastic centrifuge tube, and centrifuged for 60 min at 100,000 **g** in a horizontal field. After centrifugation, most of the chromaffin granules were at the bottom of the tube, whereas most of the mitochrondria were present as a band above the 1·8 M sucrose solution. The tube was cut below the mitochondrial layer with a special guillotine and the mitochondria were removed with a Pasteur pipette. The large granule pellet at the bottom of the tube was resuspended in water and kept at — 35 °C until used. A detailed description of the separation of mitochrondria and chromaffin granules has been given previously.[5]

Incubation procedure

Each flask contained the following ingredients in a volume of 4 ml: 3 ml of adrenal medulla homogenate, 1 ml of 0·2 M potassium phosphate buffer (pH 7·4) containing 120 μg (0·008 μc) of dopamine β-^{14}C, 1·5 mg of ATP, and 1·5 mg of fumaric acid. Fumarate was added because Levin *et al.*[6] had shown that, in a partially purified

dopamine β-oxidase system, the conversion of dopamine to noradrenaline was enhanced by the addition of ATP and fumarate. Uniformity of composition, especially of specific activity, of both the test and the control, was assured by first putting all the ingredients of both 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 TM 10 or β-methyl TM 10 was added to the other. The final concentrations of TM 10 were $1{\cdot}75 \times 10^{-2}$ M, 4×10^{-2} M, $4{\cdot}4 \times 10^{-2}$ M, $1{\cdot}75 \times 10^{-1}$ M and of β-methyl TM 10 $8{\cdot}2 \times 10^{-4}$ M, $1{\cdot}6 \times 10^{-3}$ M, $4{\cdot}1 \times 10^{-3}$ M, $8{\cdot}2 \times 10^{-3}$ M, $2{\cdot}1 \times 10^{-2}$ M, $4{\cdot}1 \times 10^{-2}$ M, $8{\cdot}2 \times 10^{-2}$ M and $1{\cdot}6 \times 10^{-1}$ M, respectively. The flasks containing the test and control samples were incubated in a shaking incubator at 37 °C. After incubation for 5 hr, the reaction was stopped by the addition of 6·5 ml of perchloric acid (0·8 N), 6 μmoles of carrier noradrenaline were added to the flasks, the precipitated protein was removed by centrifugation, a few milligrams of ascorbic acid were added to each sample to prevent oxidation of the catechol amines, and the supernatant fractions were neutralized to pH 6·8 with a small volume of 40 per cent potassium hydroxide. The supernatant solutions were filtered onto the ion-exchange column for chromatographic separation of the noradrenaline from adrenaline and dopamine.

Similar experiments were carried out in which Marsilid (*iso*propyl *iso*nicotinyl hydrazide) was added to the incubation mixture instead of TM 10. The concentrations of Marsilid used were $1{\cdot}6 \times 10^{-3}$ M and $6{\cdot}1 \times 10^{-3}$ M with homogenates of bovine adrenal medulla and $5{\cdot}4 \times 10^{-3}$ M with a homogenate of human pheochromocytoma.

The effect of TM 10 on the conversion of ^{14}C-dopamine to noradrenaline was also studied in similar incubation experiments in which homogenates of human pheochromocytoma or suspensions of chromaffin granules, isolated from ox adrenal medulla or from human pheochromocytoma, served as the source of the dopamine β-oxidase. The concentrations of TM 10 used in these experiments are shown in Tables 3 and 4.

Ion-exchange separation of dopamine, noradrenaline and adrenaline

The procedure is based on the report by Ellman[7] that it is possible to separate these three catecholamines by elution from Dowex-50 with hydrochloric acid. By trial and error, several satisfactory systems were developed. For most of the experiments reported in this paper, the catecholamine-containing solution, adjusted to pH 6·8, was filtered onto a 68 × 1·0-cm column of Dowex 50 × 8, 250-mesh, in the hydrogen-form; elution was carried out with 0·3 N hydrochloric acid. After from 1 to 1·5 l. of the solution of hydrochloric acid had passed through the column, 10-ml fractions of eluate were collected. The noradrenaline and adrenaline were completely eluted by the time 3 l. of the 0·3 N hydrochloric acid had passed through the column; a further 1·5 to 2 l. were required to elute the dopamine. However, once the adrenaline had been eluted, the dopamine could be rapidly and completely eluted with a much smaller volume, by passing 3 N HCl into the 500-ml reservoir containing the 0·3 N HCl, so that a constantly increasing hydrochloric acid gradient was produced. Some of the advantages of this system over the carboxylic resin system[8] are: (1) the noradrenaline, adrenaline and dopamine are more clearly separated; (2) the operation of the column at low pH prevents autoxidation of the catecholamines on the columns; and (3) the

only other material in the aqueous adrenaline and noradrenaline eluate is hydrochloric acid, which on evaporation leaves pure adrenaline, noradrenaline or dopamine hydrochloride.

After elution from the column, the concentrations of noradrenaline and adrenaline in the fractions of eluate were determined spectrophotometrically by measuring the absorption of each fraction at 280 mμ, at which wavelength the molar absorbancy of adrenaline and noradrenaline is $2{\cdot}80 \times 10^{-3}$.

For estimation of the radioactivity of each fraction, samples of 0·5, 1·0 or 2·0 ml were evaporated to dryness on stainless steel planchets and counted at infinite thinness in an automatic gas flow counter.

In every experiment the identity of the noradrenaline was confirmed by paper chromatography of a small amount of the noradrenaline eluate in a phenol hydrochloric acid system. In some experiments the association of radioactivity with the noradrenaline spot was confirmed by autoradiography of the paper chromatograms.

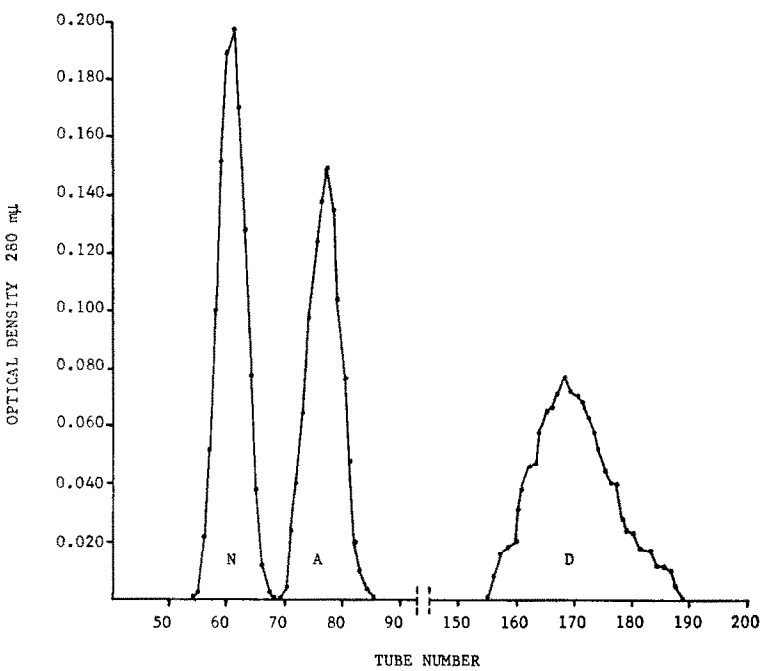

FIG. 1. Portions of a chromatogram obtained by eluting a mixture of 3 μmoles each of noradrenaline (N), adrenaline (A), and dopamine (D) from a 35 × 0·7-cm column of Dowex-50 resin with 0·3 N HCl. The recoveries were noradrenaline 2·8 μmoles, adrenaline 3·0 μmoles, and dopamine 3·1 μmoles. Each tube collected 7·0 ml of eluate. The identity of the amines was confirmed by paper chromatography in a phenol–HCl system.

Because in any one experiment the total amount of noradrenaline in all incubation flasks was identical, the greater the conversion of ^{14}C-dopamine to ^{14}C-noradrenaline the higher was the specific activity of the noradrenaline recovered, and vice versa. Hence, the specific activity of the test samples, as compared to that of the control samples, was a measure of the stimulation or inhibition of the conversion of dopamine to noradrenaline by the agents added to the test flasks.

Amine oxidase activity was measured manometrically[9] using the dialysed adrenal mitochondrial fraction as a source of the enzyme,[10] tyramine (0·002 M) as substrate, and oxygen as the gas phase. The initial linear rate of oxygen uptake was taken as a measure of the activity of this enzyme, and the inhibition of oxygen uptake by the agents added (TM 10 or β-methyl TM 10) as a measure of the inhibition of the enzyme; the oxygen consumption of the mitochondrial fraction also was measured, in the absence of substrate, and appropriate corrections were made.

RESULTS

The effect of different concentrations of TM 10 *and β-methyl TM* 10 *on the conversion of* ^{14}C-*dopamine to* ^{14}C-*noradrenaline by homogenates of bovine adrenal medulla* (*Table* 1). Concentrations of TM 10 between 8×10^{-2} M and $1{\cdot}75 \times 10^{-2}$ M stimulated the conversion to noradrenaline of ^{14}C-dopamine. However, the same concentration of TM 10 (4×10^{-2} M) caused different degrees of stimulation with different preparations of homogenate. A concentration of TM 10 of $1{\cdot}75 \times 10^{-1}$ M (50 mg/ml) caused some inhibition; however, at a concentration of $1{\cdot}75 \times 10^{-2}$ M the conversion was not modified appreciably.

TABLE 1. THE EFFECT OF DIFFERENT CONCENTRATIONS OF TM 10 ON THE CONVERSION OF ^{14}C-DOPAMINE TO NORADRENALINE BY HOMOGENATES OF BOVINE ADRENAL MEDULLA

Experiment number	Molar concentration of TM 10	Specific activity of noradrenaline (counts/min per μmole)	% activation or inhibition
1	0	784	
	8×10^{-2}	8568	+ 993
2	0	1120	
	4×10^{-2}	7168	+ 539
3	0	3136	
	4×10^{-2}	10,332	+ 229
4	0	3612	
	4×10^{-2}	6664	+ 84·5
5	0	5012	
	$1{\cdot}75 \times 10^{-2}$	4900	− 2·2
6	0	2576	
	$4{\cdot}4 \times 10^{-2}$	4816	+ 87·2
	$1{\cdot}75 \times 10^{-1}$	2016	− 21·8

The effect of β-methyl TM 10 *on the conversion of* ^{14}C-*dopamine to noradrenaline* (*Table* 2). Concentrations of β-methyl TM 10 from $4{\cdot}1 \times 10^{-3}$ M to $2{\cdot}1 \times 10^{-2}$ M stimulated the conversion. At lower concentrations, $3{\cdot}2 \times 10^{-4}$ M, the stimulation was insignificant; higher concentrations, $8{\cdot}2 \times 10^{-2}$ M and $1{\cdot}6 \times 10^{-1}$ M, produced inhibition.

The effect of TM 10 *on the conversion of* ^{14}C-*dopamine to noradrenaline by bovine chromaffin granules* (*Table* 3). At the concentrations studied, $3{\cdot}5 \times 10^{-2}$ M and 1×10^{-2} M, no significant effect and a 10 per cent inhibition, respectively, were observed.

Human pheochromocytoma tissue. When incubated with homogenates of human pheochromocytoma tissue the concentrations of TM 10 studied, $1{\cdot}75 \times 10^{-2}$ M,

$3{\cdot}5 \times 10^{-2}$ M and 7×10^{-2} M, all caused inhibition of the conversion of ^{14}C-dopamine to noradrenaline (Table 4). A concentration of TM 10 of $3{\cdot}5 \times 10^{-2}$ M slightly inhibited the conversion by human chromaffin granules (Table 4).

Effect of Marsilid on the conversion of ^{14}C-dopamine to ^{14}C-noradrenaline by homogenates of bovine adrenal medulla and by a homogenate of human pheochromocytoma (*Table* 5). Marsilid at concentrations of $1{\cdot}6 \times 10^{-3}$ M and $6{\cdot}1 \times 10^{-3}$ M stimulated the conversion 72 and 235 per cent, respectively, in homogenates of bovine adrenal

TABLE 2. THE EFFECT OF β-METHYL TM 10 ON THE CONVERSION OF ^{14}-C-DOPAMINE TO NORADRENALINE IN HOMOGENATES OF BOVINE ADRENAL MEDULLA

Experiment number	Molar concentration of β-Me TM 10	Specific activity of noradrenaline (counts/min per μmole)	% activation or inhibition
7	0	1176	
	$8{\cdot}2 \times 10^{-4}$	1232	+ 4·8
	$1{\cdot}6 \times 10^{-3}$	1428	+ 21·4
	$2{\cdot}1 \times 10^{-2}$	1876	+ 59·7
8	0	2576	
	$4{\cdot}1 \times 10^{-3}$	3640	+ 41·4
	$4{\cdot}1 \times 10^{-2}$	4172	+ 62·0
9	0	2296	
	$8{\cdot}2 \times 10^{-3}$	3192	+ 39·1
	$8{\cdot}2 \times 10^{-2}$	2016	− 12·2
	$1{\cdot}6 \times 10^{-1}$	728	− 68·3

TABLE 3. EFFECT OF TM 10 ON THE CONVERSION OF ^{14}C-DOPAMINE TO NORADRENALINE BY ISOLATED BOVINE CHROMAFFIN GRANULES

Experiment Number	Molar concentration of TM 10	Mean specific activity of noradrenaline (counts/min per μmole)	% activation or inhibition
12	0	23,184	
	$3{\cdot}5 \times 10^{-2}$	23,828	+ 2·8
13	0	3080	
	$7{\cdot}0 \times 10^{-2}$	2744	− 10·9

medulla. A concentration of $5{\cdot}4 \times 10^{-3}$ M caused 15·3 per cent stimulation in a homogenate of human pheochromocytoma.

Effect of TM 10 *and of Marsilid on amine oxidase of bovine adrenal medulla.* A concentration of TM 10 of $1{\cdot}1 \times 10^{-1}$ M caused 47 per cent inhibition and a concentration of $1{\cdot}1 \times 10^{-1}$ M caused complete inhibition. A Marsilid concentration of between

$1{\cdot}3 \times 10^{-3}$ M and $2{\cdot}5 \times 10^{-3}$ M produced 50 per cent inhibition; complete inhibition was produced by a concentration of $5{\cdot}6 \times 10^{-3}$ M.

No significant amine oxidase activity could be detected in the mitochrondria isolated from the human pheochromocytoma.

TABLE 4. EFFECT OF TM 10 ON THE CONVERSION OF ^{14}C-DOPAMINE TO NORADRENALINE BY A HOMOGENATE OF HUMAN PHEOCHROMOCYTOMA OR BY ISOLATED HUMAN CHROMAFFIN GRANULE

Experiment number	Molar concentration of TM 10	Mean specific activity of noradrenaline (counts/min per μmole)	% activation or inhibition
14	* 0	8652	
	* $1{\cdot}75 \times 10^{-2}$	6776	− 21·7
	* $3{\cdot}50 \times 10^{-2}$	5768	− 33·4
	* $7{\cdot}00 \times 10^{-2}$	2940	− 66·0
16	† 0	1624	
	† $3{\cdot}50 \times 10^{-2}$	1400	− 13·8

* Whole homogenate.
† Isolated chromaffin granules.

TABLE 5. EFFECT OF MARSILID ON THE CONVERSION OF ^{14}C-DOPAMINE TO NORADRENALINE BY A HOMOGENATE OF BOVINE ADRENAL MEDULLA OR OF HUMAN PHEOCHROMOCYTOMA

Experiment number	Molar concentration of Marsilid	Mean specific activity of noradrenaline (counts/min per μmole)	% activation or inhibition
10	* 0	784	
	* $1{\cdot}6 \times 10^{-3}$	1344	+ 72
11	* 0	1120	
	* $6{\cdot}1 \times 10^{-3}$	3752	+ 235
15	† 0	8792	
	† $5{\cdot}4 \times 10^{-3}$	10.036	+ 15·3

* Bovine adrenal medulla.
† Human pheochromocytoma.

DISCUSSION

The concentration of TM 10 (4×10^{-2} M) used in the first experiments was suggested by the report of Bain and Fielden,[4] which indicated that this concentration of TM 10 inhibits the conversion of ^{14}C-dopamine to ^{14}C-noradrenaline by homogenates of human pheochromocytoma. Far from the finding of an inhibition, however, a stimulation of the conversion of ^{14}C-dopamine to noradrenaline by

homogenates of ox adrenal medulla was observed with TM 10 at a concentration of 4×10^{-2} M. Only with very high concentrations of TM 10, 0·0175 M and 0·175 M, was any inhibition observed and, with the latter concentration, this inhibition amounted to only 21·8 per cent. With β-methyl TM 10, stimulation of the conversion of ^{14}C-dopamine to noradrenaline was likewise observed with concentrations below $8{\cdot}2 \times 10^{-2}$ M. Higher concentrations certainly produced inhibition, 0·16 M causing 68·3 per cent inhibition. No significant stimulation was seen when ox adrenal chromaffin granules served as the source of dopamine β-oxidase.

With human pheochromocytoma tissue, TM 10 at a concentration of $1{\cdot}75 \times 10^{-2}$ M produced 21·7 per cent inhibition; higher concentrations produced greater inhibition.

Since adrenal medulla contains an active amine oxidase,[11] an enzyme whose activity is inhibited by TM 10,[12] the possibility was considered that the lower concentrations of TM 10 and of β-methyl TM 10 used might produce the observed stimulation by inhibiting the amine oxidase, in this manner preventing the destruction of dopamine by the amine oxidase. If this were the case, the concentration of the ^{14}C-dopamine would be expected to remain high and to be available for conversion to noradrenaline throughout the incubation period. For this reason the effect of the well-known amine oxidase inhibitor, Marsilid (*iso*propyl *iso*nicotinyl hydrazide),[13] on the conversion of ^{14}C-dopamine to noradrenaline was studied. Like TM 10 and β-methyl TM 10, Marsilid stimulated the conversion of ^{14}C-dopamine to noradrenaline in whole homogenates of adrenal medulla. A concentration of Marsilid of $5{\cdot}6 \times 10^{-3}$ M produced 100 per cent inhibition of adrenal medulla amine oxidase. A similar concentration, $6{\cdot}1 \times 10^{-3}$ M, caused 235 per cent stimulation of the conversion of ^{14}C-dopamine to noradrenaline. A concentration of TM 10 of 4×10^{-2} M, which gave between 70·3 and 87·5 per cent inhibition of amine oxidase, caused 229, 839 and 84·5 per cent stimulation of the conversion of ^{14}C-dopamine to noradrenaline.

In view of these findings it is probably justifiable to suggest that the stimulation of the conversion of dopamine to noradrenaline by the lower concentrations of TM 10 and β-methyl TM 10 can be attributed to the inhibition of the amine oxidase in the adrenal medulla homogenates by concentrations of these agents which are inadequate to inhibit dopamine β-oxidase. This prevents the destruction of dopamine and permits a high concentration of dopamine to be available as a substrate for dopamine β-oxidase throughout the whole incubation period. On the other hand, still higher concentrations do inhibit the dopamine β-oxidase and thus directly reduce the conversion of ^{14}C-dopamine to noradrenaline. This conclusion is further supported by the experiments in which isolated chromaffin granules served as the source of the dopamine β-oxidase. The amine oxidase of adrenal medulla is known to be localized in the mitochondria and absent from the chromaffin granules.[5] With this latter preparation no significant stimulation of the conversion of ^{14}C-dopamine to noradrenaline by TM 10 was observed. On the other hand, no stimulation of the conversion of ^{14}C-dopamine to noradrenaline was observed with homogenates of human pheochromocytoma, but inhibitions of between 21·7 and 66 per cent were observed with all concentrations of TM 10 tested. This might be explained by assuming that the amine oxidase of human pheochromocytoma is much less active than that of ox adrenal medulla or that the dopamine β-oxidase is more sensitive to the TM 10. The former hypothesis is supported by our inability to detect any significant amine oxidase activity in the mitochrondria derived from the pheochromocytoma.

It is most unlikely that such high concentrations of TM 10 as are required partially to inhibit the dopamine β-oxidase of ox adrenal medulla or of human pheochromocytoma tissue could ever be present in any of the body fluids of animals receiving these drugs in doses such as would inhibit sympathetic activity. Thus, both TM 10 and β-methyl TM 10 must act to inhibit sympathetic nervous activity by some other mechanism than by inhibiting dopamine β-oxidase.

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