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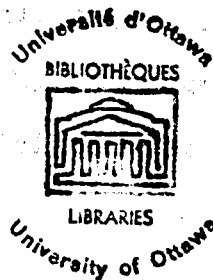


OCT 28 1964

ARCS

**METABOLISM OF dl-ADRENALINE- $\beta$ -C<sup>14</sup>  
IN THE RAT TREATED WITH VARIOUS DRUGS**

by K. MISSALA



Thesis submitted to the Faculty  
of Medicine, in partial fulfil-  
ment of the requirements for the  
degree of Master of Science.

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September 1964



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This work was suggested and directed by  
Prof. Dr. A. D'IORIO to whom I am greatly  
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I N T R O D U C T I O N

PRINCIPAL METABOLIC PATHWAYS OF CATECHOLAMINES.

The discovery of adrenaline and its pharmacological properties goes back to the end of the last century.

In 1895, OLIVER and SCHAFFER observed that the injection of the extracts from adrenal glands increased arterial blood pressure and caused the resulting vasoconstriction [OLIVER 1895]. The same year CYBULSKI reported that, especially under stress, the adrenal vein blood contained a substance responsible for the vasopressive activity [CYBULSKI 1895]. During the next few years, many investigators tried to purify and to isolate the "active principle" of the adrenal medulla [ABEL 1899; TAKAMINE 1901]. Finally, in 1904, STOLTZ succeeded in synthesizing adrenaline, the first hormone obtained chemically, and its non-methylated homologue: nor-adrenaline [STOLTZ 1904].

ELLIOTT, in a series of experiments, used a small dose of adrenaline and confirmed the results obtained by OLIVER. Also, the physiological effects of adrenaline were found to be similar to those observed following the stimulation of sympathetic nerves [ELLIOTT 1905]. Thus, he identified adrenaline with the chemical transmitter.

BARGER and DALE also tried to identify adrenaline with the sympathetic transmitter and advanced the hypothesis that the single methyl group on the nitrogen is a basic factor in the balance between the excitatory and inhibitory actions of

catecholamines [BARGER 1910].

Twenty years after, however, the observations of ELLIOTT were contested by CANNON and ROSENBLUETH. These authors provided evidence that adrenaline and sympathin did not act in the same way. They postulated the existence of two various, active substances which, when liberated by adrenergic nerve fibres, possessed different properties:

- (1) Inhibitory - Sympathin I;
- (2) Excitatory - Sympathin E (CANNON 1933).

The Sympathin I was identified hypothetically with adrenaline, and Sympathin E with noradrenaline (BACQ 1934).

The discovery of iproniazid, an inhibitor of monoamine oxidase (MAO) (ZELLER 1952), advanced the study of this enzyme and its role in the metabolism of catecholamines.

SCHAYER and SMILEY observed that the use of this inhibitor increased the amount of adrenaline labeled in methyl position excreted in urine. This compound was also found to be very active in VITRO (SCHAYER 1953).

However, the inhibition of this enzyme did not increase the duration of action of endogenous or exogenous catecholamines (BURN 1954).

For many years the oxidative deamination was considered to be the most important step in the metabolism of catecholamines (BLASCHKO 1952; 1954; SCHAYER 1953).

SCHAYER found that, the conjugation with sulfuric or

glucuronic acid, insignificant under physiological conditions, became important when a large quantity of catecholamine was ingested.

The discovery of O-methylation (ARMSTRONG 1957) was a very important step in the study of the metabolic pathway of catecholamines. The identification by ARMSTRONG et al. of 3-methoxy-4-hydroxymandelic acid, as a urinary metabolite of adrenaline, directed later research towards the O-methylation. That same year, AXELROD demonstrated the importance of O-methylation in the metabolism of catecholamines and PELLERIN et al. showed that catecholacids are also O-methylated (AXELROD 1957; PELLERIN et al. 1957). In 1958, AXELROD isolated an enzyme from rat liver responsible for this reaction.

The inhibition of MAO increased twofold the excretion of metanephrine, suggesting that monoamine oxidase deaminates a considerable amount of metanephrine to 3-methoxy-4-hydroxymandelic acid, and that O-methylation of catecholamines occurs before deamination (AXELROD 1958; KOPIN, AXELROD, GORDON 1961; LABROSSE, AXELROD 1961).

The inhibition of catechol-O-methyl transferase blocks the methylation of adrenaline (KOPIN 1961). But the combined blockage of both enzyme systems results in the formation of a new metabolite of adrenaline (KIRSHNER 1960), and in the increased production of acidic, mainly conjugated catabolites,

unidentified (DESCHAEFDRYVER, KIRSHNER 1961).

Recently, KOPIN and his colleagues demonstrated that binding plays an important role in the inactivation of catecholamines. In rat and mouse, the injected adrenaline or noradrenaline disappears from the whole animal and is excreted in two phases:

- 1/ in the first phase, the major route of metabolism is O-methylation;
- 2/ in the second phase, after several hours, the bound catecholamine is slowly released, and it seems that it is metabolized by monoamine oxidase (KOPIN et al. 1962).

In 1954, VON EULER and his co-workers advanced the hypothesis that adrenaline is probably not metabolized before it is released into the circulation, so that injected adrenaline may follow the same metabolic pathway as the endogenous hormone. (KOPIN 1963). Nevertheless, noradrenaline may be formed and metabolized in the tissue without reaching the circulation.

The deviation of the metabolism of adrenaline, resulting in the formation of adrenochrome or of its autooxidation, was considered by certain researchers to be involved in schizophrenia (HOFFER 1957). However, SZARA and AXELROD could not detect any formation of adrenochrome in VIVO (SZARA et al 1958).

Using the hallucinogenic drugs and various inhibitors of catechol-O-methyl transferase in VITRO and in VIVO, we tried to verify the existing hypothesis, in connection with the action of these compounds, on the metabolism of injected adrenaline in a rat, in the light of the recent findings.

It is useful to review shortly the different pathways of the metabolism of catecholamines, and the role played by various enzymatic systems.

The mechanism most often involved in the catabolism of catecholamines are as follow:

- 1/ Oxidative deamination by monoamine oxidase (MAO);
- 2/ Conjugation of catecholamines and their O-methylated metabolites with glucuronic or sulfuric acid;
- 3/ Methylation by catechol-O-methyl transferase (COMT);
- 4/ Binding of exogenous amines.

Fig. 7 presents the metabolic pathway of injected epinephrine in a rat, such as proposed by KOPIN, and based on the most recent experiments (KOPIN 1961).

#### MONOAMINE OXIDASE.

A new approach to the study of catecholamines came with the discovery by HARE of an enzyme able to deaminate oxidatively tyramine. In this reaction, ammonia is released and the corresponding aldehyde is formed. The highest acti-

vity of this enzyme was found in the mitochondria fraction of liver (HARE 1928).

Several investigators have tested the oxidative deamination of adrenaline and other amines using rat liver and brain cortex slice techniques (PUGH 1937; QUASTEL 1933; BLASCHKO 1937; 1933; 1937 a; 1937 b). As a result, two new enzymes were discovered: aliphatic amine oxidase and adrenaline oxidase. Shortly after, it was shown that all three enzymes described above are similar in the chemical reactions they catalyse, their behaviour towards inhibitors and their distribution (KOHN 1937; BLASCHKO et al. 1937). Experiments of GADDUM and KWIATKOWSKI showed that the same enzyme may also oxidize adrenaline in VIVO, since its effects are potentiated by ephedrine (GADDUM 1938). In 1941, ZELLER proposed to name these enzymes monoamine oxidase (MAO), because all of them act upon aliphatic and phenylethyl amines, which have an amino group attached to a terminal carbon atom. (BLASCHKO 1953), and are semicarbazide resistant. This would differentiate it from diamine oxidase (DAO), acting upon short aliphatic diamines, and affected by semicarbazide (ZELLER 1941).

It was shown that about  $2/3$  of the enzymatic activity of MAO is present in the mitochondria, and only  $1/3$  is associated with the microsome fraction (HAWKINS 1951) of the liver (HAWKINS 1952). The occurrence of monoamine oxidase in

all tissues was demonstrated by BLASCHKO. However, the same author could not find any activity of this enzyme in blood (BLASCHKO 1952).

The work of SCHAYER and his associates proved conclusively that MAO plays an important role in the metabolism of adrenaline in VIVO (SCHAYER 1951; 1952; 1953). Using adrenaline- $\beta$ -C<sup>14</sup> and adrenaline methyl-C<sup>14</sup>, these authors were able to demonstrate that, in the inactivation of adrenaline, the  $\beta$  and the methyl carbon follow different metabolic patterns. About 90% of  $\beta$ -C<sup>14</sup> was present in urine; however, at the same time only 60% of methyl-C<sup>14</sup> was excreted. This fact indicated that the molecule was split between those two carbons.

The potentiation of adrenaline-like action is suggested to be due to monoamine oxidase inhibition (BLASCHKO 1954).

Oxidative deamination is considered to be a naturally occurring process in animals. PELLERIN and D'IORIO and ARMSTRONG and SHAW found the deaminated acidic compound, 3-homovanillic acid, excreted in urine after injection of 3,4-dihydroxyphenylalanine (DOPA) (PELLERIN 1955; 1957; SHAW 1956; 1957). Also, ARMSTRONG and M<sup>C</sup>MILLAN isolated the 3-methoxy-4-hydroxymandelic acid from the urine of a patient with pheochromocytoma. This acid was recognized to be a metabolite of both adrenaline and noradrenaline, since both catechol-

amines are substrates for the same enzyme - monoamine oxidase (ARMSTRONG 1957). Then, ARMSTRONG proposed the hypothesis that 3-methoxy-4-hydroxymandelic acid (VMA) might be formed by the action of monoamine oxidase (MAO) upon adrenaline or noradrenaline followed by methylation of the resulting 3,4-dihydroxymandelic acid (DHMA) (ARMSTRONG 1957). The occurrence in urine of DHMA was supported by VON EULER (1958).

The oxidative deamination was especially studied by ZELLER (1958). He observed that the potentiation and the protection of catecholamines by iproniazid is strong evidence in favour of the theory that the catecholamines are attacked first by monoamine oxidase, ... The lack of potentiation is an indication that methylation is involved in the process of inactivation (ZELLER 1959; ZELLER, BLANKSMAN et al. 1959). However, by pretreating animals with iproniazid, there was a twofold increase in the urinary excretion of metanephrine. These results support the hypothesis that in VIVO O-methylation of adrenaline occurs before the deamination (AXELROD 1958); ZELLER, BLANKSMAN et al. 1959), and that the injected adrenaline retains the methyl group in the chain. In man, 91% of the infused adrenaline  $\beta$ -C<sup>14</sup> was recovered from urine, while this recovery only amounted to 32% when the hormone was labeled in the methyl position. These results suggested that approximately 2/3 of the molecules of infused adrenaline lose the N-methyl group during

the metabolism (RESNICK 1959).

In 1960, KOPIN and AXELROD identified 3,4-dihydroxyphenyl glycol as a compound derived from the deamination of adrenaline and noradrenaline, and which was supposed to serve as a precursor of 3-methoxy-4-hydroxyphenyl glycol. AXELROD demonstrated that iproniazid does not affect the rate of disappearance of catecholamines. It blocks only the deamination of their O-methylated products (AXELROD 1960), and reduces the radioactivity in 3-methoxy-4-hydroxymandelic acid and its non methylated analogue (GOODALL 1958). KIRSHNER confirmed these results (KIRSHNER 1960). The results obtained by BACQ and his associates suggested that the minor fraction of catecholamines, which is deaminated before it is O-methylated, is totally inactivated in the first reaction (BACQ et al. 1961).

BRUNJES and his colleagues reviewed the metabolism of catecholamines and concluded that when oxidative deamination is the first step, the intermediate metabolite formed from adrenaline is 3,4-dihydroxymandelic acid, which in turn may be metabolized by O-methylation to yield 3-methoxy-4-hydroxymandelic acid. This enzyme is also required for the conversion of metanephrine to 3-methoxy-4-hydroxymandelic acid (BRUNJES 1963).

The experiments reported above were performed mostly by using the inhibitors of MAO, so that the physiological

conditions were generally modified. The results observed were sometimes contradictory, and they do not help in an evaluation of the real importance of the role played by this enzyme in the metabolism of catecholamines in VIVO.

However, the discovery of the O-methylation contributed to the generally accepted opinion that monoamine oxidase (MAO) plays only a minor role in the biological inactivation of catecholamines.

#### CONJUGATION.

It is known that conjugation is involved in the detoxification mechanism in VIVO, and that it takes place in the intestine and the liver (PELKAN 1922; LUND 1951; BEYER 1956). The liver is also very effective in conjugating circulating adrenaline (ELLIOTT 1905; TRENDLENEBURG P. 1929; HARTUNG 1946). In VIVO under physiological conditions, the conjugation of a simple phenol is generally incomplete and a portion of it may be eliminated in unchanged form (NOVELLO 1925).

In man, after ingestion of a large dose of adrenaline, the combined adrenaline is excreted in urine within 5 hours (RICHTER 1940). The same investigator obtained evidence that conjugation may be more rapid than oxidation by monoamine oxidase (MAO); he also suggested that this catecholamine may be inactivated by esterification with sulfuric acid (RICHTER

1941; HARTUNG 1946; BACQ 1949), or, by combining through one of its phenolic hydroxyl group with glucuronic acid (CLARK 1951). Thus, by conjugation adrenaline may lose its pressor activity. However, more recently, it was reported that both in men and rats adrenaline is not conjugated with glucuronic acid (AXELROD 1957; SCHMIDT 1958).

In dog, the conjugate isolated from urine, after ingestion of a large quantity of adrenaline, was found to be a glucuronide (BEYER 1945). However, adrenaline injected intravenously was inactivated and eliminated as sulfuric ester (BACQ, FISCHER 1949). From similar experiments, DOGSTON and co-workers concluded that in rabbits, after oral ingestion of d-adrenaline, only an insignificant amount of sulfconjugate was excreted. At the same time the glucuronide represented 21% (DOGSTON 1947; CLARK, DRELL 1954). Previously, it was observed that l-adrenaline suppressed glucuronic acid conjugation in liver slices (LIPSCHITZ 1939).

The evidence obtained by TORDA, demonstrated that cocaine inhibits the esterification of phenols in VIVO, and of the phenol sulfur esterase in VITRO. Thus, it potentiates the action of adrenaline, and may be regarded as indirectly supporting the theory of RICHTER, that adrenaline is inactivated partly by esterification of the phenol ring (TORDA 1943)(a), (b).

More recently, it has been observed that sulfates of

adrenaline and noradrenaline are present in the urine of man and rabbits, and they are conjugated by cell-free extracts of rat liver (GREGORY 1961).

The amount of the conjugated hormone, excreted in urine under physiological conditions, is small. It was shown that after an acid hydrolysis the conjugated adrenaline or noradrenaline was liberated, increasing the level of biologically active amine (VON EULER 1951). Also, it was pointed out that between 1 and 4% only of the intravenously injected catecholamine is excreted in unchanged form (HOLTZ 1947; ELMADJIAN 1956; VON EULER 1954; BACQ 1961). Moreover, SCHAYER found no significant increase in the free adrenaline level, following acid hydrolysis, of urine from rats injected intravenously with a small dose of this hormone. This observation favours the opinion that conjugation is a minor pathway of inactivation of catecholamines under physiological conditions. Nevertheless, its role increases significantly when a large dose is involved (SCHAYER 1951). Confirming this, AXELROD found a large amount, 21%, of conjugated metanephrine glucuronide in the urine of rats injected intraperitoneally with 5 mg of epinephrine bitartrate (AXELROD 1958).

In 1959, the same researcher obtained evidence that the enzyme responsible for conjugation of metanephrine is present in the microsomal fraction of rat liver (AXELROD 1959; 1960). However, DESCHAEPDRYVER and KIRSHNER found that

that in man, metanephrine is not conjugated with glucuronide, but with a sulfate, since incubation of urine with glucuronidase did not give metanephrine (DESCHAEFDRIYVER 1961).

#### O-METHYLATION.

MACLAGAN and WILKINSON first showed that phenolic groups could be methylated in man. After ingestion of 3,5-diiodo-4-hydroxy benzoate, they isolated from urine the corresponding compound methylated in 3-hydroxy position (MACLAGAN 1951).

A few years later, ARMSTRONG, SHAW and WALL studying human urine of patients with phenylketonuria found a wide variety of phenolic acids methylated in meta position (ARMSTRONG 1956). In the following year, the isolation of 3-methoxy-4-hydroxymandelic acid from the urine of patients with pheochromocytoma was reported, and this acid was identified as a major metabolite of adrenaline and noradrenaline (ARMSTRONG 1957; 1959).

PELLERIN and D'IORIO found homovanillic and dihydroxyphenylacetic acids in the urine of rabbits previously given a large dose of DOPA. As well, from the urine of rats injected intraperitoneally with DOPA-C<sup>14</sup>, these investigators separated, by chromatography, four compounds including DOPA, DOPAMINE, 3,4-dihydroxyphenylacetic and 3,4-dihydroxyphenylpyruvic acids (PELLERIN 1955).

In 1957 and 1958, two groups of researchers showed the existence of an enzymatic system capable of methylating catecholacids (PELLERIN 1957), and catecholamines (AXELROD 1957; 1958) in VITRO. This enzyme was found to be present in the soluble fraction of rat liver and kidney homogenate, and to require methionine, ATP and  $Mg^{++}$ ; further, that it is inhibited by sulfhydryl (SH) binding group reagents (AXELROD 1958b). The enzyme described above was named catechol-O-methyl transferase (AXELROD 1958), and was observed to act on any compound that had two hydroxy groups adjacent to one another (AXELROD 1960 c). Metanephrine and normetanephrine were found to be methylated metabolites of adrenaline and noradrenaline respectively (AXELROD 1957), and were present in urine as free and conjugated compounds. Their excretion in urine is increased twofold after pretreatment with iproniazid, suggesting that under physiological conditions, a considerable amount of O-methylated amine is deaminated by monoamine oxidase to give 3-methoxy-4-hydroxymandelic acid (AXELROD et al. 1958). These results support the hypothesis that methylation precedes oxidative deamination of adrenaline. Furthermore, metanephrine and normetanephrine were found in normal, human urine, indicating that O-methylation was a major pathway of the metabolism of catecholamines in man (LABROSSE 1958; KIRSHNER et al. 1958).

The inhibitors of catechol-O-methyl transferase were

shown to prolong the physiological effects of adrenaline probably by competing for this enzyme (AXELROD et al. 1959). AXELROD and KOPIN (1959) demonstrated that, in rats, the 3-methoxy-4-hydroxyphenyl glycol sulfate is an important metabolite of adrenaline and is found in urine; however, in man it represents only a small fraction of the methylated metabolites (AXELROD, KOPIN 1959; KOPIN 1960). It is possible that MHPG might be partly formed by prior deamination of the catecholamines to DHPG, since only a part of MHPG is formed by the deamination of the methylated catecholamines (AXELROD et al. 1960 b).

The conversion of metanephrine to normetanephrine was observed in VITRO when metanephrine was incubated with an enzyme from a microsome fraction of rabbit liver. Similarly, conversion could not be demonstrated in VIVO (AXELROD 1960 b). While, the enzymatic O-demethylation of metanephrine to adrenaline occurs only in VITRO, (AXELROD, SZARA 1958), BACQ does not exclude the possibility that this reaction, to a smaller extent, may occur also in VIVO (BACQ et al. 1961).

In cats, the distribution of urinary metabolites of adrenaline differs from that found in man (KIRSHNER 1960).

In man, the administration of metanephrine resulted in excretion of VMA and MHPG sulfate, indicating that a large portion of it is deaminated.

GOLDSTEIN and co-workers (1960a) using the double labeling technique studied the N-methylation of noradrenaline to adrenaline in rats. They demonstrated that this reaction is irreversible, and that no demethylation of epinephrine occurs in cats (GOLDSTEIN, FRIEDHOFF et al. 1960). However, more recently, VERLY, KOCH et al. demonstrated that in cat, 13% of radioactivity after intravenous injection of adrenaline-H<sup>3</sup> was found in plasma as noradrenaline. Thus, these authors provided evidence that demethylation of adrenaline occurs in VIVO (VERLY 1962).

The experiments of KOPIN, AXELROD and GORDON (1961), KIRSHNER et al. (1961) and LABROSSE et al. (1961) confirm the results obtained previously by KOPIN (1960) and by AXELROD (1959), that O-methylation of adrenaline to metanephrine, which is then deaminated and conjugated, or deaminated and oxidized, constitutes the principal pathway of metabolism of this catecholamine. O-methylation is also considered as a primary factor for the biological inactivation of sympathetic mediators (BICKEL et al. 1961).

#### BINDING OF EXOGENOUS AMINES.

In 1959, AXELROD proposed the theory that methylation of adrenaline in VIVO occurs in two phases. One part of the hormone is immediately methylated, but some of it is bound to the tissue from which it is released and then methylated.

AXELROD and TOMCHICK then suggested that this binding to the tissue prevents enzymatic alteration of the amines (AXELROD et al. 1960), and potentiates the sympathomimetic effects of the catecholamines (BLASCHKO 1954). IVERSEN and WHITBY supported the theory of AXELROD, and demonstrated that the rate of disappearance of adrenaline is slower when low doses are used, than is the case with high doses. In 1961, HERTTING observed that blockage of the binding sites should accelerate the rate of metabolism of catecholamines, which become more accessible for the enzyme action. Therefore it seems that the binding into the tissue constitutes a very important step in the metabolism of both hormones.

EXPERIMENTAL

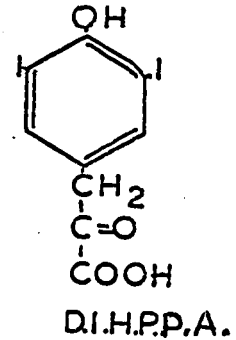
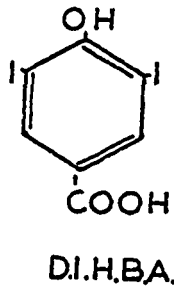
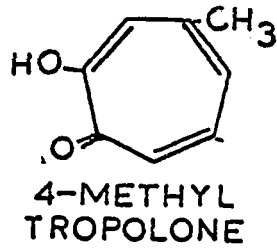
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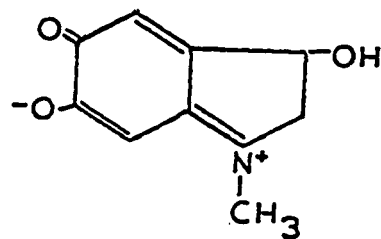
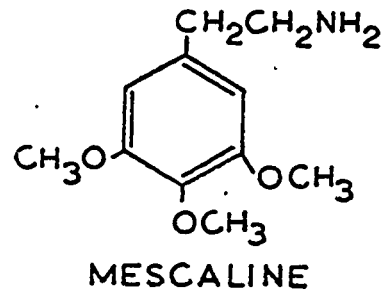
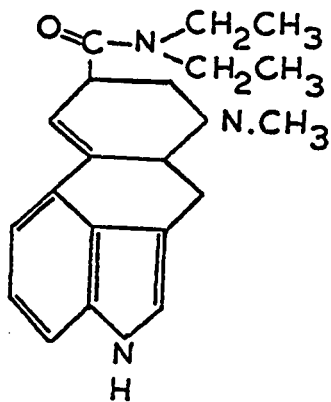
Fig. 1

KEY STRUCTURE OF SUBSTANCES USED IN THE EXPERIMENTS

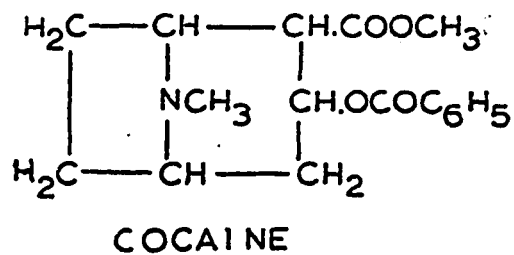
A.



B.



C.



THE EFFECT OF 4-METHYL TROPOLONE, AN O-METHYL TRANSFERASE  
INHIBITOR ON THE METABOLISM OF ADRENALINE IN VIVO.

A/ INTRODUCTION:

It is well known that O-methylation (ARMSTRONG 1957; AXELROD 1957; PELLERIN 1957) together with oxidative deamination (BLASCHKO 1953) is involved in the metabolism of catecholamines, and the enzyme responsible for this is catechol-O-methyl transferase.

It was found that in VIVO a compound such as pyrogallol inhibits this enzyme reversibly and blocks the O-methylation of adrenaline (BACQ 1936; AXELROD, LAROCHE 1959).

More recently it was demonstrated that a compound with a tropolone ring, 2-hydroxycycloheptatrienone (BELLEAU 1961) is a highly active competitive inhibitor of rat liver methyl transferase (MAVRIDES 1964) and that in VIVO it is much more effective than pyrogallol (BELLEAU; BURBA 1961). A low dose of this inhibitor potentiates the action of adrenaline on the nictitating membrane of a cat (BELLEAU, PINDELL as cited in BELLEAU 1961), but at a dose 10 times higher it led to a marked decrease in the sensitivity of  $\beta$ -receptors to isopropyl-noradrenaline (MURNAGHAN. M. et al. 1963). In rats, the urine of animals treated with tropolone (40 mg/kg) contained 50 to 100% more of the catechol and much less of the corresponding methoxy compounds. In VITRO

experiments 10 mg of tropolone inhibits in 30 min. 96% of COMT activity in the heart (POTTER, AXELROD 1963).

Experiments with mice showed that a high dose of tropolone 50 mg/kg or 100 mg/kg protects the animal for at least 2 hours against lethal doses of adrenaline. The LD<sub>50</sub> of tropolone in mice is 585 mg/kg. In small doses, 10 mg/kg, it increases adrenaline mortality (MURNAGHAN M. et al. 1963). These observations suggested that the in VIVO effects of  $\beta$ -methyl-tropolone are due to the inhibition of catechol O-methyl transferase as well as to the blocking of both alpha and beta receptor sites (BELLEAU 1961).

We were very fortunate in obtaining some of this compound from Dr. BELLEAU and testing it for inhibitory action in VIVO in rats.

The following pages will describe the effect of 4-methyl-tropolone on the metabolism of adrenaline - $\beta$ -C<sup>14</sup> in rats.

#### B/ EXPERIMENTAL PART:

For all our experiments young male rats of SPRAGUE-DAWLEY strain, weighing between 150 and 200 g were used.

The rats were divided into four groups of six animals each, except for Group IV which was composed of four rats only. They were placed in individual metabolic cages. All of them were treated by two subcutaneous injections:

1. of 10 mg per kg of body weight of dibenamine-HCl

in water (pH slightly acidic), to reduce the toxicity of adrenaline (RAAB et al. 1946);

2. and of 1.5 mg per kg of body weight of marplan in M/15 phosphate buffer. Marplan is a benzylhydrazine analogue of iproniazid - an amine oxidase inhibitor, which effectively blocks the in VIVO oxidative deamination of adrenaline ( RANDALL et al. 1959).

GROUP I - CONTROL: received two injections: dibenamine and marplan;

GROUPS: II, III and IV additionally were injected with an O-methyl transferase inhibitor: 4-methyl-tropolone in M/15 phosphate buffer and the pH was adjusted to 7.4 with 0.1 N NaOH;

GROUP II: received 10 mg/kg of 4-methyl-tropolone;

GROUP III: 30 mg/kg of this compound;

GROUP IV: 60 mg/kg of the same drug.

One hour later all animals were treated by intraperitoneal injection of adrenaline - $\beta$ -C<sup>14</sup> bitartrate in water equal to 4 mg/kg of adrenaline base. Its specific activity was 1.1  $\mu$ c/mM.

Urine was collected during 48-hour periods. To avoid the contamination of the urine by food, the animals fasted during the experiment, but were allowed to drink water "ad libitum". We observed that the rats drank less water than usual. The volume of urine was very small.

The urine collected during a 48-hour period, together with the washings of collecting pans with 0.01 N acetic acid, was made up to a final volume of 100ml.

The percentage of total  $C^{14}$  excreted in urine after administration of  $\beta-C^{14}$  adrenaline is shown in TABLE I.

The urine was evaporated to dryness in a rotatory evaporator at  $40^{\circ}C$  and the residue was dissolved with 5 ml 0.1 N HCl. The insoluble matter was discarded by centrifugation at low speed.

TABLE I

Group	% of radioactivity recovered in urine
Control	66.3
4-methyl-tropolone:	
10 mg/kg	64.0
30 mg/kg	47.3
60 mg/kg	43.9

Results are expressed as a mean percentage of administered dose excreted.

1/ Hydrolysis:

The hydrolysis was performed at two different concentrations: 1 N HCl and 5 N HCl. To one ml of concentrated

urine, conc. HCl was added and placed in tubes which were sealed and heated at 130°C for 15 min. After the tubes were opened, the hydrolyzed urine was adjusted at pH 2.5 and the insoluble material was eliminated by centrifugation.

## 2/ Chromatography:

Among the various methods for separation of urinary metabolites of adrenaline one of the most commonly used is descending paper chromatography. We tested several varieties of paper and solvent. We observed a poor resolution when catecholamines and catecholacids possessing very close  $R_f$  values were run on Whatman # 1. In order to obtain better separation of urinary metabolites of adrenaline ion exchange papers were tested. This technique has the advantage of eliminating neutral and acidic compounds from cation exchange papers, and amines from anion exchangers.

Among the ion exchange papers tested by us were: carboxy-methylcellulose, cellulose phosphate PC-20 and Amberlite papers WA-2 and IRC-50, all in cation form; diethylaminoethylcellulose DE-20 and aminoethylcellulose AE-30 were in anion form.

Amberlite paper, cellulose impregnated with resins, was used with acetate buffers at pH 4.6 and 6.0. The separation was unsatisfactory: the catecholamines appeared to be absorbed too completely.

With PC-20 satisfactory separation of catecholamines

and their metabolites was obtained in the buffer system as well as in the organic solvent system normally used: Bu:HAc:water (7:1:2) and (4:1:1).

In the buffer the resolution was very rapid, but the spots were quite large. Bu:HAc:water solvent mixture gave sharper spots. The pH of the sample influences the rate of migration. When the pH was too acidic the amines were retained on the starting line. A good rate of migration was observed when the sample to be spotted was adjusted at pH 2.5.

During one experiment we noticed that there was a marked difference between the two batches of PC-20 we used. The faster paper was 1 meq/g; the slower 2.2 meq/g.

In our case, to obtain better partition of the catecholamines in urine, the chromatograms were carried on PC-20 for 36 and, depending on the paper used, 60 hrs. and the solvent front and all acid derivatives (if any) ran off the paper. All chromatograms were made in duplicate. In a preliminary experiment adrenaline was added to urine and chromatographed in parallel with a normal (control). We have found that in urine there is a tendency for the amines to flow slightly faster than they do in a pure solution.

We observed in the synthetic mixture that the spots were regular, but that the same synthetic mixture added to hydrolyzed urine gave the spots in V form. These anomalies are easily explained by the presence of an excess of salts

in the sample. The  $R_f$  value is influenced by the high concentration of salts.

Papers AE-30 and DE-20 were used for the separation of acidic catabolites of adrenaline.

TABLE II gives the  $R_f$  values for the different catecholamines and catecholacids in Bu:HAc:water system (7:1:2) using Whatman I, (a), PC-20 (b), DE-20 (c), and AE-30 (d).

TABLE II

COMPOUND	PURE SOLUTION				MIXTURE		
	A	B	C	D	A	B	C
L-ADRENALINE 1/	0.24	0.05	0.32	0.46			
DL-METANEPHRINE. HCl	0.41	0.19	0.52	0.66	0.43		0.59
DL-NORMETANEPHRINE.HCl	0.35	0.10	0.46	0.58	0.35		0.45
DOPAMINE.HCl	0.33	0.06	0.40	0.55			
DL-NORADRENALINE. HCl	0.21	0.03	0.28	0.38			
DL-3-METHOXY-4-HYDROXYMANDELIC ACID	0.69	0.85	0.16	0.12	0.69		0.17
3-4-DIHYDROXYPHENYL-ACETIC ACID	0.79	0.91	0.38	0.38	0.80		0.29
3-4-DIHYDROXYMANDELIC ACID	0.51	0.65	0.05	0.03	0.51		0.06
METHADREN 2/		0.144				0.147	
ADRENALINE		0.088					
PARANEPHRINE		0.197				0.196	
METANEPHRINE		0.220				0.250	

1/ PC-20 1 meq/g; Bu:HAc:water 7:1:2

2/ PC-20 1 meq/g; Bu:HAc:water 4:1:1

Between 10 and 25 lambdas of hydrolyzed urine were applied on paper cut into strips 2 cm wide and 56 cm long. To locate the metabolites on paper the control chromatograms were performed on urine to which was added the synthetic mixture of catecholamines.

After the necessary period of time for chromatography, which varied according to the solvent system, pH of sample, temperature and nature of the paper, the papers were removed from the chambers and suspended in a current of air and allowed to dry.

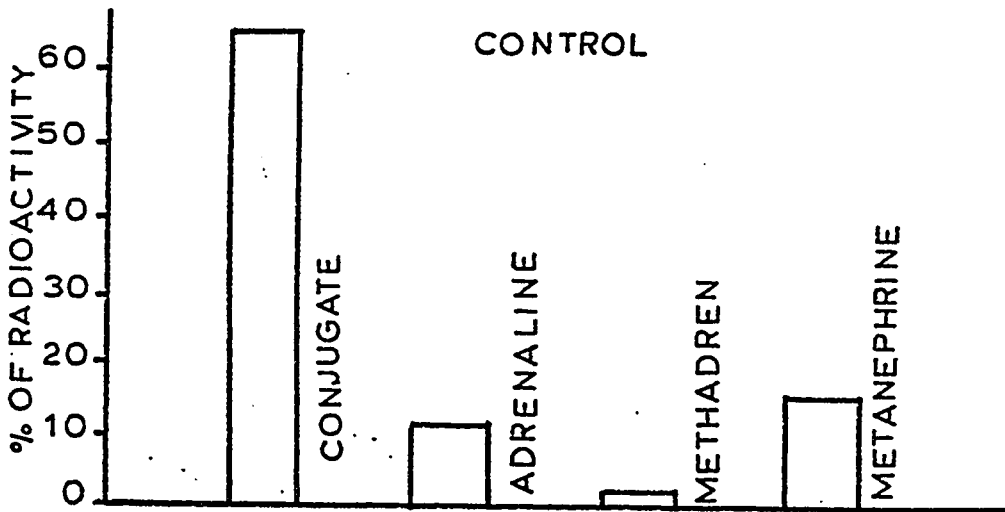
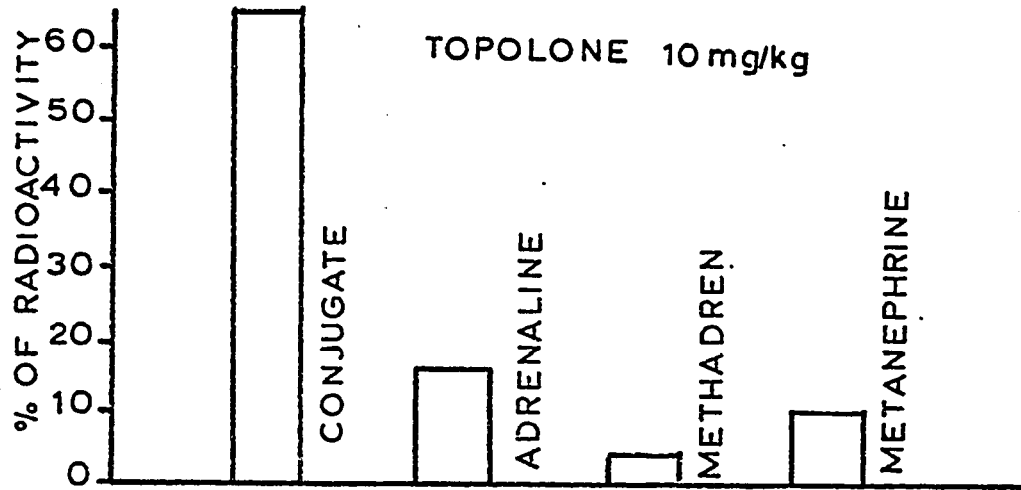
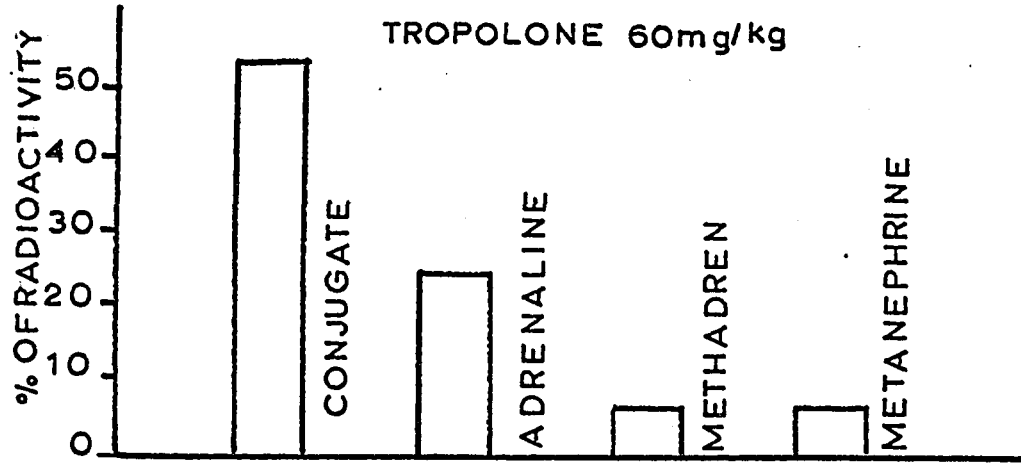
The spots on the control chromatograms were detected by spraying the dried strips with a freshly prepared solution of 0.1% p-nitroaniline in N HCl mixed with 0.2% Na nitrite and 10%  $K_2CO_3$  (10:10:25) at 5°C (as in SMITH I.). This color reaction gives an intense violet color with 3-methoxy-4-hydroxymandelic acid and metanephrine, blue-violet with paraneprine, pink-violet with adrenaline and pink-grey with N-methyl-adrenaline (methadren) and 3,4-dihydroxymandelic acid.

All chromatograms were cut into small strips one cm long, on which the radioactivity was measured.

### 3/ Radioactivity measurement:

At the beginning of this series of experiments radioactivity was measured with low background counter. The ra-

Fig. 2



URINE BEFORE HYDROLYSIS

radioactive material was eluted from each centimeter of chromatographic strip with 0.01 N HCl into small metal planchets and evaporated to dryness under an infra-red lamp. We abandoned this method for two reasons:

1. It was very difficult to wash out all radioactivity from PC-20;
2. The radioactivity measured with this counter represented only 30% of the total, so the samples with low radioactivity could not be precisely counted.

Liquid scintillation method appeared to be more satisfactory. The small pieces of paper were placed directly in vials containing toluene, PPO and POPOP (Bull. Nuclear Chicago N.11) and counted for 10 min. in a Nuclear Chicago Scintillation counter.

The distribution of radioactivity among urinary metabolites of adrenaline- $\beta$ -C<sup>14</sup> in normal rats and in animals pretreated with 4-methyl-tropolone is shown in the following tables.

TABLE III and Fig. 2 show the average values for radioactive adrenaline and its metabolites in concentrated urine before hydrolysis.

---

PPO - 2,5-diphenyloxazole

POPOP - 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene

---

TABLE III

Group	% of total radioactivity			
	Conjugate	Adrena- line	Metha- dren	Meta- nephrine
Control	65.1	10.9	1.8	14.9
Tropolone 10mg/kg	64.8	14.8	3.3	9.7
Tropolone 60mg/kg	53.1	23.6	5.3	6.0

It appears that in case of treated animals:

1. The amount of adrenaline excreted in urine is higher than in the Control group;
2. The value for metanephrine is lower and represents only 40% of the metanephrine found in normal urine;
3. The free catecholamines represent only 30% of the total radioactivity.

4/ Conjugate:

The hydrolysis in N HCl did not furnish the expected results. The conjugate represented a high percentage of the urinary radioactivity in Control as well as in tropolone treated animals. The spot of conjugated catabolites was submitted to a second and more vigorous hydrolysis as follows.

One ml of urine hydrolyzed with 1 N HCl was chromato-

Fig. 3

Distribution of radioactivity in urine after hydrolysis with 1 N HCl.

AE-30; Bu:HAc:water (7:1:2); 39 hr.

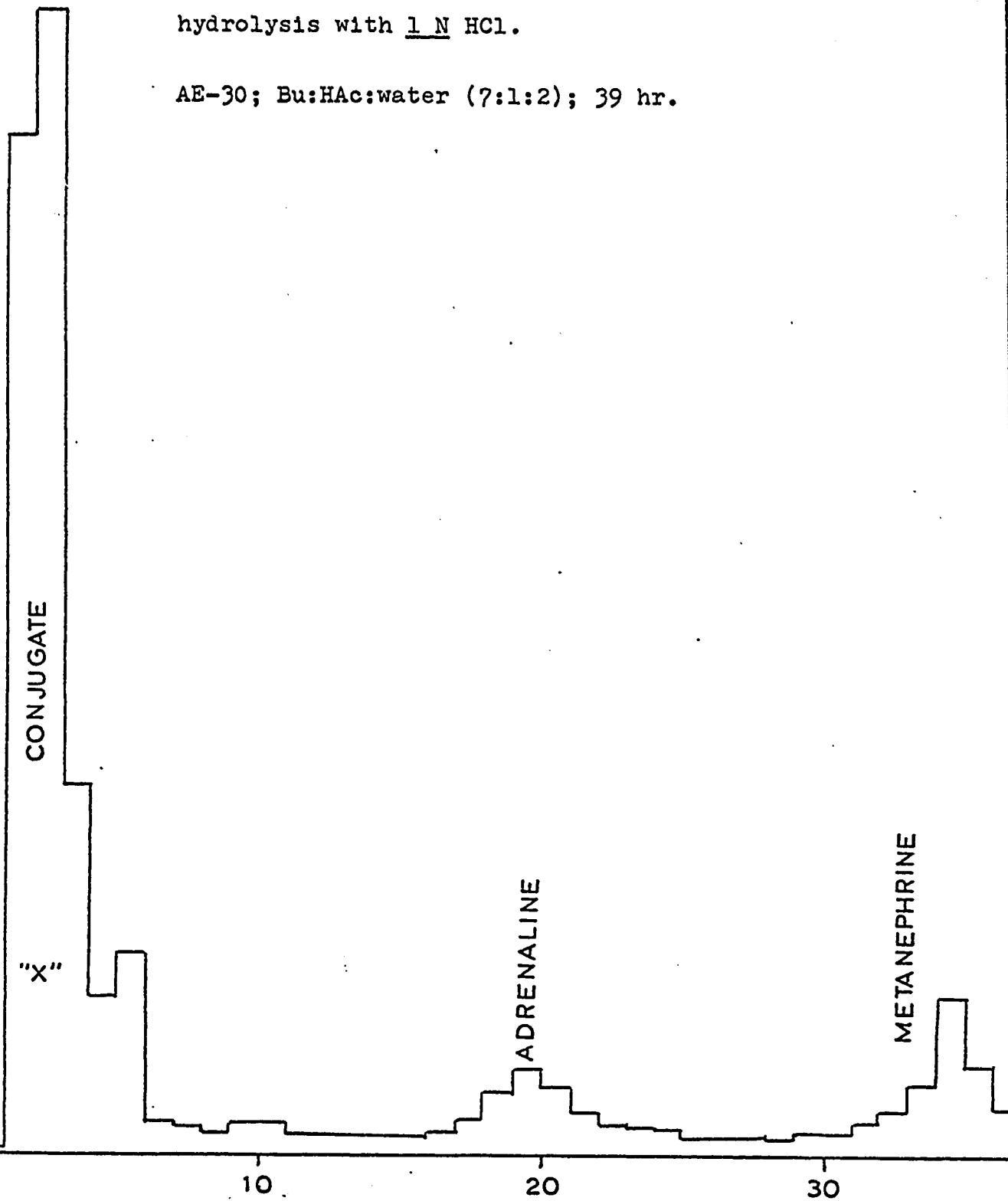
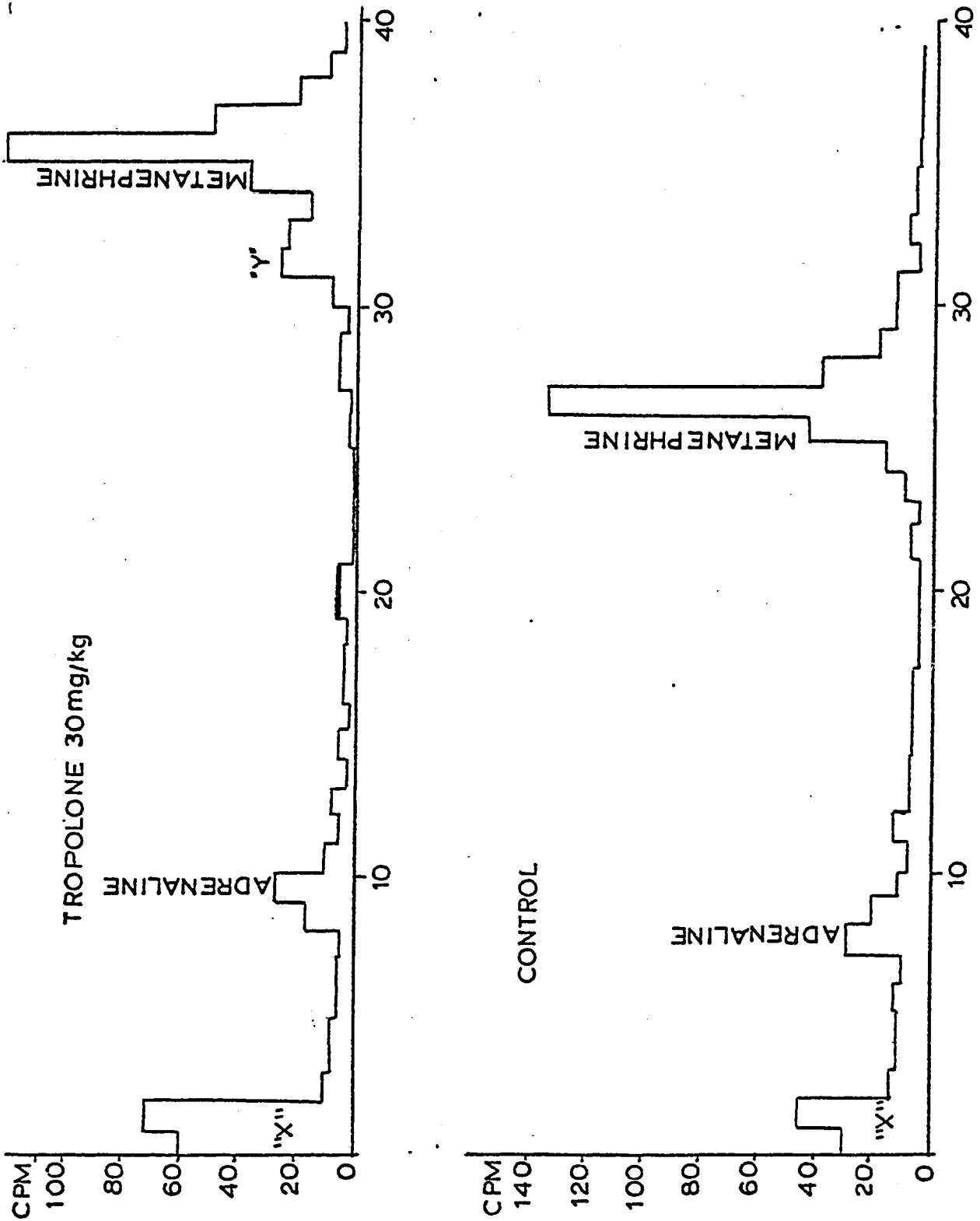


Fig. 4

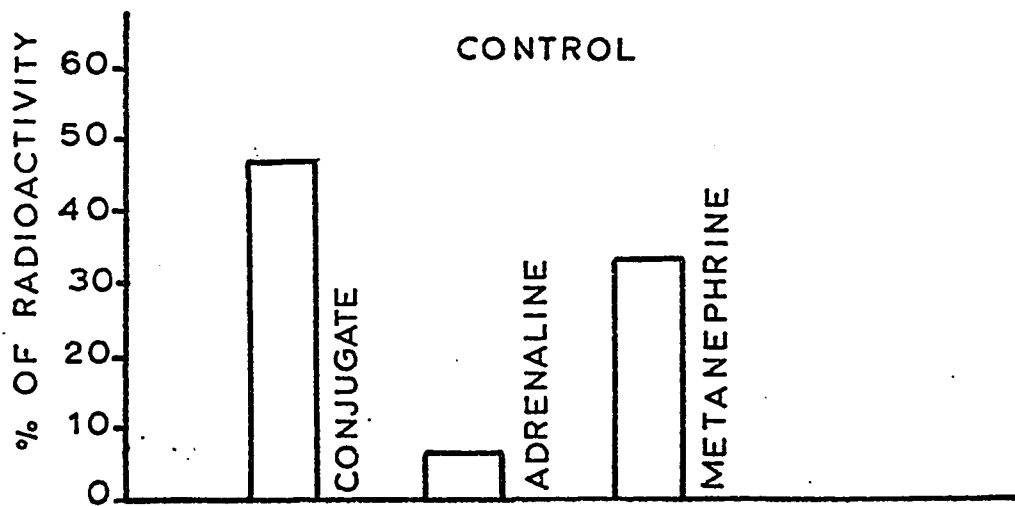
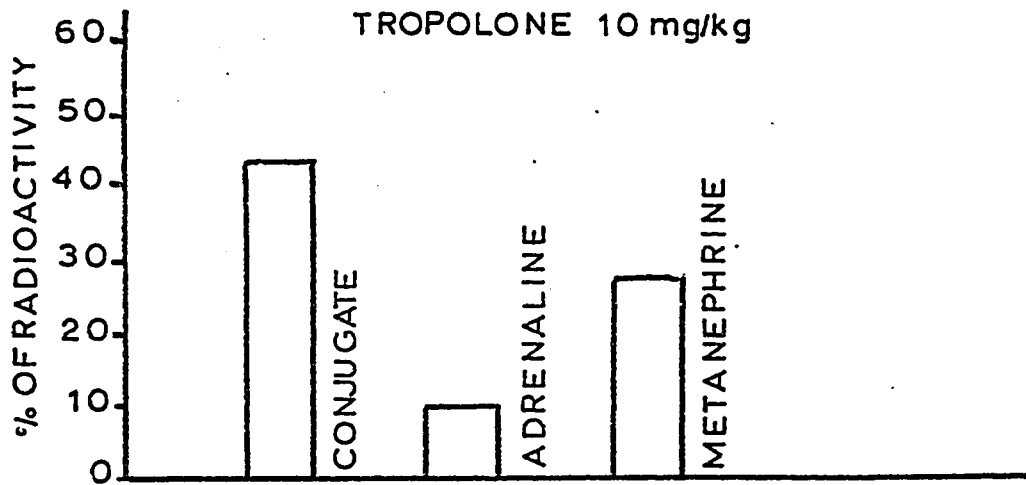
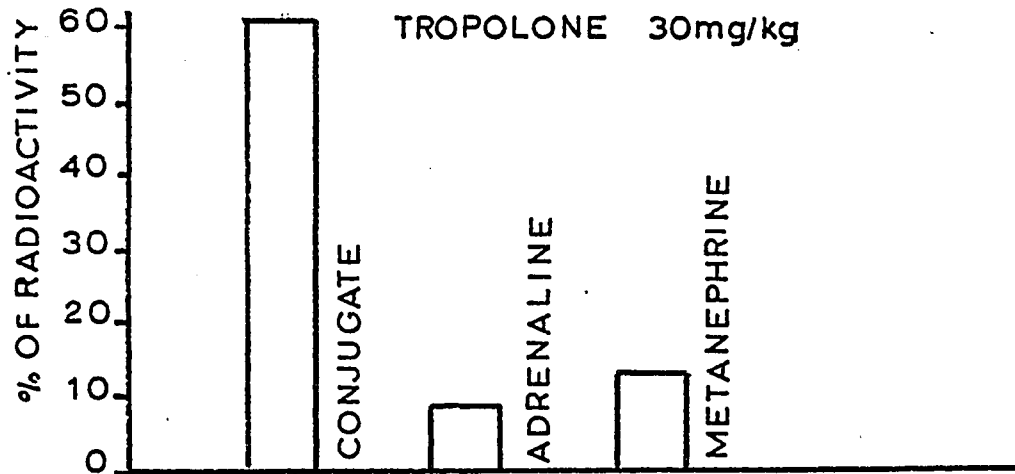


Distribution of radioactivity in the conjugate fraction after acid hydrolysis with 5 N HCl.

graphed on paper 23 cm wide (AE-30) in Bu:HAc:water system for 39 hrs. The distribution of the radioactivity on a strip 1.0 cm wide, cut from the centre of the paper, is shown in Fig. 3. The peak X, corresponding to the conjugate, contained 66.5% of total radioactivity counted on the paper. From the rest of the paper the zone corresponding to the conjugate was cut into strips 2 cm wide and eluted with 0.01 N HCl. The eluates combined were concentrated to one ml. 25 lambdas of it was used to determine radioactivity. The remaining volume was divided into two parts for hydrolysis in 5 N HCl for 15 min. at 130°C in a sealed tube under nitrogen. The hydrolyzed sample was adjusted to pH 2.5 and 25 lambdas of it were used for chromatography on PC-20 in Bu:HAc:water system. Control chromatograms with synthetic mixture were run at the same time. After the papers were dried, the controls were sprayed with p-nitroaniline reagent to localize the principal amines. The chromatograms containing radioactive compounds were cut into small strips and their radioactivity was counted as described above.

Fig. 4 represents the distribution of radioactivity in "conjugate" fraction of control urine and of the tropolone-treated animals after hydrolysis.

Fig. 5



URINE AFTER HYDROLYSIS (IN HCl)

TABLE IV

The distribution of radioactivity in conjugate fraction after hydrolysis.

Compound	% of radioactivity	
	Control	Tropolone 30 mg/kg
Unhydrolyzed conjugate	17.5	21.4
Adrenaline	13.1	11.2
Metanephrine	51.8	33.1

TABLES V and VI record the results obtained with hydrolyzed urine:

1/ TABLE V as well as Fig. 5 show the urinary metabolites of  $\beta$ -C<sup>14</sup>-adrenaline after hydrolysis with N HCl;

TABLE V

Group	% of radioactivity		
	Conjugate	Adrenaline	Metanephrine
Control	45.9	5.3	31.6
Tropolone 10 mg/kg	42.7	9.4	26.9
30 mg/kg	61.4	8.6	12.8

2/ TABLE VI shows the distribution of urinary metabolites of adrenaline after hydrolysis with 5 N HCl.

TABLE VI

Group	% of total radioactivity							
	Conj.	B	C	D	E	F	G	H
Control	-	7.1	7.9	8.9	30.6		25.2	8.8
Tropolone 60 mg/kg	6.9	11.8	6.8	6.2	13.7	13.9	16.6	4.2

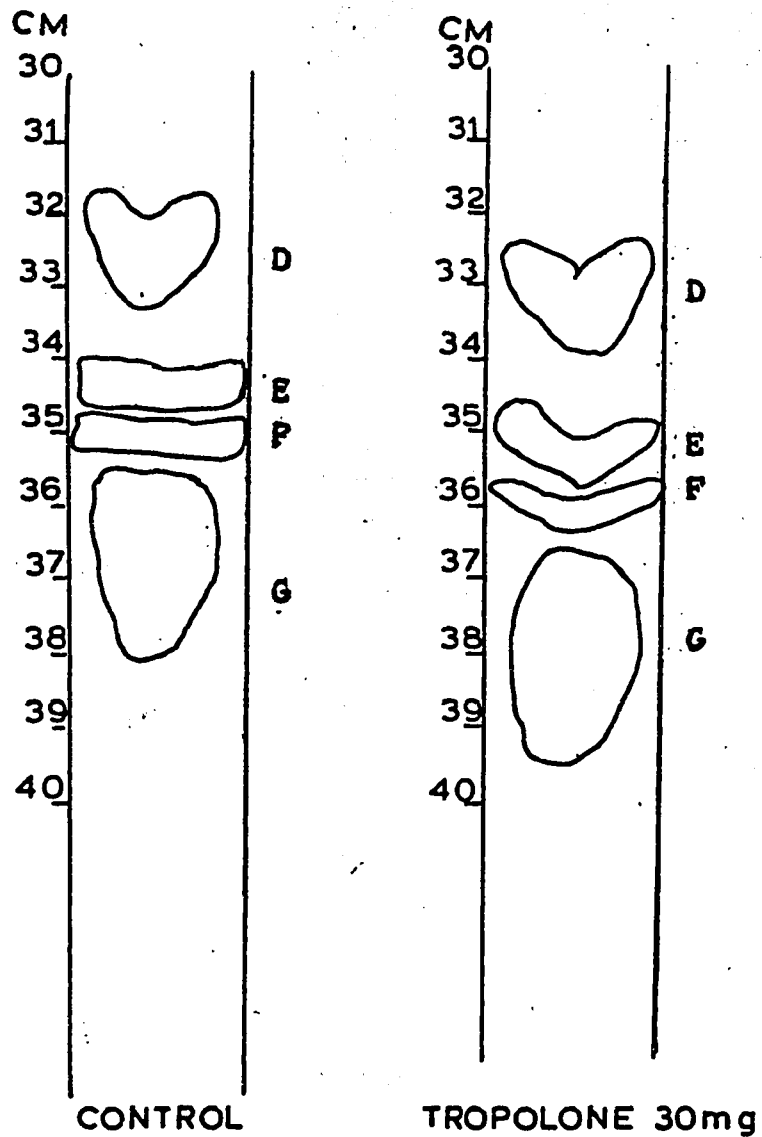
From TABLE V it appears that there is an important reduction of metanephrine (60%) in the urine of animals treated with tropolone, and that in the same group the conjugate represents a higher percentage of total radioactivity.

The hydrolysis with 5N HCl caused some destruction. In both urines, control - and tropolone-treated animals, the conjugate is totally hydrolyzed. In general the separation lacks uniformity, and many peaks in the control group are composed of a mixture of two or more metabolites. The peaks involved are C-D and E-F.

In tropolone-treated animals the separation of peaks E and F seems to be better. We have some evidence for believing that these peaks correspond to N-methyl-adrenaline and paranephrine respectively. Synthetic paranephrine added to

Fig. 6

Chromatography of the synthetic mixture added to the radioactive urines after acid hydrolysis with 5 N HCl.



D/ ADRENALINE  
E/ METHADREN  
F/ PARANEPHRINE  
G/ METANEPHRINE

hydrolyzed urine gives a color spot which corresponds to radioactive peak F (Fig. 6). Here again the radioactivity corresponding to peaks G and F: of metanephrine and of an unidentified metabolite is higher in the control group. The radioactivity of all unidentified metabolites represents nearly 40%.

In TABLE VII we have assembled some of the  $R_f$  values for urinary metabolites of adrenaline, calculated on the basis that  $R_f$  adrenaline is equal to 1.

TABLE VII

Peak	$R_f$ values	
	Control	Tropolone 60 mg/kg
A	0.05	0.06
B	0.21	0.18
C	0.93	0.92
Adrenaline	1.00	1.00
E	1.09	1.12
F	1.15	1.12
G	1.23	1.21
H	1.39	1.39

5/ Extraction of methylated compounds from hydrolyzed urine:

The extraction of methylated metabolites was carried out according to the BELLEAU method (BELLEAU, BURBA 1961). By this method after three repetitions only 40 % of the metanephrine could be extracted.

One ml of hydrolyzed urine adjusted to pH 10.0 with 0.5 M borate was extracted 3 times for 30 min. with 10 ml of ethylene dichloride. The organic phase was evaporated to dryness at 50°C, using nitrogen. The residue was then dissolved in 0.1 ml of the same solvent and 50 lambdas were used for chromatography.

Control extractions were performed at the same time on synthetic compounds of adrenaline, N-methyl-adrenaline, paranephrine and metanephrine. Only metanephrine and paranephrine after extraction could be detected by chromatography.

TABLE VIII shows the presence of two or three methylated metabolites extracted from urine. These results must be considered as qualitative only. Nevertheless, there is evidence that urinary O-methylated metabolites of adrenaline  $\beta$ -C<sup>14</sup> following the tropolone injection represent much lower radioactivity than in the control group.

TABLE VIII

Group	% of total radioactivity		
	Metanephrine	Methoxy-x	Methoxy-y
Control	12.8	1.1	5.2
Tropolone 30 mg/ kg	9.2	1.4	-
Tropolone 60 mg/ kg	2.9	1.0	1.9

From these experiments it may be concluded that 4-methyl-tropolone seems to act on O-methyl transferase in rats and to inhibit to a certain extent the O-methylation of injected adrenaline in VIVO.

#### DISCUSSION.

Using  $C^{14}$  labeling, the metabolism of injected adrenaline was studied in normal and in-tropolone-treated rats. The results show a distinct difference in the metabolic product of exogenous catecholamine between the two groups of animals. In the control group the O-methylated metabolites of adrenaline represent a much higher percentage of radioactivity than in the tropolone group.

The techniques adopted in this work permitted a satisfactory separation of catecholamines. The use of ion exchange papers for this purpose proved much more satisfacto-

ry than the use of the common Whatman #1 paper.

Chromatography of the urine on PC-20 paper in butanol:acetic acid:water and subsequent determination of radioactivity indicated the presence of at least three radioactive compounds, containing about 90% of the excreted radioactivity. One corresponds to a conjugate, one to adrenaline, and the last spot to free metanephrine.

After chromatography on PC-20 of acid hydrolyzed urine, we were able to obtain more radioactive zones than are usually found on Whatman # 1.

Tentative identification has been achieved and the two zones E and F (in TABLE VI) correspond to N-methyl-adrenaline and paranephrine respectively.

N-methylation of adrenaline and the natural occurrence of N-methyl-adrenaline in adrenal glands was already reported by AXELROD (AXELROD 1960a). After an intravenous injection of this compound he found in the urine large quantities of N-methyl-metanephrine: free and conjugated.

The occurrence of para-O-methylation in VIVO is still a controversial subject. Our results, unfortunately, have not been directly confirmed by other workers.

In VITRO, both adrenaline and noradrenaline form para- and meta-O-methyl ethers. However, the para-compounds represent only 10 to 15% of the methylation mixture (DALY, AXELROD and WITKOP 1959).

Recently, (MASRI 1964) it was reported that O-methyl transferase from rat liver may selectively O-methylate in the meta- as well as in the para- position. In a series of experiments with tri-hydroxy and dihydroxy-phenolic acids, these investigators have shown that in the case of a caffeic acid (3,4-dihydroxycinnamic) both para- and meta-O-methylation occurred. In the case of 2,3,4-trihydroxybenzoic acid only meta-O-methylation took place, but in the case of 3,4,5-trihydroxyphenolic compound the methylation occurred in the para-position. This group of workers has suggested that phenolic substrate, having three adjacent hydroxyl-groups, is methylated on the middle hydroxyl group, regardless of whether this group is in the para or meta-position.

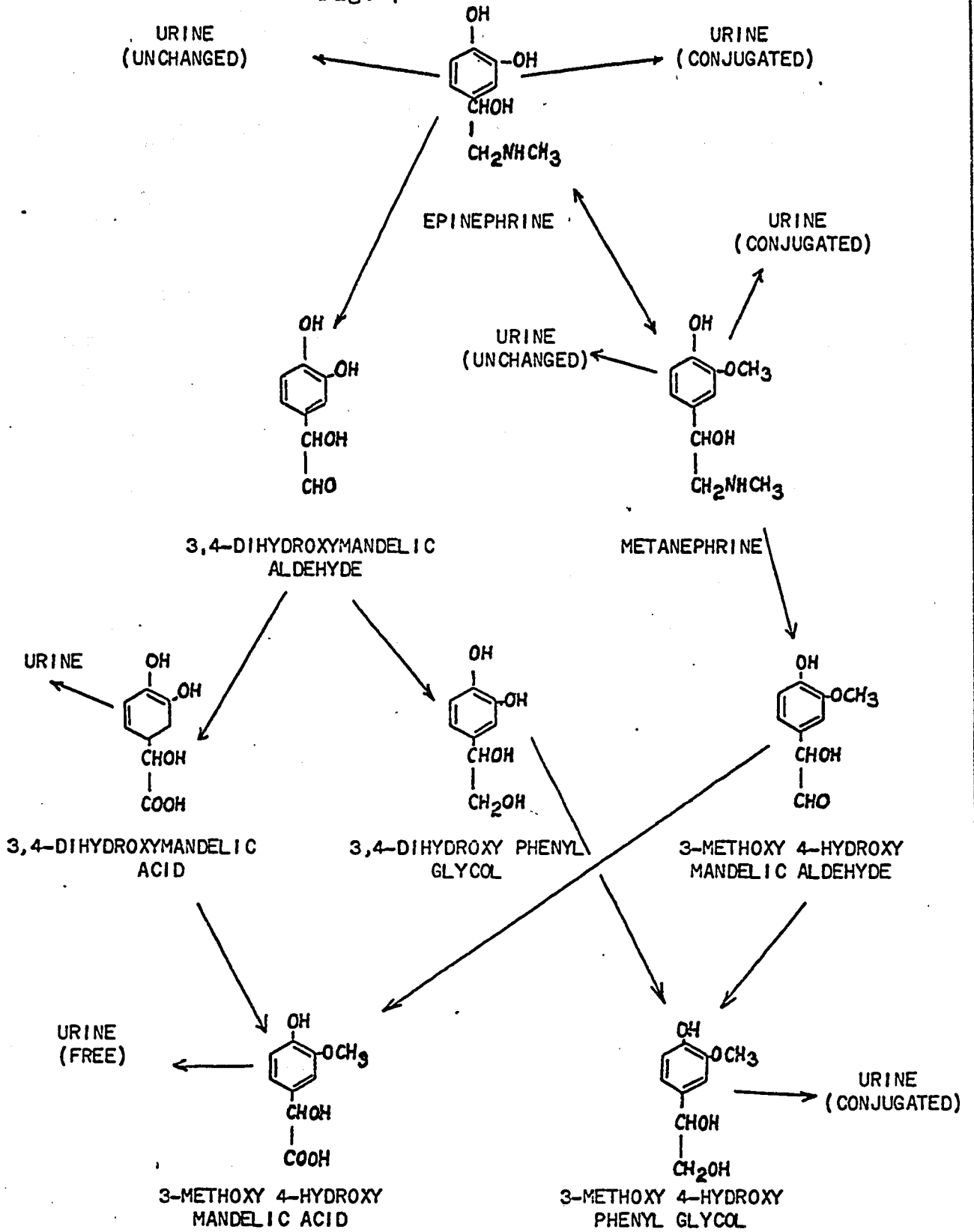
Para-O-methylation of adrenaline in VIVO could not be demonstrated by DALY, AXELROD and WITKOP with or without MAO inhibitors. This study showed no detectable paranephrine. On the other hand, the intraperitoneal administration of metanephrine and paranephrine to rats does not lead to the interconversion of these compounds. In both cases the recoveries were the same (DALY, AXELROD, WITKOP 1959). However, those in VIVO experiments cannot be regarded as definitive on this point.

From a recent observation of MASRI and co-workers, it now appears probable that the animal preparation of O-methylating enzyme contains two O-methyl transferases: meta- and

para- with varying specific activities toward different substrates (MASRI 1964).

From our experiments it may be concluded that 4-methyl-tropolone seems to act on O-methyl transferase in rats and to inhibit to a certain extent the O-methylation of injected adrenaline- $\beta$ - $C^{14}$  in VIVO.

Fig. 7



METABOLISM OF EPINEPHRINE

METABOLISM OF EPINEPHRINE- $\beta$ -C<sup>14</sup> BITARTRATE IN A NORMAL RAT.

In the following experiments two separate groups of control rats were injected with radioactive adrenaline.

The Control for lysergic acid diethylamide, mescaline and adrenochrome was performed on a group of nine male rats of the Sprague-DAWLEY strain, each weighing between 160 and 180 g. All rats received pretreatment with dibenamine HCl one hour prior to the intramuscular injection of adrenaline- $\beta$ -C<sup>14</sup>.

Urine was collected for 48 h. and adrenaline and its metabolites were isolated from it, using KOPIN's method (KOPIN 1961), slightly modified by us.

The Control group for rats injected with cocaine were treated in a way similar to that described above, the only difference consisting in the mode of administration of the adrenaline. In this group (four rats only) radioactive adrenaline was administered intraperitoneally. In the experiments reported here, an attempt has been made to differentiate between the metabolism of adrenaline injected intramuscularly and intraperitoneally into a normal rat.

From the following TABLES, TABLE IX and X it appears that the main difference in the metabolism of adrenaline injected in the two ways is in the metanephrine fraction. The total metanephrine, free and conjugated, in both experiments remains the same. However, the proportion between

TABLE IX  
 Distribution of radioactivity among the metabolites of adrenaline- $\beta$ -C<sup>14</sup> injected intramuscularly, after pretreatment with dibenamine - Control group.

Compound	R a t #									Average SD
	1	2	3	4	5	6	7	8	9	
Adrenaline free conj.	2.72	2.48	3.51	3.56	3.54	2.64	4.85	4.64	4.55	3.61 ± 0.90
	6.01	8.79	9.80	8.57	9.84	7.08	8.29	9.57	9.44	8.60 ± 1.31
DHMA + DHPG free conj.	0.40	0.38	0.90	0.18	0.60	0.36	0.72	0.58	0.60	0.52 ± 0.22
	1.16	1.18	1.06	1.72	1.80	1.08	2.72	2.08	2.74	1.73 ± 0.67
Metanephrine free conj.	10.71	10.53	12.91	11.40	10.58	10.86	9.22	17.90	8.85	11.44 ± 2.69
	38.62	38.39	34.67	36.00	38.09	39.25	37.46	29.39	36.53	36.49 ± 3.03
VMA	5.80	6.84	7.62	6.98	6.66	7.73	4.59	6.91	5.19	6.48 ± 1.07
MHPG hydrolyzable unhydrolyzable	16.09	14.28	18.97	19.52	14.06	14.50	14.45	14.55	16.13	15.84 ± 2.08
	17.72	14.35	12.61	9.07	15.09	17.90	10.62	11.96	9.83	13.24 ± 3.25

TABLE X

Excretion of the intraperitoneally injected adrenaline- $\beta$ -C<sup>14</sup> and its metabolites in rats pretreated with dibenamine. The results are expressed as a percentage of radioactivity excreted in the urine.

Compound	R a t #				Average	SD
	1	2	3	4		
Adrenaline						
free	2.03	3.69	1.57	3.03	2.58	± 0.98
conj.	5.21	5.65	6.35	5.55	5.69	± 0.48
DHMA + DHPG						
free	0.52	0.54	0.22	0.48	0.44	± 0.15
conj.	3.36	4.50	1.46	1.50	2.70	± 1.49
Metanephrine						
free	4.58	4.50	3.25	3.02	3.83	± 0.82
conj.	44.45	41.58	44.78	-	43.60	± 1.76
VMA	4.16	4.20	7.71	-	5.36	± 2.04
MHPG						
hydrolyzable	14.70	17.15	17.10	-	16.35	± 1.40
unhydrolyzable	19.44	18.84	13.79	-	17.36	± 3.10

free and conjugated catecholamine varies significantly. After intraperitoneal injection there is less of the free metanephrine and more of the conjugated compound. On the contrary, after intramuscular administration the level of free metanephrine is about three times as high as in the urine from animals injected intraperitoneally. Both values for adrenaline, free and conjugated, are slightly lower in the case of the intraperitoneally injected rats.

From this observation it seems that the intraperitoneal injection of adrenaline affects the conjugation of catecholamines, but has no significant effect on their total amount.

THE EFFECT OF THE PSYCHOMIMETIC DRUGS ON THE METABOLISM  
OF  $\beta$ -C<sup>14</sup>-ADRENALINE IN RATS.

A/ INTRODUCTION:

It is well known that a number of naturally occurring compounds are capable of inducing toxic psychoses and hallucinations in man. These compounds are characterized by the predominance of their effects on mental and psychic functions (JACOBSEN 1963) and are classified in three main groups:

1. The lysergic acid group;
2. The mescaline group;
3. The tryptamine group.

Because of the similarity of the syndromes caused by hallucinogenic compounds to that of schizophrenia, they are quite often used as research tools in psychiatry (BECKMAN 1961). All known hallucinogens are readily absorbed from the intestinal tract, and no major difference was found between the effective oral, subcutaneous and intravenous doses (JACOBSEN 1963).

In our research we tested only the first two groups and their effect on the metabolism of adrenaline in VIVO.

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LYSERGIC ACID DIETHYLAMIDE - LSD<sub>25</sub>.

The lysergic acid diethylamide, LSD<sub>25</sub> is a synthetic derivative of lysergic acid, one of the principal alkaloids of ergot (STOLL, HOFMANN 1938). Its psychic effects were discovered quite accidentally by HOFMANN (HOFMANN 1959). This compound was revealed to be the most potent of presently known hallucinogens, and to cause mental intoxication, changes in behaviour and visual and auditory hallucinations (FREDERKING 1955; ROTHLIN 1957a; ISBELL 1955). On the other hand, during experimental psychosis contact with reality is not lost (HOFMANN 1959). Since then many investigators have confirmed HOFMANN's observations and have produced "model psychosis" and mental disturbances in normal subjects (ROTHLIN 1957 b).

In 1953 a group of workers studied the clinical and biochemical effects of LSD<sub>25</sub> and metamphetamine given intravenously to patients with mental disorders (LIDDELL 1953). It was found that after administration of both drugs the level of the peripheral plasma adrenaline was increased. More recently HOLISTER compared the clinical syndromes of a drug, induced psychosis with schizophrenic reactions and observed certain basic differences (HOLISTER 1962).

The similarity of effects between LSD<sub>25</sub> and 1-nor-adrenaline in man was observed by RINKEL and his co-workers.

This group suggested that the action of LSD<sub>25</sub> may be related to its interference with adrenaline metabolism (RINKEL 1954).

The action of LSD<sub>25</sub> in chronic schizophrenic patients and in subjects with maniac-depressive psychosis was studied by ELMADJIAN and his colleagues. It was observed that all patients except the schizophrenics, showed positive responses with a dose 100 - 150 ug of LSD<sub>25</sub>; anxiety, rapid respiration and hallucinations. Urine analysis indicated an increase in adrenaline and noradrenaline excretion. However, to get similar response in schizophrenic patients, it was necessary to increase the LSD<sub>25</sub> dose up to 300 ug. Nevertheless urine analysis showed insignificant changes in the catecholamine level (ELMADJIAN 1958).

The observation of GADDUM in VITRO that LSD<sub>25</sub> is a potent antagonist of serotonin on smooth muscle led to the hypothesis that the hallucinogenic agent produces its aberrant mental effects by interfering with the normal function of serotonin in the brain (GADDUM 1953). This hypothesis was extended by the suggestions of WOOLLEY and SHAW that mental disturbances following the administration of a small amount of LSD<sub>25</sub> may result from the antimetabolic action of this compound on serotonin and most probably from its decrease in the brain or from its increase by competing for MAO (WOOLLEY 1954 a). The same authors postulated that clinical psychoses may have their origin in an accumulation

or in a decrease of serotonin level in the brain.

In 1954 GRAHAM and KHALDI found that in VITRO lysergic acid diethylamide inhibits the stimulating action of adrenaline and noradrenaline on smooth muscle preparations (GRAHAM 1954). A few years later, experiments with the perfused ear of a rabbit demonstrated that:

1. At low concentration ( $10^{-9}$  M) LSD antagonized serotonin without altering the response to adrenaline; but
2. The higher concentration ( $10^{-8}$  M) caused slight vasoconstriction and increased the response to adrenaline (GADDUM 1957; GINZEL 1953; SAVINI 1956).

It was observed that lysergic acid diethylamide at a dose of 1  $\mu$ g/kg does not induce mental changes in man by direct action upon the brain, but probably by a metabolite produced by a primary disturbance of the metabolism in the liver (ROTHLIN 1957 b).

This discovery by COSTA (COSTA 1959) that LSD<sub>25</sub> is not an inhibitor of serotonin but that it potentiates the serotonin contractions of the rat uterus could not be confirmed by other workers (CERLETTI 1958). Work with homogenates of rat brain furnished some evidence to support WOOLLEY's point of view that LSD<sub>25</sub> inhibits the destruction of serotonin (CERLETTI 1958) by competing with it for MAO (SLOCOMBE 1957).

Experiments with animals showed that the "maximum"

dose of LSD<sub>25</sub> tolerated by animals varied according to species. In a rat LD<sub>50</sub> was 16.5 mg/kg and the "maximum" tolerated dose was 3.2 mg/kg. In a mouse LD<sub>50</sub> was 46 mg/kg, but in a rabbit it was as low as 0.3 mg/kg (ROTHLIN 1957 b). Following the intravenous injection to rats, most of LSD<sub>25</sub> disappeared rapidly from blood circulation (STOLL 1955; LANZ 1955). In a mouse the biological half life of LSD<sub>25</sub> after intraperitoneal administration of 2 mg/kg was 7 min. The drug is very rapidly bound to the plasma proteins but somehow it is released later because it is found in various organs. The liver seems to be the only tissue where the drug is metabolized and converted to 2-hydroxy-LSD, biologically inactive compound. Most of this compound is excreted into the bile but nevertheless is reabsorbed from the intestine (AXELROD 1957).

More recently it was reported that the increase of the serotonin level in the brain depends on the amount of the drug administered. In an albino rat the dose of 100 mg/kg of LSD<sub>25</sub> did not produce any significant effect, but a dose a little larger, such as 130 mg/kg, showed a statistically significant augmentation of serotonin in the brain (FREEDMAN 1962). It was also observed that this psychomimetic drug stimulates the repletion of the serotonin following the reserpine release.

For several years LSD<sub>25</sub> was believed to produce its

hallucinogenic and other central effects by interference with the action of the brain serotonin (WOOLEY 1954 b; GADDUM 1954). Present evidence indicates that the central effects of the drug have nothing to do with its anti-serotonin action (COSTA 1959) and that the main action is the stimulation of the central adrenergic receptors (COSTA 1962).

B/ EXPERIMENTAL PART:

Some promising evidence obtained by LEDUC (LEDUC 1959) that psychomimetic drugs such as mescaline, LSD<sub>25</sub> and adrenochrome have a visible effect on the metabolism of adrenaline- $\beta$ -C<sup>14</sup> in VIVO were the starting point of the present work.

Among the various existing methods for separation of catecholamines and their metabolites (KIRSHNER 1960; SCHAYER 1953), the most satisfactory, though very long and elaborate appeared to be that of KOPIN (KOPIN 1961). His method consists of the use of alumina absorption and ion exchange columns, followed by extraction in an organic solvent (Fig.8), and finally determination of the radioactivity in a liquid scintillation counter.

For the first experiment with hallucinogenic drugs we used lysergic acid diethylamide, the most potent of the psychomimetic agents.

A group consisting of six SPRAGUE-DAWLEY rats, the male weighing between 150 and 180 g received two injections:

TABLE XI

Distribution of radioactivity among the metabolites of adrenaline- $\beta$ -C<sup>14</sup> after the injection of lysergic acid diethylamide (LSD-25) 0.2 mg/kg in urine of 9 Sprague-Dawley rats.

Compound	R a t #									Average	SD
	1	2	3	4	5	6	7	8	9		
Adrenaline free conj.	2.09 9.14	2.33 7.03	2.41 8.01	2.99 7.34	2.16 7.68	2.43 8.68	2.98 8.06	1.02 4.61	3.79 8.36	2.46 ± 0.74 7.66 ± 1.31	
DHMA + DHPG free conj.	0.58 3.82	0.48 1.50	0.80 3.48	0.64 4.90	0.52 3.40	0.82 1.94	0.86 1.56	0.26 0.44	1.02 1.62	0.55 ± 0.26 2.52 ± 1.44	
Metanephrine free conj.	9.12 34.74	11.28 35.08	12.19 31.80	9.89 32.86	9.57 34.85	8.02 34.60	9.78 30.30	8.04 37.48	10.35 26.73	9.80 ± 1.37 33.16 ± 3.20	
VMA	7.93	6.78	7.40	8.24	7.55	6.47	7.63	6.59	7.51	7.34 ± 0.61	
MHPG hydrolyzable unhydrolyzable	25.22 5.33	22.18 9.73	22.20 9.78	24.80 5.44	23.70 7.90	21.23 12.47	20.60 10.35	20.00 15.20	21.33 7.41	22.36 ± 1.84 9.29 ± 3.21	

one subcutaneous of 0.2 mg/kg of body weight of LSD<sub>25</sub> in saline and the second intramuscularly of 10 mg/kg of body weight of dibenamine-HCl. One hour later all animals were injected with 4 mg/kg of body weight of adrenaline- $\beta$ -C<sup>14</sup> in water, (specific activity 1.1  $\mu\text{c}/\text{mM}$ ). Immediately after, the rats were placed in separate metabolic cages and only drinking water was given to them. Urine was collected for a period of 48 hours in the same way as in the case of the tropolone-treated animals, as described in the previous section. The quantity of urine was very small. It was difficult to know whether this was due to any effect of LSD<sub>25</sub> on diuresis or simply to the fact that the fasting animals, drank less water.

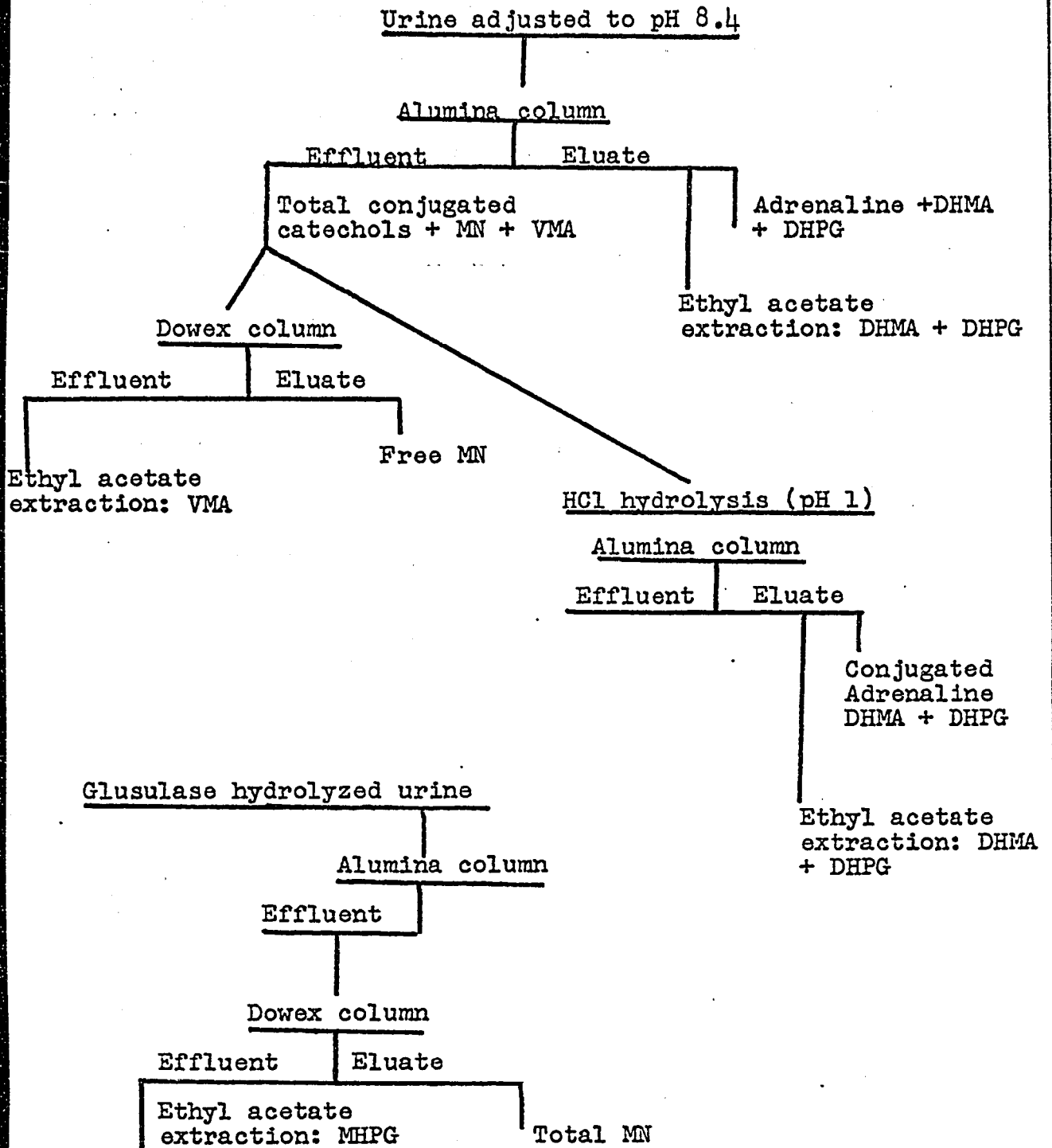
The same experiment was repeated with a smaller group of animals, using only 4 rats.

In TABLE XI the separation of urinary metabolites of adrenaline- $\beta$ -C<sup>14</sup> is shown in rats injected with 0.2 mg of lysergic acid diethylamide (LSD<sub>25</sub>).

#### 1. Extraction procedure.

A portion of 48 hour urine, diluted to 100 ml with N/100 acetic acid, was used for the determination of catechols, free metanephrine (MN) and 3-methoxy-4-hydroxymandellic acid (VMA). The conjugated catechols were separated after acid hydrolysis of a portion of effluent from an alumina column; the conjugated O-methylated metabolites -

Fig. 8



Flow sheet of extraction proceedings

MN and NHPG (3-methoxy-4-hydroxyphenylglycol) - after hydrolysis of diluted urine with glucuronidase. We reported a few minor modifications, especially concerning the extraction and the final determination of radioactivity.

a/ Separation of adrenaline, 3-4-dihydroxymandelic acid and 3-4-dihydroxyphenylglycol.

A 15 ml portion of diluted urine, adjusted to pH 8.4 with 1N NaOH and 0.5M Na<sub>2</sub>CO<sub>3</sub> using the pH-meter, was passed through a column containing 0.7 g of washed Woelm, non alkaline alumina (WEIL-MALHERBE and BONE 1952) buffered at pH 8.4 with 0.2 M sodium acetate solution. After absorption of the sample the column was washed with 5 ml of 0.2M sodium acetate followed by 5 ml of distilled water. The effluent combined with washings was used to estimate the conjugated catechols, VMA and free MN.

Adrenaline and DHMA + DHPG were then eluted from the alumina column with 25 ml of 0.2N HCl. For the most part this step was extremely slow, and in several cases we were obliged to apply pressure. The radioactivity of the free catechols was measured on 50 lambdas of the eluate, and those of the non-amine catechols after extraction with an organic solvent. A 10 ml aliquot of the eluate was acidified with 2 ml of 6N HCl and saturated with NaCl, then DHMA + DHPG were extracted into ethyl acetate. Three successive extractions with 1.5 volume of ethyl acetate were

carried out by shaking mechanically for 15 min. each time. The combined organic phases were centrifugated and evaporated to dryness in a water bath at 40°C under stream of nitrogen. The residue was dissolved in 0.2 ml of ethyl acetate and 50 lambdas of it was used to determine the radioactivity. This fraction contained both DHMA and DHPG. We were able to separate these compounds by paper chromatography. The method used for this purpose will be described later in this section.

Radioactivity due to adrenaline was calculated by subtracting the non-amine catechol radioactivity from the total catechol radioactivity.

b/ Conjugated catechols.

The conjugated catechols were determined after acid hydrolysis of 10 ml of the combined effluent and washings from the alumina column. This sample was acidified to pH 1.0 with 6 N HCl and hydrolyzed in a boiling water bath for 15 min. After cooling, the sample was readjusted to pH 8.4 and passed through an alumina column, as described above. The released catechols were first absorbed on the alumina and then eluted with 0.2 N HCl. The radioactivity of the total conjugated catechols was measured on 50 lambdas of the eluate, the conjugated non-amine catechols were determined after extraction into ethyl acetate, and the radioactivity due to the conjugated adrenaline was calculated

by the difference.

c/ Free metanephrine.

The separation of free metanephrine (MN) was performed by using a Dowex 50 CG (X4) column prepared by suspending the resin in 3 N  $\text{NH}_4\text{OH}$  overnight followed by washing with distilled water until the pH of the effluent was neutral.

An aliquot of 10 ml of the effluent from the alumina column adjusted to pH 6.5 was passed through the Dowex column and the resin was washed with 20 ml of water. The MN was eluted with 15 ml of 3 N  $\text{NH}_4\text{OH}$ . 50 lambdas of the eluate was used to determine the radioactivity.

d/ 3-Methoxy-4-hydroxymandelic acid (VMA).

VMA was extracted from the combined effluent and washings obtained from the Dowex 50 column. A 10 ml sample was adjusted to pH 1.0 and extracted 3 times with 1.5 volumes of ethyl acetate. The organic phase was evaporated to dryness and the residue was redissolved in the same solvent. One portion of it was tested for radioactivity.

e/ Total MN and 3-methoxy-4-hydroxyphenyl glycol (MHPG).

The O-methylated derivatives were isolated after enzymatic hydrolysis. To an aliquot of 10 ml of diluted urine, 10 ml of 0.5 M sodium acetate buffer pH 6.0 and 0.2 ml of glucuronidase (Endo) were added. The pH of the sample was adjusted to 5.2 and the mixture was incubated at 37°C for

24 hrs. The gluculase used in our experiments was a commercial preparation (ENDO) of  $\beta$ -glucuronidase and sulfatase and it contained per ml 100,000 units of the first and 50,000 units of the second enzyme. The optimum pH for  $\beta$ -glucuronidase is about 5.0, and that for sulfatase is close to pH 6.0. Maximum activity for sulfatase in 0.5 M acetate buffer is at pH 6.1 (FROMAGEOT in "THE ENZYMES" by SUMNER and MYRBACK). Because the acidity of all diluted urines was not the same, we decided to control the pH of samples for enzymatic hydrolysis, keeping them constant in all our experiments.

The MN was isolated by successive absorption of the sample on alumina and on Dowex columns. Both free and conjugated amines were eluted with 15ml of 3 N ammonia. The radioactivity of the eluate was determined.

MHPG was isolated after extraction with ethyl acetate from Dowex column NaCl saturated effluent and washings. Its radioactivity was measured as above.

The conjugated MN was calculated by subtracting the free MN from the total.

f/ Determination of radioactivity.

Prior to being counted in a Nuclear Chicago Scintillation counter, all samples were spotted on a small strip of filter paper, dried from solution and then immersed in a vial containing 10 ml of scintillation solvent (toluene,

QUENCH CORRECTION CURVE FOR ADRENALINE- $\beta$ -C<sup>14</sup>

QUENCHING AGENTS: + - URINE  
                          o - - ACETONE

R = ratio of counts on channel 1 / counts  
on channel 2

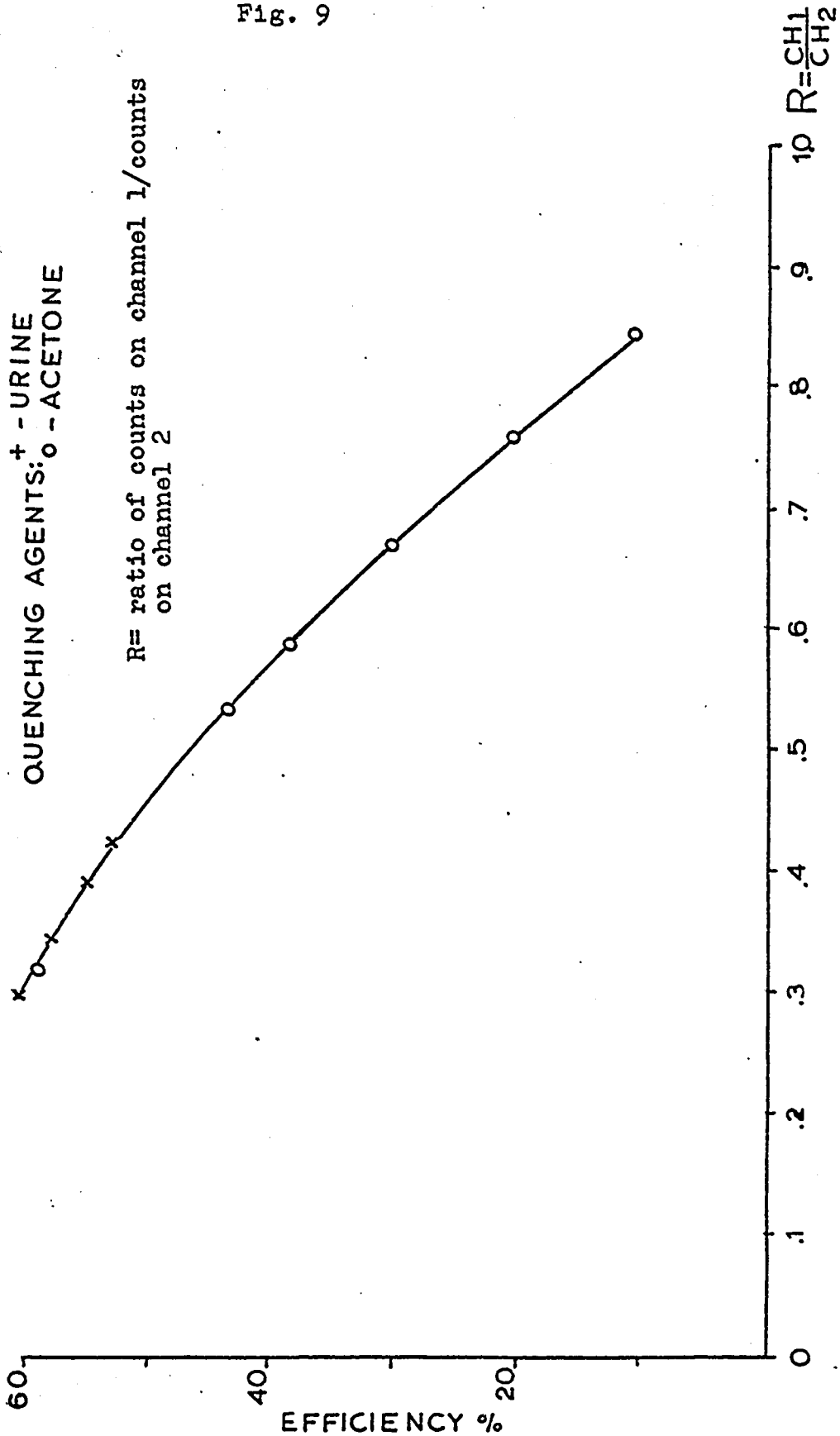


Fig. 9

added 0.3 % PPO (a) and 0.01 % POPOP (b). We found this paper counting method quite satisfactory. As the radioactive spots were insoluble in toluene (very slight variations in ratio Ch1/Ch2) we could count all our samples with the same efficiency. We controlled all changes in channel ratio very closely. Uniform drying of the paper was found to be very important, because the moisture content of a sample could lead to quenching. As some of our samples were slightly colored we made a quench correction curve for adrenaline spotted on paper. The addition of 10 lambdas of non-radioactive concentrated urine (yellow color) had no noticeable effect on the quench curve. Fig. 9 shows such a curve for adrenaline obtained with different quenching agents. The working conditions adopted were as follows: counts on channel 1 were from Ch 1 = base - level 1; counts on channel 2 were from Ch 2 = level 1 - level 2; voltage: Data 1050 v; Gate 1150 v; and temperature of the counting chamber 14<sup>o</sup>F. Under such conditions the counting efficiency for C<sup>14</sup> varied between 55 and 60%.

---

(a) PPO - 2,5-diphenyloxazole

(b) POPOP - 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene

---

g/ Separation of 3,4-dihydroxymandelic acid and  
3,4-dihydroxyphenyl glycol by paper chromatography.

Separation of DHMA and DHPG from a mixture presents serious difficulties. On Whatman # 1 those compounds could be separated only after previous enzymatic conversion to corresponding O-methylated acid and glycol (KOPIN 1961).

Trying the different ion-exchange papers and solvent systems, we were able to separate both radioactive compounds from alumina column eluate extract without having recourse to methylation procedure. We found that two types of paper, Whatman AE-30 and DEAE-20 give very good separation. On PC-20 the resolution was very poor.

The solvent system previously adopted for the separation of catecholamines was used in this case also. The chromatograms were run in Bu:HAo:water (4:1:1) for 15 hrs. The chromatograms of the extract added to the synthetic mixture composed of DHMA, DHPG, VMA and MHPG were performed parallel to the control which contained only synthetic compounds. Under such conditions the colored spots of DHPG and DHMA corresponded to the radioactive zones.

TABLE XII

R<sub>f</sub> values of DHMA, DHPG, VMA and MHPG on Whatman AE-30 and DEAE-20.

Compound	R <sub>f</sub>	R <sub>f</sub>
	AE-30	DEAE-20
DHMA	0.04	0.07
VMA	0.12	0.19
DHPG	0.35	0.45
MHPG	0.65	0.77

The solvent system used: Bu:HAc:water  
running time: 15 hrs.

MESCALINE.

Mescaline is another psychomimetic drug (KETTY 1957) of increasing interest to psychiatrists (HOCH 1951). Structurally it resembles catecholamines. These compounds likewise possess certain functional similarities. Because of the ability of mescaline to produce symptoms analogous to those of schizophrenia, (HOCH 1951), OSMOND (OSMOND 1952) suggested that a disturbance in adrenaline metabolism might lead to the formation of a toxic amine responsible for causing mental disorders and psychoses.

Chemically synthesized (SPAETH 1919) mescaline is a 3,4,5-trimethoxyphenylethylamine. This compound occurs in nature in the "mescal" buttons of Mexican cactus *Anhalonium Lewinii* Hennings and in *Trichocereus Trescheckii* Parmentier. For ages, dried tops of this plant were used in religious rites of North American Indians (KLUVER 1928) and are well known as having hallucinogenic (CLAUDE 1934) and certain curative properties (SLOTKIN 1956). In man, the administration of mescaline causes visual and auditory hallucinations (FISHER 1951; SOURKES 1956). But as a psychomimetic agent it is much less effective than LSD<sub>25</sub>.

Studies of the metabolism of mescaline have demonstrated that this compound is metabolized in the body into its corresponding acids, which are biologically inactive (SLOTA 1936). Experiments with animals showed that mescaline is

tolerated by rabbits in doses many times larger than a dose which is toxic in man (SLOTA 1936). In VITRO, it was shown that monoamine oxidase from guinea-pigs' liver attacks mescaline slowly (BLASCHKO 1937; PUGH 1937). But BERNHEIM (BERNHEIM et al. 1938) obtained evidence that preparations from rabbit liver contained an enzyme system which readily oxidized mescaline. Differences between the oxidative deamination of mescaline (which is inhibited by cyanide) and a typical substrate of monoamine oxidase suggest that two different enzymes are involved (BLASCHKO 1944).

In the rat, the intraperitoneal LD<sub>50</sub> of mescaline sulfate for an unfasted animal was found to be 370 mg/kg (SPECK 1957). The dose 411 mg/kg caused death in less than 30 min. But the same animal may tolerate a dose as high as 534 mg/kg when injected subcutaneously (HOSHIKAWA 1962). In man, the dose normally used is 6 mg/kg. This dose combined with insulin might therefore kill a rat (FISHER 1958).

It was demonstrated that a single injection of mescaline causes peripheral vasoconstriction, slows heart rate and produces hypoglycemia (SPECK 1958). Fasting or a previous injection of adrenaline has a protective action on the animal by reducing the degree of hypoglycemia and bradycardia produced by the mescaline (SPECK 1957). But when adrenaline was given one-half hour after the mescaline was administered, the hyperglycemic action of adrenaline was

partially blocked.

The routes of metabolism followed by mescaline in man and in experimental animals are different (HARLEY-MASON 1957). In man, its hallucinogenic action is probably due to the formation of an acidic metabolite (HARLEY-MASON 1958). In the dog, after oral administration, the deamination of mescaline led to the formation of 3,4,5-trimethoxyphenylacetic acid, which is excreted in the urine (SLOTA 1936). But a quite significant percentage of unchanged drug is also excreted (SPECTOR 1961).

The inactivation of mescaline in VIVO may follow three different pathways:

- 1/ Through a monoamine oxidase (MAO);
  - 2/ Through a diamine oxidase (ZELLER 1958);
- or
- 3/ Through demethylation (DALY 1962).

The potentiation of the effects of mescaline may be induced by blocking either MAO with iproniazid, or DAO with a semicarbazide (ZELLER 1958).

In mice, mescaline is mostly (in 80%) excreted in unchanged form (BLOCK et al. 1952). In rats, after intraperitoneal administration of radioactive mescaline, 72% is excreted in urine as 3,4,5-trimethoxyphenylacetic acid and only 9% as unchanged mescaline (BLOCK 1952).

In 1962 a group of workers (FRIEDHOFF, GOLDSTEIN 1962) found 3,4,5-methoxyphenylethanol in the urine of rats

after the administration of mescaline. Normally, mescaline is converted primarily to 3,4,5-trimethoxyphenylacetic acid, and very little 3,4,5-trimethoxyphenylethanol is formed.

These authors suggested that mescaline is at first metabolized to aldehyde and then oxidized to acid by aldehyde dehydrogenase (FELLMAN 1959), or reduced to alcohol. They obtained certain evidence that mescaline itself is not a hallucinogenic drug and that, rather, alcohols or perhaps aldehydes are toxic.

In a series of experiments, using enzyme-blocking agents, it was demonstrated that the metabolites of mescaline-methoxyphenylaldehyde and methoxyphenylethanol - produce an extremely potent biological effect at doses much lower than are required for mescaline itself. A dose of 50 mg per kg of body weight of mescaline produces very moderate effects and changes in a rat (FRIEDHOFF 1962).

A few years ago, AXELROD (AXELROD 1956) reported that mescaline undergoes deamination in VITRO.

In connection with the findings that O-methyl transferase from rat liver methylates one of the OH groups of adrenaline (AXELROD 1957), FISHER proposed the hypothesis that mescaline with three methoxy groups may inhibit the methylation of noradrenaline in the brain. Then the resulting increase in the brain of this hormone level may be responsible in part for the psychoses caused by mescaline

TABLE XIII

Effect of mescaline (50 mg/kg) on the excretion of injected adrenaline- $\beta$ -C<sup>14</sup> and its urinary metabolites in rats pretreated with dibenamine.

Compound	Rat #						Average	SD
	1	2	3	4	5	6		
Adrenaline free conj.	2.85 7.29	2.20 6.32	3.61 6.69	2.64 5.70	3.11 8.72	2.77 7.56	2.77 $\pm$ 7.05 $\pm$	0.47 1.06
DHMA + DHPG free conj.	1.32 3.06	0.72 2.84	0.64 3.50	1.08 2.66	0.82 2.38	0.64 2.08	0.90 $\pm$ 2.75 $\pm$	0.25 0.50
Metanephrine free conj.	7.76 38.07	6.34 41.23	8.62 35.76	9.67 35.14	8.30 35.02	8.14 34.76	8.13 $\pm$ 36.66 $\pm$	1.09 2.54
VMA	6.89	7.87	7.58	8.01	8.49	7.85	7.78 $\pm$	0.53
MHPG hydrolyzable unhydrolyzable	14.41 15.68	14.24 18.94	21.52 11.06	17.51 16.31	16.29 13.03	16.23 12.03	16.70 $\pm$ 14.51 $\pm$	2.68 2.98

TABLE XIV

Effect of mescaline (100 mg/kg ) on the excretion of intramuscularly injected adrenaline- $\beta$ -C<sup>14</sup> and its urinary metabolites in rats pretreated with dibenamine.

Compound	R a t #			Average	SD
	1	2	3		
Adrenaline free	3.90	8.96	5.62	6.16	± 2.57
conj.	7.37	12.72	10.31	10.09	± 2.68
DHMA + DHPG free	0.44	0.78	0.32	0.51	± 0.24
conj.	0.86	1.08	1.52	1.15	± 0.34
Metanephrine free	11.27	7.96	8.82	9.35	± 1.72
conj.	33.50	23.08	28.29	28.29	± 5.21
VMA	7.75	7.45	8.24	7.81	± 0.40
MHPG hydrolyzable	28.15	27.26	18.63	24.68	± 5.25
unhydrolyzable	5.64	0	7.35	4.33	± 3.85

(FISHER 1958).

From more recent studies on enzymatic O-methylation and O-demethylation of this drug (DALY 1962), it may be concluded that the main metabolic product of mescaline in a rat is 4-O-methylated amine: 3,5-dihydroxy-4-methoxyphenylethylamine.

#### EXPERIMENTAL PART.

Experiments were performed on two groups of animals. One group was composed of six male rats, weighing between 160 and 180 g, the second group of four rats of smaller weight. To reduce the toxic effect of adrenaline the rats were pretreated one hour before with dibenamine.HCl. At the same time as the first group received 50 mg/kg of mescaline, the second group was administered with 100 mg/kg of the same drug.

The urine was collected as described above for LSD<sub>25</sub> treated rats, and the metabolites of injected adrenaline were separated according to the KOPIN method.

In TABLE XIII the separation of urinary metabolites of labeled adrenaline is shown in rats injected with 50 mg/kg of mescaline while TABLE XIV shows similar results for animals treated with 100 mg/kg of mescaline.

#### ADRENOCHROME:

In 1952 a Saskatchewan group (OSMOND 1952; HOFFER

1954) reported that adrenochrome and adrenolutine are the substances which, when injected into normal subject, produce psychoses. Adrenolutine, immediately derived from adrenochrome, is the more stable of the two compounds.

The same group of workers suggested the following possibilities:

- 1/ that in the CNS of schizophrenic patients the adrenaline was not completely oxidized;
- and, 2/ that the resulting accumulation of abnormal metabolites, due to a certain defect in enzymatic breakdown of adrenaline, might be the cause of the mental disturbance.

A few years later, the presence of adrenochrome in the plasma of normal subjects, in an amount of 50 micrograms per litre, was reported (HOFFER 1958). This investigator observed that the administration of lysergic acid diethylamide increased the blood level of adrenochrome. From these findings he concluded that adrenochrome plays an important role in "experimental" schizophrenia.

This hypothesis was not generally accepted. Many workers have been unable to confirm HOFFER's results (RINKEL 1954; SZARA et al. 1958).

It is well known that in VITRO adrenaline may be oxidized to adrenochrome (GREEN 1937) in two different ways:

- A/ By different enzymatic systems:

- (1). cytochrome oxidase (GREEN 1937);
- (2). catechol oxidase (BLASCHKO 1940);
- (3). phenolase (BACQ 1949);
- (4). ceruloplasmin (HOLMBERG 1951);
- (5). catecholamine oxidase from the cat salivary gland (AXELROD 1964),

or

B/ By autoxidation on standing in solution.

In the reaction of formation of adrenochrome, three possibilities may be involved:

1. The formation of adrenoerythrine as intermediate (BALL 1933);
2. The formation of leuco-adrenochrome before adrenochrome itself (GREEN 1937);
3. The existence of a semiquinone as intermediate (HARLEY-MASON 1948), because the adrenochrome itself is very unstable (BRACONIER 1943).

The second reaction (above) is catalyzed by the cytochrome indophenol oxidase system present in all tissues (GREEN 1937).

In 1940 MANN and QUASTEL showed that a number of amines including adrenaline compete with one another for amine oxidase in the brain, and that aberrant amine metabolism may be involved in mental disease (MANN 1940). The oxidation of adrenaline in VITRO is often compared with

oxidation by catechol oxidase (BACQ 1949).

In VIVO, the autooxidation of adrenaline and the formation of adrenochrome are very unlikely. The various reducing agents, such as ascorbic acid and glutathione, normally present in the tissues, protect the catecholamines against autooxidation (BLASCHKO 1940). Nevertheless, BACQ has obtained certain evidence that adrenaline may be metabolized in VIVO by indolization to adrenochrome by phenolases (BACQ 1949). But this point of view is in complete disagreement with the views of BLASCHKO (BLASCHKO 1940).

The experimental results of SCHAYER obtained with radioactive adrenochrome are in agreement with generally accepted opinion that adrenochrome does not play any role in the metabolism of adrenaline in VIVO (SCHAYER 1953). Recently, AXELROD observed that in VIVO the formation of adrenochrome in the salivary gland is negligible (AXELROD 1964).

Experiments performed on animals have shown that in dog the intravenously injected adrenochrome passed into the urine and was eliminated in 45 min. after injection. However, in a rabbit the same compound was not excreted after administration (LECOMTE 1959). Certain investigators consider adrenochrome as a biologically inactive compound (ISSEKUTZ 1950).

In this experiment we used five male rats, weighing

TABLE XV

Effect of adrenochrome on the excretion of injected adrenaline- $\beta$ -C<sup>14</sup> and its urinary metabolites in rats pretreated with dibenamine.

Compound	R a t #					Average	SD
	1	2	3	4	5		
Adrenaline							
free	6.11	7.58	8.33	4.90	5.39	6.48	$\pm$ 1.45
conj.	7.22	6.77	6.69	7.68	6.80	7.03	$\pm$ 0.42
DHMA + DHPG							
free	0.44	0.47	1.08	0.50	0.41	0.58	$\pm$ 0.28
conj.	1.80	1.14	1.14	1.18	1.16	1.28	$\pm$ 0.40
Metanephrine							
free	9.30	13.05	11.24	11.05	10.54	11.04	$\pm$ 1.35
conj.	32.38	28.72	32.52	34.18	34.15	32.39	$\pm$ 2.22
VMA	6.39	6.78	5.85	5.24	6.66	6.18	$\pm$ 0.64
MHPG							
hydrolyzable	16.80	13.68	12.23	15.61	19.34	15.53	$\pm$ 2.76
unhydrolyzable	14.68	17.90	20.33	15.53	12.83	16.25	$\pm$ 2.93

between 145 and 160 g. All of these were injected with dibenamine.HCl and with an aqueous solution of adrenochrome (pH 7.4) 25 mg/kg of body weight. One hour later radioactive adrenaline was administered.

Urine was collected over a period of 48 hours and the separation of adrenaline and its radioactive metabolites was performed, using KOPIN's (KOPIN 1961) method, previously described.

TABLE XV shows the distribution of radioactivity in rat urine, following injection of adrenochrome and radioactive adrenaline.

#### DISCUSSION.

TABLE XVI summarizes the effects of psychomimetic drugs on the metabolism of injected adrenaline- $\beta$ -C<sup>14</sup>. To reduce the toxic effect of adrenaline dibenamine.HCl was administered one hour prior to injection of the adrenaline. In all our experiments in this section we used the same group of control animals.

The average recovery of injected radioactivity in our experiments is lower than that found by SCHAYER (SCHAYER 1953) and ELMADJIAN (ELMADJIAN 1956), but is in fairly close agreement with results reported by KOPIN (KOPIN 1961).

In LSD<sub>25</sub> and mescaline (50 mg/kg) treated animals the average amount of adrenaline was the same or slightly

TABLE XVI

Excretion of the intramuscularly injected adrenaline- $\beta$ -C<sup>14</sup> and its metabolites in the urine of rats pretreated with:

A/ dibenamine;

B/ dibenamine and LSD-25;

C/ dibenamine and mescaline (50 mg/kg);

D/ dibenamine and adrenochrome.

E/ dibenamine and mescaline (100 mg/kg).

The results are expressed as average values of the percentage of radioactivity excreted in the urine.

Compound	A		B		C		D		E
	Av.	SD	Av.	SD	Av.	SD	Av.	SD	
Adrenaline	free	3.61 ± 0.90	2.46 ± 0.74		2.77 ± 0.47		6.48 ± 1.45		6.16 ± 1.15
	conj.	8.60 ± 1.31	7.66 ± 1.31		7.05 ± 1.06		7.03 ± 0.42		10.09 ± 1.15
DHMA + DHPG	free	0.52 ± 0.22	0.55 ± 0.26		0.90 ± 0.25		0.58 ± 0.28		0.51 ± 1.15
	conj.	1.73 ± 0.67	2.52 ± 1.44		2.75 ± 0.50		1.28 ± 0.40		1.15 ± 1.15
Metanephrine	free	11.44 ± 2.69	9.80 ± 1.37		8.13 ± 1.09		11.04 ± 1.35		9.35 ± 1.15
	conj.	36.49 ± 3.03	33.16 ± 3.20		36.66 ± 2.54		32.39 ± 2.22		28.29 ± 1.15
VMA		6.48 ± 1.07	7.34 ± 0.61		7.78 ± 0.53		6.18 ± 0.64		7.81 ± 1.15
MHPG	hydrolyzable	15.84 ± 2.08	22.36 ± 1.84		16.70 ± 2.68		15.53 ± 2.76		24.68 ± 1.15
	unhydrolyzable	13.24 ± 3.25	9.29 ± 3.21		14.51 ± 2.98		16.25 ± 2.93		4.33 ± 1.15

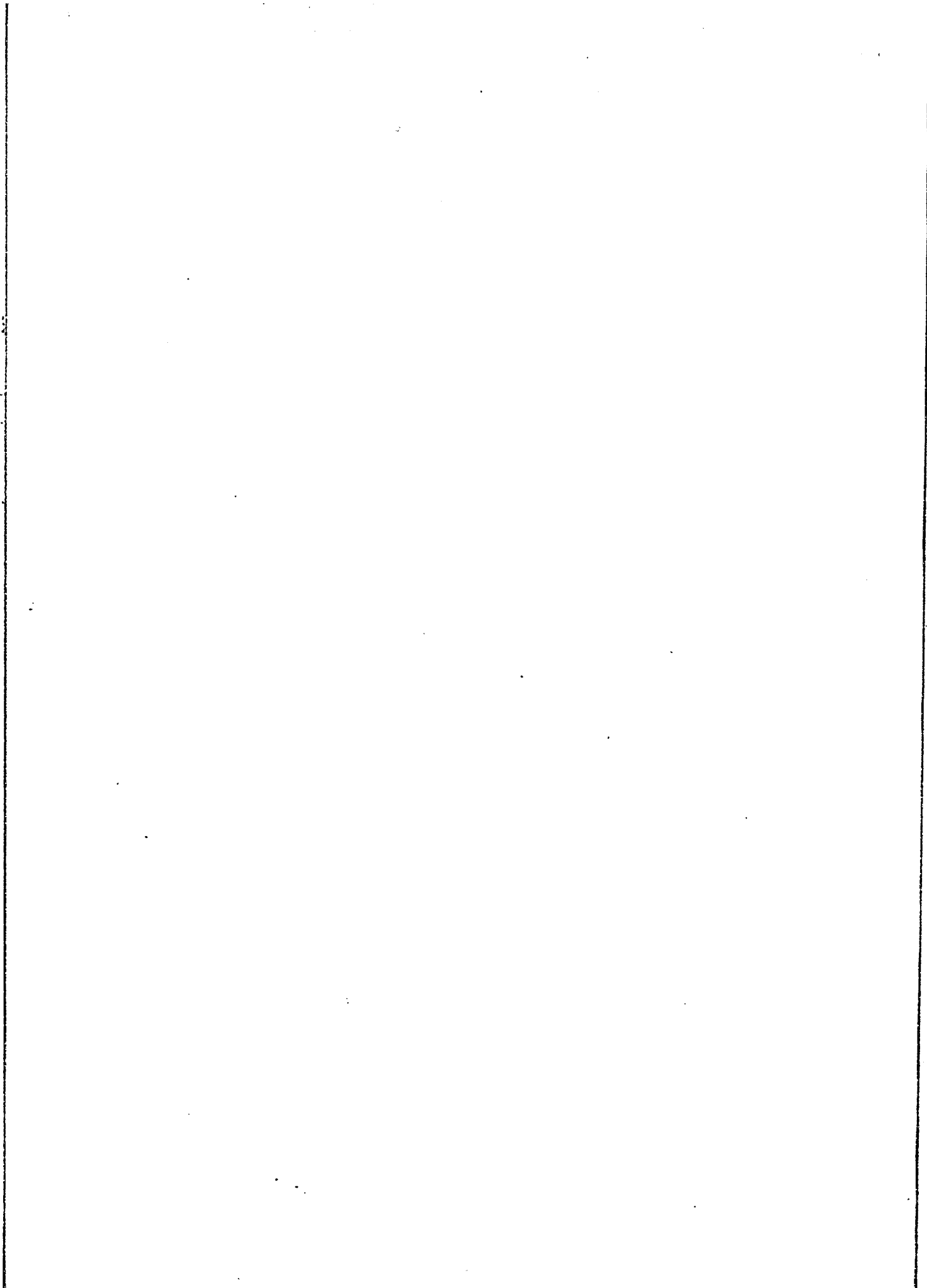


TABLE XVI

cretion of the intramuscularly injected adrenaline- $\beta$ -C<sup>14</sup> and its metabolites in the urine of rats pretreated with:

A/ dibenamine;

B/ dibenamine and LSD-25;

C/ dibenamine and mescaline (50 mg/kg);

D/ dibenamine and adrenochochrome.

E/ dibenamine and mescaline (100 mg/kg).

The results are expressed as average values of the percentage radioactivity excreted in the urine.

Compound	A		B		C		D		E	
	Av.	SD	Av.	SD	Av.	SD	Av.	SD	AV	SD
adrenaline free conj.	3.61 ± 0.90		2.46 ± 0.74		2.77 ± 0.47		6.48 ± 1.45		6.16 ± 2.57	
	8.60 ± 1.31		7.66 ± 1.31		7.05 ± 1.06		7.03 ± 0.42		10.09 ± 2.68	
MA + DHPG free conj.	0.52 ± 0.22		0.55 ± 0.26		0.90 ± 0.25		0.58 ± 0.28		0.51 ± 0.24	
	1.73 ± 0.67		2.52 ± 1.44		2.75 ± 0.50		1.28 ± 0.40		1.15 ± 0.34	
tanephrine free conj.	11.44 ± 2.69		9.80 ± 1.37		8.13 ± 1.09		11.04 ± 1.35		9.35 ± 1.72	
	36.49 ± 3.03		33.16 ± 3.20		36.66 ± 2.54		32.39 ± 2.22		28.29 ± 5.21	
A	6.48 ± 1.07		7.34 ± 0.61		7.78 ± 0.53		6.18 ± 0.64		7.81 ± 0.40	
PG hydrolyzable nonhydrolyzable	15.84 ± 2.08		22.36 ± 1.84		16.70 ± 2.68		15.53 ± 2.76		24.68 ± 5.25	
	13.24 ± 3.25		9.29 ± 3.21		14.51 ± 2.98		16.25 ± 2.93		4.33 ± 3.85	



lower than in the control group. While in the adrenochrome-treated rats this value was twice as high as in the control. In each group the values for different metabolites were very much the same from rat to rat.

Because the specific activity of adrenaline- $\beta$ -C<sup>14</sup> is very low (1.1 mc/mM) we were obliged to inject the animals with a very high dose of radioactive catecholamine. It is generally known that the metabolism of endogenous and of exogenous amine, when injected within physiological limits, follow identical metabolic pathways. In our experiments very high doses of adrenaline were injected so that the urinary metabolic pattern is not necessarily that of endogenous adrenaline.

From the data in this TABLE, about 84% of radioactivity found in urine appears as O-methylated compounds: both free and conjugated. The conjugated metanephrine represents 1/3 of it. The value of the free metanephrine varies between 8% in the case of mescaline (50 mg/kg) treated rats and 9.8 to 11.5% for other groups, including the control. The difference between these figures is highly significant ( $P < .001$ ) and indicates that O-methylation of adrenaline to metanephrine (free) is inhibited by nearly 30%.

The reported results show also that in the case of mescaline (50 mg/kg) the values for the fraction containing DHMA and DHPG are higher than for the control group ( $P < .001$ ).

However, the designated catechols represent only a very small portion of the excreted radioactivity.

On the other hand, in the case of animals injected with 100 mg/kg of mescaline, the most notable change was observed in excreted adrenaline: free and conjugated, also conjugated metanephrine. The value for the free adrenaline was twice that of the control group; the conjugated adrenaline was 20 % higher, but the conjugated metanephrine was about 25 % lower than in the control.

Because of the limited number of animals used and because of the individual variations, the reported values are more qualitative than quantitative in nature. Nevertheless, the results suggest that the inhibition of COMT takes place and that the degree of this inhibition depends on the dose of the drug used.

In the case of LSD<sub>25</sub>-treated rats, we noticed an increase in the hydrolyzed fraction of MHPG ( $P < .001$ ). However, the total radioactivity of this metabolite, including both hydrolyzed and unhydrolyzed MHPG, present the same percentage of excreted radioactivity as does the control group. This increase in the hydrolyzed fraction of MHPG may be due to the fact that we used different batches of glucuronidase for the hydrolysis. It could be that the activity of this enzyme varied from batch to batch, and in consequence, this difference in the MHPG level between

Test and Control groups is due to incomplete hydrolysis.

Metanephrine, free and conjugated, is slightly lower, and the value for conjugated DHMA and DHPG is higher in the LSD<sub>25</sub> group. The difference for free metanephrine is not significant; however for conjugated metanephrine the P value is small ( $P < .01$ ). P is of the same order of magnitude in the DHMA and DHPG.

This study is not conclusive; however it leaves no doubt that LSD<sub>25</sub>, given in an amount of 0.2 mg/kg to a fasting rat, has not a very marked effect on inhibition of COMT. However, the possibility that higher doses of this drug may inhibit O-methylation in VIVO is not excluded. Also it is possible that under certain physiological conditions or in the case of using labeled adrenaline of higher specific activity in smaller doses, the quantity of LSD<sub>25</sub> used in these experiments may be sufficient to change the metabolic pattern of catecholamines in the rat.

For adrenochrome-treated rats, the most striking difference aside from that in the free adrenaline level (twice as high as in the control group) was the slight decrease of conjugated metanephrine. The inhibition in conjugation was of the order of 12 to 14% only; however, the P value was quite significant ( $P < .002$ ).

Nevertheless, from the data presented here it has been difficult to demonstrate marked differences in the

metabolism of exogenous adrenaline following injection of adrenochrome.

In this group the rats received adrenaline- $\beta$ -C<sup>14</sup> bitartrate one hour after the administration of adrenochrome. Meanwhile it was noted that part of the injected adrenochrome was already eliminated in the urine. So, at the time of the injection of adrenaline, adrenochrome was present in the circulation in much smaller concentration than was expected. Thus, we consider it probable that its effect was partially reduced and that our results do not present a true picture of the effect of this compound on the metabolism of injected adrenaline in VIVO. In consequence, the present results should be interpreted with that point in mind.

#### DISCUSSION OF METHODS.

The separation techniques used in our experiments, both column and paper chromatography, have proved to be adequate and reproducible.

The method described previously for separation of DHMA and DHPG is not only confined to these two compounds. Under the same conditions all catecholacids may be distinctly separated. The catecholacids are more strongly retained on papers AE-30 and DEAE-20 than their phenylglycol analogues. The radioactivity and colour reactions for each of these compounds, coincided in one spot, which migrated exactly as did the authentic reference compounds.

The tint and intensity of the colours obtained by spraying the chromatograms with p-nitro-aniline were found to depend, to some extent, on the presence of the  $\text{OCH}_3$  group in catechol ring in meta-or para-position. 3-methoxy-4-hydroxymandelic acid, 3-methoxy-4-hydroxy-phenylglycol, metanephrine and paranephrine give the strongest color reaction of all the metabolites of adrenaline.

The use of cation exchange papers has already been discussed in a previous section.

The alumina and the Dowex 50 column separation methods (KOPIN 1961); WEIL-MALHERBE, BONE 1952), were found to be as satisfactory as expected. However, a few minor modifications would be advisable. At the present the adsorption and the elution of adrenaline and free catechols from the alumina column demand several hours and even then are often incomplete. The recovery of adrenaline varies from column to column or from sample to sample. The use of batch process for this purpose (as in the photofluorometric method) instead of that of WEIL-MALHERBE and BONE may save much time and give better recovery of free catechols and adrenaline.

EFFECT OF 3,5-DIIODO-4-HYDROXYBENZOIC ACID AND 3,5-DIIODO-4-OH-PHENYLPYRUVIC ACID ON THE METABOLISM OF INJECTED ADRENALINE IN RATS.

Already it has been shown that DIHBA acts as an inhibitor of COMT (D'IORIO, MAVRIDES 1962). More recently the same group of investigators reported that both iodophenols, DIHBA and DIHPPA produce an inhibition of COMT in VITRO (D'IORIO 1963).

It was felt that both these compounds should be tested for their in VIVO action.

The experiments with DIHBA were performed, using two different concentrations of this compound. Group "A" received 30 mg/kg of body weight and Group "B" 15 mg/kg of this drug dissolved in M/15 phosphate buffer, adjusted to pH 7.4.

Each group consisted of six male, Sprague-DAWLEY rats, weighing between 160 - 190 g. and 175 - 200 g. respectively. All rats received Dibenzamine at the same time as DIHBA, and a half hour later were injected with radioactive adrenaline. The rats injected with 30 mg/kg of body weight of DIHBA stood on their hind legs, immobile for several minutes. After the injection of labeled adrenaline, transitory tachycardia and hyperventilation was noted. After a short period of time the animals fell again into inactivity and catonia and remained motionless for several hours. During this time they did not drink any water and 24-hour specimens of urine

TABLE XVII

Excretion of the intramuscularly injected adrenaline- $\beta$ -C<sup>14</sup> and its metabolites in urine of rats pretreated with:

A/ dibenamine;

dibenamine and:

B/ 3,-5-diiodo-4-hydroxybenzoic acid (15 mg/kg);

C/ 3,5-diiodo-4-hydroxybenzoic acid ( 30 mg/kg);

D/ 3,-5-diiodo-4-~~Cl~~-phenylpyruvic acid (30 mg/kg).

The results are expressed as average values of the percentage of radioactivity excreted in the urine.

Compound	A		B		C		D	
	Av.	SD	Av.	SD	Av.	SD	Av.	SD
Adrenaline free conj.	3.07 ± 0.51		3.15 ± 0.80		4.99 ± 2.48		3.66 ± 1.12	
	8.35 ± 1.53		5.82 ± 1.41		8.35 ± 2.20		4.78 ± 0.96	
DHMA + DHPG free conj.	0.47 ± 0.25		0.46 ± 0.50		0.84 ± 0.55		0.62 ± 0.34	
	1.33 ± 0.34		2.30 ± 0.34		3.24 ± 1.38		2.81 ± 0.24	
Metanephrine free conj.	11.16 ± 2.91		7.08 ± 2.78		8.63 ± 1.37		10.62 ± 1.72	
	37.50 ± 1.77		37.71 ± 3.72		31.25 ± 5.25		31.75 ± 3.41	
VMA	6.94 ± 0.70		7.07 ± 2.26		7.24 ± 1.44		7.41 ± 1.00	
MHPG hydrolyzable unhydrolyzable	16.24 ± 2.44		23.15 ± 3.44		28.81 ± 2.04		20.82 ± 3.10	
	14.55 ± 2.41		13.00 ± 3.55		9.40 ± 2.03		13.57 ± 3.87	

were extremely small.

The reaction of rats injected with a lower dose of DIHBA (15 mg/kg) was normal. The volume of urine collected for a period of 48 hours was twice as large as in the previous experiment.

In the case of DIHPPA we used only one group of six rats. The animals were injected with a dose of 30 mg/kg of DIHPA in phosphate buffer, at the same time receiving dibenamine. A half-hour later the rats were given labeled adrenaline intramuscularly.

The separation of adrenaline and its metabolites was performed by using KOPIN's method, already described (KOPIN 1961).

TABLE XVII represents the mean values for adrenaline and its urinary metabolites for all groups of rats described above. The control group used here is composed of six rats instead of nine as for other experiments.

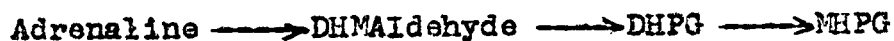
The results obtained with iodophenols show a reduction in the metanephrine level. The rats treated with 15 mg/kg of DIHBA show the greatest variation of free metanephrine. In the group receiving the higher dose of this drug, both free and conjugated metanephrine are decreased by 18%; but in the case of animals given DIHPPA, only the amount of conjugated catecholamine is diminished. At the same time we noticed an increase in DHMA and DHPG fraction and, in

the case of injection of 30 mg/kg of DIHBA, also an increase in free adrenaline.

These findings present support to a limited extent the in VITRO observations on the inhibition of O-methylation.

However, the mean value for VMA is surprisingly constant in all groups, including the control. In rats treated with iodophenol compounds the most striking difference is observed in the MHPG fraction, total and hydrolyzable. Calculation from our data reveals that this increase represents 25% in the case of rats injected with 30 mg/kg of DIHBA, and 17% in the case of those receiving 15 mg/kg of this drug.

These results suggest that there is a disturbance in the normal metabolic pathway of adrenaline. A large amount of adrenaline was probably first deaminated and then followed the metabolic route as follows:



instead of:



In the case of DIHPPA this evidence is less noticeable, nevertheless a decrease in the conjugated metanephrine level is also observed.

Our findings seem to be in full agreement with results obtained by D'IORIO and MAVRIDES (D'IORIO 1963) in VITRO

and confirm that DIHBA, depending on the dose used, can act as an inhibitor of COMT in VIVO.

EFFECT OF COCAINE ON THE METABOLISM OF ADRENALINE.

A/ INTRODUCTION:

Cocaine was isolated in 1860 by NIEMAN. In 1887 von ANREP, studying the pharmacological action of this alkaloid observed that after subcutaneous injection the skin overlying the infiltrated area becomes insensitive. Cocaine constricts the blood vessels and therefore prevents its own absorption. It has a powerful action on the central nervous system (GOODMAN 1958).

In 1910 FROHLICH observed for the first time that cocaine is a strong potentiator of the action of adrenaline on blood pressure and on certain sympathetically innervated effector organs. Since then many hypotheses have been proposed and many investigators have tried to clarify the nature of this potentiation.

Studying the inactivation of adrenaline by blood and tissues (BAIN 1937) BAIN observed that the addition of cocaine did not produce potentiating action on the effects of catecholamines. The experimental results of several workers (ROSENBLUETH et al. 1932; BACQ 1936), using the nictitating membrane of a cat, demonstrated that cocaine increases the action of adrenaline. The same investigators (CANNON 1933) suggested, a few years later, that cocaine might prevent the destruction of injected adrenaline or of a trans-

mitter substance released from the adrenergic nerves. This hypothesis was supported by TRENDELENBURG (TRENDELENBURG 1959).

Two explanation of the potentiating action of adrenaline and noradrenaline on a nictitating membrane by cocaine have been suggested:

1. that cocaine increases the sensitivity of the smooth muscle to adrenaline and noradrenaline and causes an effect similar to that of denervation, often referred to as "pharmacological denervation" (FLECKENSTEIN 1953);
- and 2. that cocaine delays the inactivation of catecholamines (TORDA 1943).

The potentiating action of cocaine depends on the dosage.

GADDUM introduced the hypothesis that the potentiation of adrenaline responses by ephedrine is due to the inhibitory effect of the latter on the oxidation of catecholamine by monoamine oxidase (GADDUM 1938). This hypothesis was extended to include the action of cocaine (MAGGREGOR 1939). The experimental evidence of PHILPOT that cocaine inhibits MAO in VITRO favors the explanation that cocaine may prevent the destruction of injected adrenaline (PHILPOT 1940), and that this inhibitory effect depends on the degree of saturation of the enzyme.

However, this hypothesis has not been generally accepted. Cocaine not only potentiates the responses to adrenaline but also the responses to other sympathomimetic amines, which are not oxidized by monoamine oxidase.

The most conclusive evidence against the theory of inhibition of MAO by cocaine comes from the work of SCHMITT and his co-worker. These authors demonstrated that iproniazid, a potent inhibitor of MAO, does not potentiate the effects of adrenaline and noradrenaline on the nictitating membrane of a cat. Cocaine augments the effects of these amines even after the administration of iproniazid. And so, it has been concluded that the effect of cocaine was not due to the inhibition of MAO (SCHMITT 1956). It is quite possible that this potentiating effect may have been caused by an inhibition of some other enzyme which very rapidly inactivates adrenaline (FURCHGOTT 1955).

Discovery of O-methylation (ARMSTRONG 1957; AXELROD 1957; PELLERIN 1957) and the role it plays in the metabolism of catecholamines suggested that the inhibition of this enzyme might explain the phenomenon of potentiation. But this hypothesis, attractive though it may be, cannot be accepted. Recently many investigators (BACQ 1936; AXELROD 1960) in VITRO conditions have found that a series of polyphenols (Catechol, Pyrogallol, Adrenalone) inhibit the rate of methylation of adrenaline. Cocaine, which strongly

potentiates the adrenaline effects on smooth muscle preparation, did not, however, affect methylation (WYLIE 1960). Previously it had been reported that cocaine produces supersensitivity to amines, which are not substrates of COMT (eg. phenylephrine) (AXELROD et al. 1958), and that pyrogallol does not produce supersensitivity to adrenaline and noradrenaline.

From all these observations it may be concluded that supersensitivity, after denervation as well as after administration of cocaine, cannot be attributed to inhibition of catechol O-methyl transferase.

According to FLECKENSTEIN's theory cocaine causes supersensitivity to all catecholamines. The sensitizing action of this alkaloid is highly selective; adrenaline and noradrenaline are potentiated, and it seems that the action of sympathomimetic amines possessing the OH-group in meta-position are most susceptible to the action of cocaine. (FLECKENSTEIN et al. 1953; INNES et al. 1954; HOLTZ et al. 1960; TRENDELENBURG 1961).

BURN (BURN 1932) first advanced the idea that nerve terminals might also function as a site for uptake of sympathomimetic amines. More recently WHITEY (WHITEY et al. 1960) using the labeled  $H^3$ -noradrenaline studied the effect of cocaine upon it. They observed that cocaine markedly reduces the uptake of circulating noradrenaline into certain tissues, probably by binding the hormone (MACMILLAN

1959). This interference with the uptake increases the quantity of circulating catecholamines, but does not affect the endogenous concentration of adrenaline and noradrenaline in the organs (MUSCHOLL 1961).

In view of more recent experiments the potentiation is explained on the basis of the blocking by cocaine of nonspecific binding sites and the inhibition of a rapid uptake of catecholamines (AXELROD et al. 1960; TRENDELENBURG 1959). This comes as a result of an increase in the concentration of these hormones. Such a point of view may be supported by the following observations:

1. That cocaine decreases the uptake of catecholamines by various tissues (BURN 1961; HERTTING et al. 1961; MUSCHOLL 1961) and delays the disappearance of injected catecholamines (TRENDELENBURG 1963a);
2. That cocaine potentiates the responses to catecholamines of innervated tissues only (KUKOVETZ 1961);
3. And finally, that the uptake of catecholamines by denervated effector organs was less than that by innervated (STROMBLAD and NICKERSON 1961).

During the last twenty years certain important points regarding the mechanism of potentiation of catecholamines by cocaine have been clarified. Of many controversial hypotheses only a few are still valid. In view of recent findings, neither MAO nor COMT plays an important role in the

complicated phenomenon of potentiation; it may easily be the binding and inhibition of uptake of the catecholamines to the site stores which takes on this role.

B/ EXPERIMENTAL PART:

Two facts marked the starting point of this investigation:

1. A large number of experiments on cocaine are physiological and pharmacological, and little is known from the biochemical point of view;
2. And the evidence obtained by LEDUC (LEDUC 1959) indicating that in a rat, cocaine inhibits O-methylation.

Experiments were performed on two groups of animals. One group was composed of eight male rats SPRAGUE-DAWLEY, and the second of seven. In both groups the body weight varied between 190 and 220 g. The animals were kept in separate cages for a few days prior to the experiment.

Group one received cocaine in the form of subcutaneous injection (50 mg of cocaine base in phosphate buffer H/15 adjusted to pH 6.5 per kg of body weight) for twelve days. In spite of the fact that during this time food and drinking water were allowed, we did not observe any change in body weight. It is well known that the local action of cocaine is striking, and that especially after subcutaneous administration multiple abscesses develop at the site of

the injection (GOODMAN 1958). All our experimental animals developed such abscesses, which most probably resulted from the prolonged ischemia due to vasoconstriction caused by the action of the alkaloid.

With the final injection of cocaine the rats received dibenamine, 10 mg/kg, and one hour later they were injected intraperitoneally with labeled adrenaline bitartrate (4 mg per kg free base). The specific activity of the amine was 1.1  $\mu\text{c}/\text{m}/\text{M}$ . The animals were then placed in special metabolic cages and urine was collected for a period of 48 hrs. in one ml of 0.1 N HCl. During this time only drinking water was permitted.

As prolonged treatment with cocaine has been shown to cause abscesses, we decided also to investigate the effects of a single dose of cocaine on the metabolism of catecholamine. For this reason the second group of rats was given only one injection of cocaine, this at the same time as was debenamine. Adrenaline- $\beta$ -C<sup>14</sup> was injected as for the previous experiment, intraperitoneally, and urine, free from food and feces, was collected over a period of 48 hrs. under the same experimental conditions as described above.

Control experiments were performed at the same time. Four rats were injected with dibenamine and adrenaline- $\beta$ -C<sup>14</sup> only. Urine was collected in the usual manner.

The separation of adrenaline and its urinary meta-

TABLE XVIII

Effect of cocaine on the excretion of administered adrenaline- $\beta$ -C<sup>14</sup> and its urinary metabolites in rats pretreated with 50 mg/kg of cocaine in subcutaneous injection for 12 days.

Compound	R a t #								Average	SD
	1	2	3	4	5	6	7	8		
Adrenaline free conj.	1.98 4.57	1.95 5.47	1.96 6.99	1.45 6.22	1.57 10.05	2.35 7.92	1.76 7.63	1.61 12.31	1.82 ± 0.29 7.64 ± 2.52	
DHMA + DHPG free conj.	0.16 2.34	0.26 1.50	0.32 0.92	0.28 0.54	0.20 1.36	0.16 1.50	0.26 1.84	0.10 1.54	0.22 ± 0.07 1.44 ± 0.54	
Metanephrine free conj.	4.44 38.80	4.40 45.99	4.06 42.34	4.28 45.57	3.03 35.55	4.70 39.86	3.97 37.20	3.08 37.41	4.00 ± 0.62 40.27 ± 3.90	
VMA	6.68	5.84	6.58	8.08	7.19	7.00	9.88	6.94	7.27 ± 1.23	
MHPG hydrolyzable unhydrolyzable	11.08 21.24	13.27 22.84	12.23 15.41	13.31 17.00	11.23 24.89	16.96 13.49	13.51 20.23	13.06 23.52	13.08 ± 1.98 19.95 ± 4.10	

TABLE XIX

Effect of one injection of cocaine on the metabolism of intraperitoneally injected radioactive adrenaline in rat.

Compound	R a t #						Average	SD
	1	2	3	4	5	6		
Adrenaline free conj.	2.45	2.06	4.84	4.07	4.92	2.79	3.51 ± 1.25	
	4.22	4.32	3.74	4.61	5.97	6.12	4.83 ± 0.98	
DHMA + DHPG free conj.	0.40	0.36	0.32	0.68	0.72	0.60	0.51 ± 0.18	
	4.40	2.38	4.28	5.02	5.02	6.48	4.60 ± 1.37	
Metanephrine free conj.	3.84	3.62	7.34	6.05	6.52	4.71	5.35 ± 1.49	
	41.56	46.99	36.37	37.07	35.39	34.59	38.66 ± 4.75	
VMA	6.04	3.94	7.27	5.28	7.12	5.60	5.87 ± 1.44	
MHPG hydrolyzable unhydrolyzable	7.36	6.29	12.64	8.19	10.39	8.41	8.88 ± 2.29	
	23.24	25.50	21.76	29.96	29.05	23.18	25.44 ± 3.38	

TABLE XX

Excretion of the intraperitoneally injected adrenaline- $\beta$ -C<sup>14</sup> and its metabolites in rats pretreated with :

A/ dibenamine (Control group);

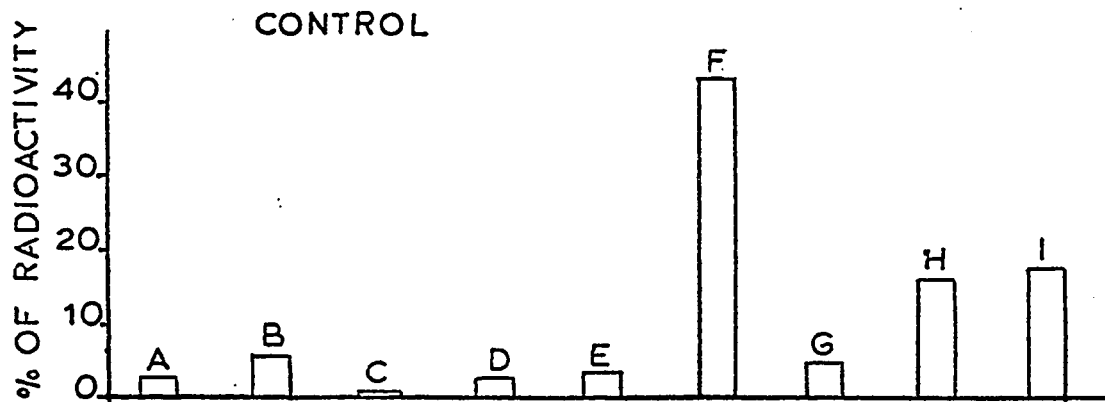
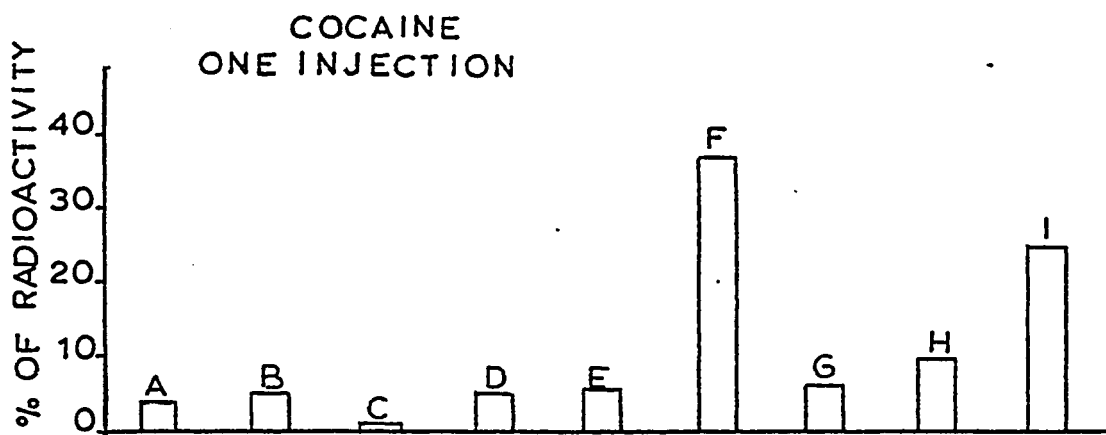
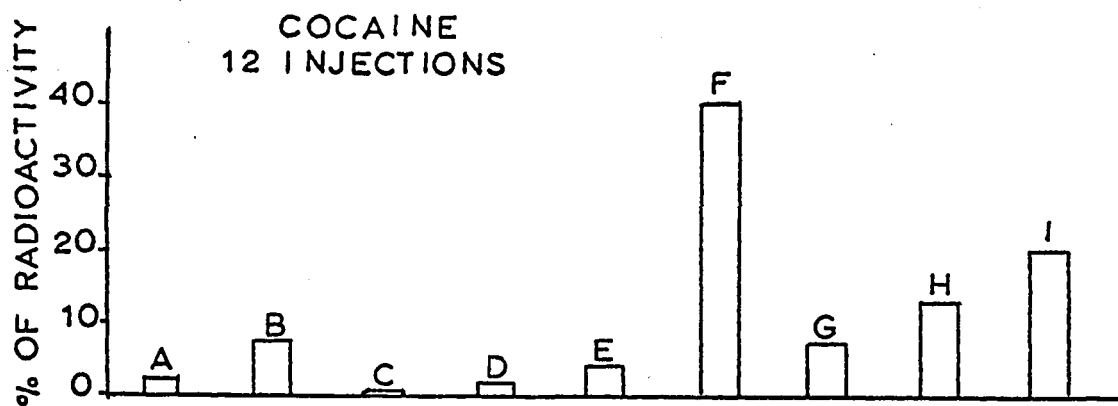
B/ dibenamine and cocaine - one injection only;

C/ cocaine - 12 injections and dibenamine.

The results are expressed as average values of the percentage of the radioactivity excreted in the urine.

Compound	A		B		C	
	Average	SD	Average	SD	Average	SD
Adrenaline						
free	2.58 $\pm$ 0.98		3.51 $\pm$ 1.25		1.82 $\pm$ 0.29	
conj.	5.69 $\pm$ 0.48		4.83 $\pm$ 0.98		7.64 $\pm$ 2.52	
DHMA + DHPG						
free	0.44 $\pm$ 0.15		0.51 $\pm$ 0.18		0.22 $\pm$ 0.07	
conj.	2.70 $\pm$ 1.49		4.60 $\pm$ 1.37		1.44 $\pm$ 0.54	
Metanephrine						
free	3.83 $\pm$ 0.82		5.35 $\pm$ 1.49		4.00 $\pm$ 0.62	
conj.	43.60 $\pm$ 1.76		38.66 $\pm$ 4.75		40.27 $\pm$ 3.90	
VMA	5.36 $\pm$ 2.04		5.87 $\pm$ 1.44		7.27 $\pm$ 1.23	
MHPG						
hydrolyzable	16.35 $\pm$ 1.40		8.88 $\pm$ 2.29		13.08 $\pm$ 1.98	
unhydrolyzable	17.36 $\pm$ 3.10		25.44 $\pm$ 3.38		19.95 $\pm$ 4.10	

Fig. 10



A-adrenaline (free); B-adrenaline (conj.); C-DHMA+DHPG (free); D-DHMA+DHPG (conj.); E-metanephrine (free); F-metanephrine (conj.); G-VMA; H-MHPG (hydrolyzed); I-MHPG (unhydrolyzed).

bolites was performed, using alumina absorption (WEIL-MALHERBE 1952) and ion-exchange column methods (KOPIN 1961) as described in the previous section.

The distribution of radioactivity among the various metabolic products of adrenaline, expressed as the percentage of the total found in urine, is shown in the following tables.

TABLE XVIII shows the urinary metabolites of adrenaline- $\beta$ -C<sup>14</sup> excreted during a period of 48 hrs. following its intraperitoneal injection in rats pretreated with cocaine for twelve days.

In TABLE XIX we have summarized the effects of a single dose of cocaine on the metabolism of C<sup>14</sup>-labeled adrenaline and the results obtained in the control group, without any further pre-treatment with cocaine, are presented in TABLE XX. Finally, Fig. 10 shows the distribution of urinary metabolites of adrenaline- $\beta$ -C<sup>14</sup> in the control group and in rats pretreated with cocaine.

#### DISCUSSION.

The results presented in TABLE 18- group of rats injected with a dose of 50 mg/kg of cocaine for a period of twelve days - do not indicate any significant differences in the metabolism of injected adrenaline- $\beta$ -C<sup>14</sup>, from those observed in the control group.

The mean value obtained for conjugated metanephrine

in this group, as well as in the control, pretreated with dibenamine before receiving an intraperitoneal injection of labeled adrenaline, varied only slightly. Also, marked stability in the free metanephrine level was observed.

In two cases only, the total metanephrine presented a slightly lower level than for the other animals in the same group.

However, the data obtained with cocaine-treated rats showed that there are quite wide variations in conjugated adrenaline from rat to rat. The mean value in this fraction is a little higher in the test than in the control group. This increase in the conjugated adrenaline fraction coincided with the slight decrease, in the total metanephrine in the two rats mentioned above. The 3,4-dihydroxymandelic acid and 3,4-dihydroxyphenyl glycol were low.

Under experimental conditions, apparently identical with dose described by LEDUC (LEDUC 1959) we have been unable to present any positive results to confirm his findings. Nor have we obtained valid evidence to support his suggestion that cocaine inhibits O-methylation in VIVO under the conditions used above.

On this score our results are rather in agreement with the recent opinion of AXELROD that "cocaine does not affect the metabolism of catecholamines in the intact animal" (AXELROD 1962; WYLIE 1960).

It is possible that the use of dibenamine modified the picture of the metabolic effects normally expected after the injection of cocaine. It is known that dibenamine provides a marked protection against the effects of adrenaline (NICKERSON 1947) and that it inhibits local vasoconstriction, permitting adrenaline to be more rapidly absorbed. On the other hand, dibenamine does not affect the destruction of adrenaline, and the adrenergic blockade produced by it is not overcome by cocaine (NICKERSON 1949).

TABLE 19 presents the effect of a single injection of 50 mg/kg of cocaine on the metabolism of exogenous adrenaline.

In this group the results varied from animal to animal. In three rats we observed an increase in both the free and in the total adrenaline level; at the same time a marked increase in conjugated fraction of catechols: DHMA and DHPG. However, a slight decrease in the conjugated and in the total metanephrine was observed.

These changes in the metanephrine level are insignificant, and we did not observe even the slightest changes in the remaining animals of the same group.

Nevertheless, the mean value for the total adrenaline was the same in the group receiving cocaine during a period of twelve days; so there was no significant variation from the control group results. The value for the total metanephrine

was also similar in both groups.

It should be noted, however, that the results with one injection of cocaine appear to differ a little from those obtained after twelve injections, especially in:

1. the slightly higher level of DHMA and DHPG (conjugated);
- and 2. the noticeable decrease of hydrolysed fraction of MHPG (about 30%).

The P value for this last compound was very small but significant:  $P < .001$ . We are not certain, however, whether the use of a new batch of glucuronidase for hydrolysis was responsible for this decrease.

In closing, it is possible that the number of rats used in our experiments in both groups - control and cocaine-treated - was not large enough to permit us to draw a definite conclusion. However, our results are such as to support the observation of AXELROD, mentioned above. And our observations indicate that in the cocaine-treated group only the conjugation is slightly affected.

SUMMARY

1. In the present work, the metabolism of adrenaline- $\beta$ -C<sup>14</sup> in a normal rat pretreated with dibenamine only, and in a rat injected with a series of various drugs is described.
2. An attempt to differentiate between the metabolism of adrenaline injected intramuscularly and intraperitoneally has been made. The main difference has been observed in the free and conjugated metanephrine fraction. Also, it seems that the intraperitoneal injection affects, to a certain extent, the conjugation.
3. The techniques for the partition of catecholamines and catecholacids by paper chromatography, using ion exchange papers and separation on columns have been described and discussed.
4. The separation of 3,4-dihydroxymandelic acid (DHMA) and 3,4-dihydroxyphenylglycol (DHPG) by paper chromatography was carried out successfully and has been described.
5. The method of determination of radioactivity in a liquid scintillation counter and in a low background counter has been described.
6. It has been shown that 4-methyl-tropolone acts as an

inhibitor of catechol-O-methyl transferase in VIVO, and that the degree of this inhibition depends on the dose used.

7. It has been shown that:

psychomimetic drugs, such as lysergic acid diethylamide, mescaline and adrenochrome have only a slight effect on the metabolism of the labeled adrenaline in the rat. However, in the case of adrenochrome-treated rats the value for free adrenaline was twice as high as in the control group; in the case of rats injected with 50 mg of mescaline, an almost insignificant decrease in the free metanephrine level and a corresponding increase in the level of conjugated 3,4-dihydroxymandelic acid and 3,4-dihydroxyphenyl glycol was observed; and, in the case of lysergic acid diethylamide-treated rats, a slight increase in the hydrolyzed fraction of 3-methoxy-4-hydroxyphenyl glycol and conjugated DHMA and DHPG was observed.

8. It has been confirmed in VIVO that 3,5-diiodo-4-hydroxy-<sup>Phenyl-</sup>benzoic and-pyruvic acids can act, depending on the dose used, as inhibitors of catechol-O-methyl transferase.

9. It has been demonstrated that in cocaine-treated animals the metabolism of adrenaline is similar to that of controls. Only the conjugation mechanism was slightly

affected. The metabolic pattern of adrenaline observed after twelve injections of cocaine differed very little from those observed after a single injection.

A P P E N D I XAbbreviated forms of some biochemical terms

COMT	- catechol-O-methyl transferase
DHMA	- 3,4-dihydroxymandelic acid
DHPG	- 3,4-dihydroxyphenyl glycol
DIHBA	- 3,5-diiodo-4-hydroxybenzoic acid
DIHPPA	- 3,5-diiodo-4-hydroxyphenylpyruvic acid
DOPA	- 3,4-dihydroxyphenylalanine
MAO	- monoamine oxidase
MHPG	- 3-methoxy-4-hydroxyphenyl glycol
MN	- metanephrine
LSD <sub>25</sub>	- lysergic acid diethylamide
VMA	- 3-methoxy-4-hydroxymandelic acid

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