THE FLUORIMETRIC MEASUREMENT OF ADRENALINE AND NORADRENALINE IN HUMAN PLASMA

- by -

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THESIS

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A. HISTORY

1) Discovery of Adrenaline and Noradrenaline

The earliest observations that the adrenal medulla produces a substance which is added to the blood stream and has powerful effects on muscular tissue in general, expecially the heart and vascular system, were made by Oliver and Schäfer in 1895, (1). They prepared extracts of the adrenal medulla and injected them intravenously into animals. The extracts had a marked effect on the arterial system, heart activity and the respiration. Extracts prepared from patients suffering from advanced Addison's disease had, on the other hand, no activity. In 1898, Abel (2) isolated the insoluble, but very potent, powder from the adrenal medulla to which he gave the name "epinephrine". Von Fürth, in 1900, (3) further purified the hormone by precipitation of the insoluble metallic salts of adrenaline and by subsequently splitting the latter to yield an even more potent amorphous powder which he called "suprarenin". A pure crystalline preparation of l-adrenaline was isolated in 1901 by Takamine (4), and in 1905, by Aldrich (5). Takamine gave it the name "adrenalin" and it was the first of the natural hormones to be isolated. Adrenaline also became the first synthesized hormone, (see Fig. 1.) as

racemic dl-3,4-dihydroxyphenylethanolmethylamine; achieved independently by Stolz in 1904 (6) and Dakin in 1905 (7). The definite establishment of the empirical formula of the hormone was actually the work of Friedmann in 1906 (8). Flächer, in 1908 (9) separated the two optical isomers.

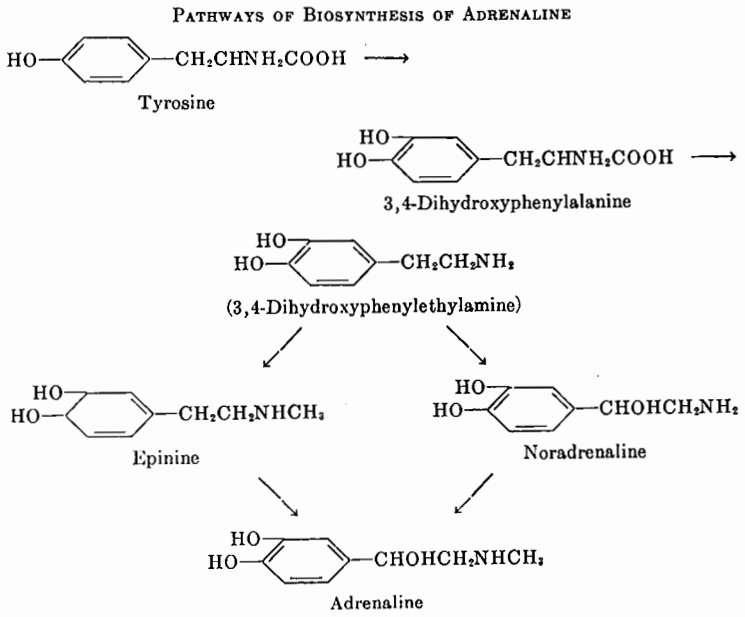


FIGURE 1: Scheme for the biological synthesis of adrenaline proposed by Blaschko (41).

Noradrenaline was also synthesized (see Fig. 1.) independently by Stolz and by Dakin. Barger and Dale in 1910, (10) examined a series of primary and secondary amines from the standpoint of their similarity in action to that of stimulating nerves of the sympathetic system. They described amines of this character "sympathomimetic" in action, rather than "adrenine-like", thus describing their activity in relation to innervation by the sympathetic system. They found that the optimum condition for sympathomimetic activity was in the structure amino-ethanol-catechol or noradrenaline. Biberfeld, in 1906 (11) had pointed out the pressor action of noradrenaline and suggested its use as a therapeutic agent, noting that it was less toxic than adrenaline. Noradrenaline, norepinephrine or arterenol is α-3,4-dihydroxyphenyl-β-amino-ethanol. Synthetic dl-noradrenaline was resolved into its optical isomers by Tullar in 1948 (12), who later isolated it from U.S.P. Standard "Epinephrine", in 1949 (13), prepared from bovine adrenal medullary extracts; at the same time, Goldenberg et al, (14) and Auerbach and Angell (15) also detected noradrenaline in the U.S.P. preparation.

In 1938, Holtz et al. (16) discovered an enzyme, l-DOPA decarboxylase, which converts 3,4-dihydroxyphenylalanine to hydroxytyramine (DOPAmine), a compound differing from noradrenaline only in an -OH group; this led to an increased

interest in the possibility of the biological occurrence of noradrenaline. Blaschko (17), in 1939, postulated that noradrenaline is formed during the biosynthesis of adrenaline from tyrosine by way of DOPA. Stehle and Ellsworth, in 1937, (18) suggested that noradrenaline might be the substance liberated on stimulation of the sympathetic hepatic nerves. In experiments with the ergotoxinized cat, they showed that noradrenaline usually caused an increase in the blood pressure similar to that produced by hepatic nerve stimulation while the pressor effect of adrenaline was reversed. Similarly, Melville, (19) demonstrated that certain benzodioxan derivatives which reverse the pressor action of adrenaline only slightly reduced the pressor effect of noradrenaline and that of splanchnic nerve stimulation. Greer et al. (20) described the effects of stimulation of the hepatic sympathetic nerves in the light of noradrenaline activity and concluded that they were strikingly similar and differed from the activity of adrenaline, in contrast.

It was not until von Euler, in 1946, demonstrated a substance having the properties of noradrenaline in extracts from sympathetic nerves and visceral organs that it could be concluded that noradrenaline is the mediator at the post-ganglionic terminations of the sympathetic nervous system.

Biological tests, colorimetric and fluorimetric reactions of purified spleen extracts (21), mammilian heart extracts (22), and extracts of various sympathetic nerves (23) established that the transmitter substance was not adrenaline but noradrenaline.

In 1947, Holtz, Credner and Kroneberg (24) reported that urine contains what appears to be a conjugated noradrenaline, "urosympathin", and that extracts of mammalian suprarenals seem to contain the same substance along with adrenaline.

2) Neurohumoural Functions of Adrenaline and Noradrenaline

Sympathomimetic amines generally elicit responses similar to those elicited by stimulation of adrenergic nerves. However, differences may occur in intensities or in qualities of response. The chromaffine material of the adrenal medulla associated with the production and storage of adrenaline and noradrenaline, although not essential to life, appears to have an important function notably in the adaptation to environmental stress. It is now known that the sympatho-adrenal system functions not only in times of stress, but that it is normally continuously active at a low level in the maintenance of homeostasis.

Many speculations concerning the nature of neurohumouralism arose in the early 1900's as a result of the findings of Oliver and Schäfer. In 1901, Langley (25) speculated on the relative activities as a consequence of the

administration of adrenal extracts and the stimulation of sympathetic nerves. He concluded that the action of the extracts was directly peripheral rather than on the sympathetic nerve endings because after degeneration of the sectioned postganglionic fibres this characteristic reaction still occurred. Following the isolation of adrenaline from the adrenal gland by Takamine, Elliot (26) characterized adrenaline by its ability to stimulate plain muscle and gland cells, in parallel with their sympathetic innervation. Denervated, this plain muscle was even more sensitive to the action of adrenaline. He found that stimulation took place at the myo-neural junction, since adrenaline was inactive on the nerve cell itself. The actions of adrenaline and that of sympathetic nerve stimulation, however, were not always consistent and this led Langley (27) to attribute the different responses to the proportion of two kinds of receptive substances (motor or inhibitory receptive substances or both) made by the cell to affect the impulse.

Loewi (28) in 1921, was able to demonstrate the presence of an "Acceleranzstoff" in the perfusion fluid from frog heart. On stimulation of the vagus nerve, the rate of a second, recipient frog heart was accelerated by this substance in the perfusion fluid. In the frog, where the vagus is a mixed nerve, its stimulation results in acceleration or inhibition depending on the frog and the season.

In the same year, Cannon and Uridil (29) disclosed that after the removal of the adrenal glands they were able to cause an acceleration of the denervated heart upon stimulation of nerves in the splanchnic area. Furthermore, this did not occur when the hepatic nerves were cut, giving evidence that this agent arose in the liver and was carried by way of the blood stream. Stimulation of the hepatic nerves after extirpation of all abdominal viscera, except the liver, caused a rise in the blood pressure outlasting the period of stimulation; this did not occur with closure of the hepatic artery and vein. Cannon and Bacq, (30) proposed that the neurohumoural substance, which has the properties of a hormone, be called "sympathin". They suggested this in view of the fact that, while adrenaline was known to arise from the adrenal gland, the immediate source of sympathin in their experiments lay elsewhere. Smooth muscle under sympathetic control was demonstrated as one of these sources.

Cannon and Rosenblueth (31) elaborated the theory of "sympathin" to include two kinds. In view of the different actions of adrenaline and sympathin especially after ergotoxin, where adrenaline causes a fall in blood pressure and sympathetic stimulation causes a rise in blood pressure, they assumed that an Excitatory sympathin and an Inhibitory sympathin

were produced by structures stimulated and inhibited by sympathetic impulses. These chemical mediators of sympathetic nerve impulses induced a typical response in the cell from which they arose and then after escaping into the blood stream induced effects elsewhere in organs innervated by the sympathetic system.

In 1934, Bacq,(31A) working in Cannon's laboratory suggested that the Inhibitory substance was adrenaline, while the Excitatory substance was noradrenaline; he later abandoned this idea, although other authors gave support to this theory.

The studies of von Euler (23) provided the first evidence that noradrenaline constitutes the sympathetic transmitter substance when he subjected extracts of adrenergic nerves to more thorough purifications and found chiefly noradrenaline (32). Extracts of various organs have been shown to contain mainly noradrenaline. Thus the relative adrenaline content is low in spleen and liver and highest in the heart and submaxillary gland, (sheep, excluding the adrenal gland) (33). This content is probably due to the nerve mediators present in nervous tissue and chromaffin cell groups (34). Euler and Purkhold, (35) confirmed the fact that sympathetic denervation diminished the amount of adrenaline-like material in organ extracts and demonstrated that it

is due mainly to a drop in noradrenaline. Goodall (36) found that on complete cervico-stellate ganglionectomy of the sheep the adrenaline and noradrenaline levels of the heart fell to very low levels, although the adrenaline content of the heart extracts shows a more irregular behaviour. Regeneration of the adrenergic nerves was found to raise the noradrenaline content to its normal range within five to six weeks.

Noradrenaline is released into the blood stream by the peripheral, and the central or reflex stimulation of adrenergic nerves (37). Adrenalectomy results in a decrease in the excretion of adrenaline, indicating that it is chiefly derived from the adrenal gland; noradrenaline excretion is not lowered but frequently increases after bilateral adrenalectomy indicating that it is chiefly derived from other sources than the adrenal gland, presumably from the adrenergic nerves (38). In view of the different roles of the two substances and the implications which have arisen in the use of the term "sympathin", von Euler suggests that the proper names for these two hormones be employed (33).

B. BIOLOGICAL SYNTHESIS OF ADRENALINE AND NORADRENALINE

When we examine the structures of adrenaline and noradrenaline from the aspect of their biological synthesis, we are at once struck by the similarity in structure with tyrosine and phenylalanine, two well known dietary constituents. It is now known that these amino acids are the primary precursors of adrenaline and noradrenaline. This is of interest from a pharmacological as well as a biochemical viewpoint, as these precursors must develop sympathomimetic properties in the course of transformation.

The conversion of phenylalanine to tyrosine was established in 1940 by Moss and Schoenheimer (39). The aromatic nucleus and side chain of adrenaline are derived from phenylalanine, as was demonstrated by Gurin and Delluva (40) in 1947; they administered labelled phenylalanine to rats and recovered radioactive adrenaline from the adrenal gland. The discovery of a specific l-DOPA decarboxylase in mammalian tissue by Holtz *et al.* (16) which converts 3,4-dihydroxyphenylalanine (DOPA) to 3,4-dihydroxyphenylethylamine (DOPAmine) led to speculation concerning the biosynthesis of the hormones. The enzyme was discovered in the kidney; it has also been found in the liver (41), in the mucous membrane of guinea-pig intestine (42), and in the adrenal medulla (43). Here we have a reaction in which

a pharmacologically inert amino acid is converted into a substance having sympathomimetic properties.

Blaschko, (17, 41) hypothesized on the biosynthesis by comparing the structures of tyrosine and adrenaline. He predicted a minimum of four steps in the conversion: 1) introduction of an additional phenolic hydroxyl in meta position to the side chain; 2) decarboxylation; 3) addition of a hydroxyl group to the β-carbon of the side chain; and 4) methylation of the nitrogen. This sequence, as can be observed in Figure 1, also involved the intermediary formation of noradrenaline. DOPAmine has been found in the urine of rabbits and humans following the administration of oral or subcutaneous DOPA, (44); and also in normal human urine, (45). DOPA has also been demonstrated in the heart and adrenal glands of thyroidectomized sheep (36).

Recently, several groups of workers have demonstrated the synthesis of adrenaline and noradrenaline from phenylalanine, tyrosine, DOPA, and DOPAmine. Radioactive noradrenaline has been isolated chromatographically after incubation of C14-labelled DOPA with bovine adrenal medullary homogenates in the presence of pyridoxal phosphate and ATP (46). Kirshner and Goodall (47) were able to demonstrate the formation of labelled adrenaline, noradrenaline and DOPAmine from labelled tyrosine by adrenal slices. Udenfriend et al. (48) showed that labelled phenylalanine, tyrosine, DOPA, and DOPAmine

were all precursors of adrenal adrenaline and noradrenaline in the rat, and that DOPAmine is a more immediate precursor than DOPA, hence justifying Blaschko's scheme. (Fig. 1).

Methylation of noradrenaline was first demonstrated by Bülbring in 1949, (49) who found that minced adrenal glands from the dog or cat methylated noradrenaline in the presence of ATP. In the same year, Bülbring and Burn (50) were able to perfuse the adrenal gland of a dog with heparinized blood and found an increase in the amount of adrenaline at the expense of endogenous and added noradrenaline. Keller, Boissonas and du Vigneaud, (51) showed the incorporation of radiocarbon into adrenaline from the adrenal glands of rats fed methyl-labelled methionine in a choline-free diet.

Inactivation of the catechol amines and their subsequent excretion has been the subject of much enquiry. Five different systems may play roles;

1) oxidative deamination by amine oxidase
2) oxidation by a phenolase type of system
3) conjugation to ethereal sulphates and glucuronides
4) excretion in free form
5) adrenalone formation

As there is little adrenaline and noradrenaline present at any one time in the body, as in fact their turnover is relatively low (48), there appears to be little need for a large supply of enzymes for formation and inactivation. The importance of amine oxidase as a physiological mechanism for

the inactivation of adrenaline and noradrenaline has been accepted since the work of Schayer and his colleagues (52); they showed that the methyl group and the β-carbon of adrenaline suffer different fates. The distribution of amine oxidase follows that of the two catechol amines in many tissues (53). To what extent the action of phenolases or catechol oxidases are of importance in the inactivation of the amines is unknown. Experiments with Corbasil (dihydroxynorephedrine),which is not oxidized by amine oxidase, have indicated that some other system than that of amine oxidase plays a significant role. Von Euler and Zetterström recently (54) found that injected Corbasil was inactivated to about the same extent as the two natural hormones. The excretion of the catechol amines in their free form or conjugated as ethereal sulphates and glucuronides does not appear to be as important as enzymatic oxidation in their metabolism. The ratio of free adrenaline and noradrenaline to their conjugated derivatives as found in the urine varies from 1:1 to 1:3. (55). The preparation of an apo-enzyme from blood which destroys adrenaline and produces what appears to be adrenalone has recently been described (56).

C. SOME PHARMACOLOGICAL ASPECTS OF ADRENALINE AND NORADRENALINE

The autonomic nervous system consists of nerves, ganglia, and plexuses which provide innervation to the heart, blood vessels, glands, viscera and smooth muscle. It is widely distributed throughout the body and controls the automatic or vegetative functions. Discharge of the sympathetic system may lead to widespread effects. Its terminals make contact with a large number of postganglionic neurones. The concept of "neurohumouralism" maintains that nerve impulses elicit responses in receptor cells through liberation of a specific chemical substance (57). Thus when postganglionic sympathetic nerves are stimulated there is a release of a chemical substance which has sympathomimetic properties at the neuro-effector junction. Noradrenaline appears to be the chief impulse mediator arising from the sympathetic nerves and the organs innervated by them. Adrenaline appears to arise chiefly from the adrenal medulla, and to re-enforce the adrenergic mediator which diffuses from structures receiving sympathetic stimulation to circulate in the blood stream and affect distant organs. Adrenaline apparently acts directly on effector cells and depending on the chemical nature of the receptor substances in the effector cell, the response may be of an inhibitory or of an augmentative or excitatory nature. The adrenaline and noradrenaline producing systems can be activated independently of each other, indicating a duality of function.

Holtz, Credner and Kroneberg (24) demonstrated some years ago that extracts of the adrenal gland contained a substance, along with adrenaline, having sympathomimetic properties similar to those of noradrenaline. Subsequently, the adrenal medulla of dog, cat and man were shown to contain considerable amounts of noradrenaline along with adrenaline. (58). The distribution of adrenaline and noradrenaline in two histochemically distinct types of adrenomedullary chromaffin cells has been described by Eränkö (59). Recently, Blaschko et al. (60) have been able, by the method of differential centrifugation, to demonstrate that the pressor amines are present in a granular fraction with sedimentation properties similar to those of mitochondria. Both adrenaline and noradrenaline are released from the adrenal medulla on stimulation of the splanchnic nerve, their proportions varying considerably.

The distribution of noradrenaline and adrenaline in other tissues and organs parallels their adrenergic nerve supply and the occurrence of chromaffin cell groups. The latter have been found in the heart (36), salivary glands (35), and all along the sympathetic nerve tracts (61).

The measurement of adrenaline and noradrenaline in human adrenal glands, blood, and urine has recently been accomplished by several experimenters. The following table may serve to illustrate the levels of the two amines found:

Source		Adrenaline	Noradrenaline
Adrenal glands: (a)	age 45 yrs.	922-988 µg[e]	172-196 µg[e]
	60 yrs.	1554-1680 µg[e]	241-298 µg[e]
	75 yrs.	2154-2354 µg[e]	149-197 µg[e]
Blood: (b)	male - plasma	1.18±0.044 µg./litre	5.29±0.233 µg./litre
	- red cells	2.81±0.065 µg./litre	2.02±0.14 µg./litre
	female - plasma	1.46±0.082 µg./litre	5.16±0.16 µg./litre
	- red cells	2.27±0.14 µg./litre	3.77±0.12 µg./litre
Plasma range (c)		0.5 - 1.0 µg./litre	2.5 - 3.5 µg./litre
Urine (d)		4.3 µg. free/24 hours (range 1.6 - 8.0)	27 µg. free/24 hours (range 15 - 50)

Total catechol amines (free and conjugated) yield about double these values

a - ref. 62
b - ref. 63
c - ref. 64
d - ref. 65

[e] - per pair of adrenals

Noradrenaline is generally considered the specific constituent of the adrenergic nerves and has been shown to be present in the entire neurone (66), but is more concentrated in the nerve endings. In general, it is considered that in situations of stress there results a sudden release of adrenaline from the adrenal medulla in response to increased metabolic needs, while adrenergic nerve discharge elicits a vasoconstrictor effect in the periphery thus increasing circulation to organs requiring it.

The most outstanding feature of noradrenaline is its vasoconstrictor effect on the blood vessels which causes an elevation of both systolic and diastolic arterial pressures. The cardiac output remains unaffected or decreases slightly and the total peripheral resistance is augmented (67). A slowing of the heart occurs due to a compensatory vagal reflex but the higher pulse pressure increases the stroke volume (67). Adrenaline is also a very potent vasopressor agent but differs from noradrenaline in that it produces vasodilation with low concentrations. It effects a greater action than noradrenaline on the venous pressure although noradrenaline elicits the greater rise in blood pressure. Adrenaline causes constriction of the arterioles and capillaries of the skin and mucosa, while the blood vessels of skeletal muscle are dilated; splanchnic vascular resistance may be decreased and the hepatic blood flow augmented (67). Noradrenaline has little effect on the skin vessels.

Adrenaline produces acceleration of the heart rate and enhanced cardiac output; at the same time the rhythm is often altered, the work of the heart decreased and the oxygen consumption of the myocardium is increased. The actions of noradrenaline, such as bradycardia are due to reflex action in response to the raised blood pressure; the pulse pressure usually increases while the cardiac output remains unchanged. Adrenaline and noradrenaline both increase

the flow in coronary vessels but it has not been determined whether the dilator action is due to a direct action on the coronary vessels or secondary to the increased heart activity and resultant metabolites.

Important in biological assay methods are the effects of adrenaline on the smooth musculature; in the gastro-intestinal tract, peristalsis and tone are diminished; the splenic capsule is contracted; certain urinary bladder muscles are relaxed and others are contracted; the ureter is stimulated; the uterine musculature varies according to the species, dose, the phase of the sexual cycle and the state of gestation.

Oxygen consumption is increased considerably by adrenaline but not by noradrenaline; both increase the respiratory volume, again the former being the more active compound.

Adrenaline has a number of important metabolic effects (67); it can mobilize glycogen from the liver and skeletal muscle to raise the blood sugar and lactate; this glycogenolysis is followed by rapid glycogenesis after the adrenaline effect has passed. A rise in serum arterial potassium, a fall in venous potassium, a fall in serum cholesterol and serum inorganic phosphate are seen with adrenaline.

D. ADRENALINE AND MENTAL ACTIVITY

A brief review of the correlation of adrenaline levels in human plasma with mental activity, as found by Weil-Malherbe and his associates using a fluorimetric method of measurement is of interest to our study. The intravenous injection of insulin brings about an immediate lowering of the plasma adrenaline levels (68, 69, 70, 71) which precedes the onset of hypoglycaemia, an apparent contradiction with the majority of observations showing that insulin stimulates sympathetic activities. Arterio-venous differences reveal that the reduction of the blood level of adrenaline is associated with a transitory increase of the arterio-venous difference which may be attributed to the increased rate of utilization of adrenaline by peripheral tissue. The restoration of consciousness also coincides with the rise of the blood adrenaline concentration. This appears to be a specific effect due to the level of glucose in the blood, since termination of coma with glucose brings about a rise above the resting level of adrenaline and preceding the rise in the blood-sugar level; and as well, this adrenaline response is seen when glucose, as in the glucose tolerance test, is given by mouth.

The therapeutic effect of convulsive treatment is generally considered due, in large part, to stimulation of the sympathetic nervous system either directly or indirectly. Weil-Malherbe and Bone (68, 72, 71) have demonstrated that a rise in

plasma adrenaline of 75% above the base level occurs immediately after the fit; an increase in noradrenaline occurs as well but may be attributed to secondary effects resulting from muscular contractions and cardiovascular changes, as the increase in noradrenaline is repressed by the use of barbiturates and/or muscle paralysants which modify the convulsion. The adrenaline response persists, however, and may be considered as due to stimulation of autonomic centres in the brain. The rise in the plasma adrenaline level is very brief but is also seen with subconvulsive doses of electrical current. The injection of barbiturates on the other hand results in a fall of the plasma adrenaline level to about 60% within one minute whereas the noradrenaline level is comparatively unaffected (72). The injection of a convulsant drug, such as "leptazol" may result in a mean increase of the plasma adrenaline to over 130% of the normal level accompanied by an increase in the noradrenaline level similar to that evoked by electroshock (also suppressed by muscle paralysants) (72). Photic stimulation in a subject susceptible to epileptic discharge as indicated by the EEG pattern, resulted in a significant rise in the adrenaline level, and a drop in the noradrenaline level (71). This effect was not seen where the EEG pattern showed no response to the stimulation; all such responses were suppressed by barbiturates.

In another series of experiments Renton and Weil-Malherbe (73) demonstrated that a lowering of the plasma

adrenaline level occurs during sleep, that it increases by a mean of 40% on waking and is 27% higher just before breakfast than during sleep. The noradrenaline level stays practically the same during sleep as in the following morning of the test, although it showed an insignificant increase of 9.5% on waking. Von Euler et al. (65) have studied the diurnal variations in urine samples and found that excretion of noradrenaline is reduced to less than half during the night, the adrenaline also being reduced.

A study was made by Weil-Malherbe (71) of a group of patients suffering from congenital mental disorders such as phenylpyruvica oligophrenia, mental deficiency and a group suffering from senile dementia. These were compared to a varied group of patients "whose intellectual faculties were not grossly impaired", but which included cases of psychosis, psychopathy, neurosis and organic diseases. Although the difference in the plasma adrenaline levels of the phenylpyruvica oligophrenic cases is statistically significantly lower than the mean of the other mentally deficient groups (a correlation is suggested with the severity of the mental defect) the difference between the two groups, oligophrenic group and the non-oligophrenic group, is highly significant. The noradrenaline levels also show a significant difference although not as striking. These results indicate a correlation between mental deficiencies and a reduced level of circulating adrenaline, possibly due to the reduced mentation processes in these patients.

E. MECHOLYL TESTING

A new pathway into the research of autonomic disorders is revealed in the Mecholyl test of Funkenstein et al. (74). The basis of this test lies in determining the blood pressure response to an injected dose of Mecholyl. Mecholyl, or methacholine, is an analogue of acetylcholine, the naturally occurring autonomic transmitter.

Acetyl-β-methylcholine Methacholine $(CH_3)_3 = \overset{+}{N}\cdot CH_2\cdot CH(CH_3)\cdot O\cdot COCH_3$ OH^-

Acetylcholine $(CH_3)_3 = \overset{+}{N}\cdot CH_2\cdot CH_2\cdot O\cdot COCH_3$ OH^-

Mecholyl exerts its characteristic actions in the body in a manner similar to that of acetylcholine. It is less readily hydrolyzed by cholinesterases and hence has a longer duration of action. The vascular actions, with which the Mecholyl test is most concerned, are such that the peripheral vessels dilate and the blood pressure falls. In man, the effects of constant intravenous infusion of methacholine are essentially identical with those produced by acetylcholine in 1/200th the dose (75). 20 mg. of Mecholyl chloride when injected subcutaneously produces an immediate fall in blood pressure, with a compensatory rise in pulse rate, flushing and a sensation of warmth within two minutes. After three minutes salivation, lacrimation, sweating, and palpitation occur. The cardiovascular responses are transient and a bradycardia develops when the blood pressure returns to normal. Complete recovery is attained within twenty to thirty minutes of the injection. Cumulative action does not occur.

Funkenstein et al. administered adrenaline and Mecholyl on separate occasions to a group of individuals, which included psychotic patient and normal controls. They observed that the degree and duration of the hypertensive and hypotensive action of these drugs, as measured by recording the systolic blood pressure, permitted classification of their subjects into a number of groups, each group displaying a characteristic response to these drugs. The adrenaline responses were either marked or moderate in all divisions, the rise in blood pressure generally falling to normal within four to eight minutes. On the other hand, the responses to Mecholyl were widely divergent. A mild reaction categorized that group whose recovery from the fall in blood pressure was over-compensated in four minutes by a rise in blood pressure. 80% of the control subjects fell into a group in which the drop in blood pressure was followed by a return to normal within ten minutes. In other groups the return to normal of the blood pressure after Mecholyl was greater or more prolonged.

Associated with this autonomic test, Funkenstein et al. have found that when the clinical picture of the patient is altered either by improvement or impairment, these changes are reflected in the blood pressure pattern of the Mecholyl test. These experimenters also establish that a relation exists between this test and the predicted effectiveness of electroshock therapy. They base this relation on the fact that the group which shows a prolonged hypotension after Mecholyl injection is lacking in sufficient sympathetic compensatory action which normally would cause a rise in the blood pressure to normal within ten minutes. This group,

having hyporeactive sympathetic centres, benefit by shock treatment and show normal responses to Mecholyl after the treatment. On the other hand, that group which shows an elevation of blood pressure which overcompensates for the depression due to Mecholyl do not show an improved clinical picture after electroshock therapy, but show instead, an aggravated sympathetic hyperreactivity. Electroshock treatment of the subjects of this group is therefore contraindicated.

Gellhorn and his associates (76) have examined this test from the point of view of its reflection of reactivity of the hypothalamic-cortical system, whose significance in emotional disturbances is generally recognized in functional psychoses (77). In experiments with the anesthetized cat, these authors demonstrated that Mecholyl or any agent lowering the blood pressure in sufficient magnitude evokes a sympathetico-adrenal discharge which compensates for the hypotension induced. They measured the blood pressure and contractions of the normal and acutely denervated nictitating membranes during the course of experimental treatment. Thus the blood pressure response is more prolonged with increasing dosage of Mecholyl, the innervated membrane is contracted through sympathetid stimulation with lower concentrations, and with higher concentrations, the denervated nictitating membrane is contracted as well, due to adreno-medullary discharge. Electrical stimulation of the autonomic centres, the medullary sympathetic centre or the hypothalamus, induced the same effects as that induced reflexly by Mecholyl although to a lesser degree. Autonomic blocking agents, such as tetraethylammonium chloride, were shown to abolish the reflex

sympathetic discharge after Mecholyl. Barbiturate anesthesia resulted in a prolongation of the hypotensive action of Mecholyl and a reduction in the contractions of the nictitating membranes; at the same time the hypothalamus showed a decrease reactivity to electrical stimulation. The prolongation of the hypotensive action of Mecholyl in such cases must result from the reduced reactivity of the autonomic centres.

Hypothalamic lesions were shown to prolong and aggravate the hypotensive response to Mecholyl, indicating loss of reactivity in reflex centres of sympathetic stimulation. In such animals, having hypothalamic lesions, the response to electrical stimulation of the posterior hypothalamus, and the responses to Mecholyl, as reflected in the resultant sympathetic reactivity are greatly diminished. This indicates that activation of the hypothalamus controls the degree and duration of the hypotensive action of Mecholyl and the reflex discharges of the sympathetico-adrenal system. Electrical or chemical stimulation of the hypothalamus evokes an increased sympathetic discharge augmenting the sympathetico-adrenal response to Mecholyl and results in a quicker return to normal of the blood pressure and sometimes concurrently an overcompensatory hypertensive phase.

The central parasympathetic system does not play a significant role in the blood pressure response to Mecholyl; thus vagotomy does not alter the hypotensive action of Mecholyl.

It thus seems that the Mecholyl test can be used as a reflection of hypothalamic activity whose disturbances are related in function to the sympathetic nervous system.

F. METHODS OF ASSAY

1. Biological Assay of Adrenaline and Noradrenaline

Until recently biological assay has been the sole method of measurement of the two catecholamines in biological tissues and fluids, where their concentrations are generally minute. Chemical methods have existed for some time but they were not sufficiently sensitive for measurement of the catecholamines except in extracts of adrenal glands. To estimate biologically the amounts of adrenaline and noradrenaline present in a mixture, it is necessary to measure the response as gauged by a change in contraction of various kinds of musculature which respond differently to the two amines and to compare this to a standard preparation of one of the amines carried through the same assay. The cat's blood pressure may be used for one determination, the activity ratios being usually in the range of 1:1, but may be up to 5:1, for noradrenaline:adrenaline (78). Ergotoxine or some other sympatholytic substance may be used to obtain some information regarding the relative concentrations of the two substances in question; in this case the blood pressure effect of adrenaline would be expected to be reversed while the pressor action of noradrenaline to be only reduced or deleted. A second reference standard could be the action of the mixture on the hen's rectal caecum where adrenaline is up to sixty times more active than noradrenaline in relaxing the musculature (78).

This procedure assumes that there are no interfering substances present; conditions must be set up to remove potential interfering substances either by purification of the extract or by the use of

blocking agents. Fairly large doses are required to elicit responses in contrast to the chemical methods. The reliability of the biological assay is not without question, error may be incurred due to variability of response by a single tissue to a single drug.

2. Chemical Reactions of Adrenaline and Noradrenaline

Preliminary to the estimation of the two catecholamines in biological fluids and extracts it is necessary to remove interfering substances such as catechol compounds with little biological activity. Extracts of tissues may be obtained by treatment with trichloracetic acid or acid-ethanol (78), but these methods lead to considerable losses with blood. Further purifications of extracts is generally necessary. As this thesis is concerned with the measurement of the catecholamines in blood, this review will be restricted to methods used for their extraction from blood.

Dialysis has been employed (79) in an attempt to separate adrenaline from the blood proteins; adsorption on Permutit (alumino-silicates) or silicic acid (80) as well as removal of interfering substances on fuller's earth (78) have been attempted, however, none of these methods have proved satisfactory, as they incur losses. Shaw found that adrenaline is quantitatively adsorbed on aluminum hydroxide at pH 8-8.5 (81) whereas no adsorption takes place at pH 4. Adsorption may also be carried out by the formation of aluminum hydroxide directly in the extract (78). The procedure of adsorption on aluminum oxide has been used by Lund (82), Crawford and Outschoorn (83), and Weil-Malherbe and Bone (84) for catechol measurement in blood plasma and serum. The catechols are eluted with acid; Lund (82) employed acetic acid and Pekkarinen employed oxalic acid (85),

neither of which dissolves the alumina appreciably. At pH 2-3, elution proceeds rapidly. Other dihydroxyphenyl compounds such as DOPAMine, DOPA, and dihydroxyphenylacetaldehyde (86), and ascorbic acid (87) are adsorbed.

When solutions of adrenaline or noradrenaline are exposed to the air or oxygen for any length of time they are liable to become oxidized. Red oxidation products may also be formed as a result of oxidation by halogens and heavy metal ions. At alkaline reaction particularly, fluorescent products may be formed.

Traces of heavy metals accelerate the autoxidation of adrenaline by complexing with it; several metals have been studied by Chaix and Pallaget (89) who found Cu^{++} the most active catalyst of the metals studied for the oxidation of adrenaline and noradrenaline: the reaction is stabilized after the consumption of 3 molecules of oxygen per molecule of adrenaline and 2 molecules oxygen per molecule of noradrenaline. Ascorbic acid has been reported to have a destructive effect on solutions of the two amines at acid pH (90); this can only be partially attributed to the formation of dehydroascorbic acid by the catalysing effect of cupric ions in the solution. Oxalic acid has also been used to stabilize solutions; it is effective probably again by complexing copper and other ions in the solutions.

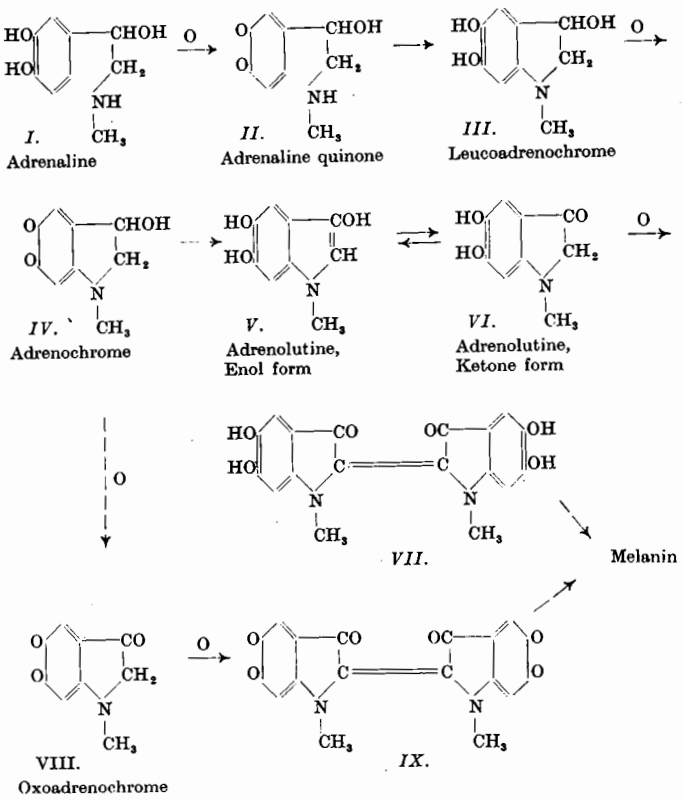


FIGURE 2: A proposed scheme for the oxidation of adrenaline to melanin (From Lund, ref. 88).

The red compound formed by the enzymatic oxidation of adrenaline with the uptake of two atoms of oxygen per molecule, was identified as adrenochrome (see Fig. 2.) by Green and Richter (91), and Richter and Blaschko (92) in 1937. They also identified the red colour which develops spontaneously in a neutral or weakly alkaline solution of adrenaline to be due to the formation of adrenochrome. Beaudet (93) described the formation of noradrenochrome in 1951. The stoichiometric oxidation of adrenaline to adrenochrome by chemical and enzymatic means has been studied by Koelle and Friedenwald (94) and Friedenwald et al. (95); they have shown that the oxidation can yield a variety of compounds at different stages of oxidation, some of which constitute reversible systems under properly controlled conditions. A red compound, called adrenoerythrine and thought to be identical with adrenaline quinone (see II, Fig. 2), has been reported by Ruiz-Gijon (96) to have retained most of the biological activity of the adrenaline from which it was formed. A noradrenaline-quinone has been described (97) on oxidation of noradrenaline at pH 0-1; the quinone is an orange-yellow colour which turns to red on addition of an alkaline buffer.

Loew was the first to observe the yellow-green fluorescent product which results from the alkaline oxidation of adrenaline, while Paget thought it was a new specific reaction for adrenaline (98). Ehrlén (99) postulated that the fluorescent product is 1-methyl-3,5,6-trihydroxyindole (see VI, Fig.2.). Lund (88, 100) was able to isolate adrenolutine by rearrangement of the adrenochrome molecule in alkaline solution in the absence of oxygen and to crystallize the lutine. He showed that a recently prepared adreno-chrome solution on irradiation with ultraviolet light becomes fluorescent.

Adrenochrome is resistant to further oxidation in neutral or weakly acidic solution whereas adrenolutine is very easily oxidized irrespective of acidity. Noradrenochrome is less readily transformed into noradrenolutine; its formation has been studied by Bu'Lock and Harley-Mason (97). Addition of alkali to a noradrenochrome solution gives a yellow-brown colouration which is not fluorescent, probably due to the removal of a proton from the nitrogen atom preventing the usual isomerization to noradrenolutine. The fluorescent compound can be stabilized by the addition of ascorbic acid to the alkali used for isomerization of the adrenochrome or noradrenochrome (101).

The further oxidation of adrenolutine appears to follow a course analogous to the formation of indigo. The di-indogenide presumably polymerizes to form melanin (88).

3. Colourimetric Methods

The colourimetric methods are based on the formation of coloured oxidation products, either of the chromes of adrenaline and noradrenaline or reduction products. Several methods are available. They differ in sensitivity, specificity and ease of use.

The most sensitive method is that of Whitehorn (80), modified by Shaw (81), the basis of which lies in the reduction of arsenomolybdic acid by the catechol nucleus with the aliphatic side chain. Adrenaline results in the production of the most intense colour, which, when it is pretreated with alkali, increases in intensity five-fold whereas the other catecholamines studied showed no change. This increase in colour after treatment with alkali gives an idea of the relative amounts of adrenaline present in

a mixture of catecholamines. Quantities of adrenaline as low as 40 mμg. with a standard error of 15 mμg. can be detected; recovery of quantities added to blood has been good although it is not sensitive enough to detect endogenous adrenaline in plasma or serum. It has been proved useful by Shaw and Raab (87) in detecting adrenaline in various tissues. Bloor and Bullen (102) were able to detect levels as low as 20 mμg. with an accuracy of 5% using this method. However, it has never been developed to differentiate adrenaline and noradrenaline quantitatively. The latter authors have labelled it a temperamental method as it is laborious and must be run with rigid control.

Auerback and Angell (15) based their method on the purplish-red colour formed by the condensation of noradrenaline with sodium-β-naptha-quinone-4-sulphonate. Since only primary amines react in this way the method does not detect adrenaline. However, noradrenaline may be detected in the presence of adrenaline. It has been used for concentrations of noradrenaline greater than 50 mμg.

Coloured chromes can be formed by a variety of oxidants such as iodine, ferricyanide, ferrichloride, marcurochloride, potassium permanganate, potassium persulphate, potassium bichromate, silver oxide and others. Adrenaline and noradrenaline may be determined differentially in a mixture by the use of iodine as oxidant (103); this yields adrenochrome and noradrenochrome and their corresponding iodochromes, red coloured compounds which may appear bluish at different pH's due to the different proportions of chromes and iodochromes formed at the different pH's (99). Differentiation of the two amines is based upon the difference in oxidation velocities; at pH4 all the adrenaline and only about 10% of the noradrenaline

has been converted to the chrome compound in one and a half minutes, whereas at pH 6, both are oxidized in three minutes. It is necessary to read the colours photometrically at a wavelength where the disproportionate quantities of chromes and iodochromes acquired through oxidation at different pH's give the same reading for a given quantity of adrenaline or noradrenaline. This wavelength has been found to be 529 mμ. Measurements are satisfactory for amounts in the range of 20-200 μg. The standard deviation is about 5-10% where one component is present in amounts of only 10-20% of the total catechol amines present, this increases with decreasing concentration of this lesser component. Oxidation by permanganate can be carried out on the same basis (104) thus avoiding the difference in colour tints of the chromes and iodochromes. These methods are useful in determination of adrenaline and noradrenaline in extracts of adrenal glands where their concentration is comparatively high.

4. Fluorimetric Methods

Gaddum and Schild in 1934 (105) reinvestigated the fluorescence of adrenaline in alkaline solution and confirmed its great sensitiveness; a concentration as low as 10^{-8} could be detected. The fluorescence, which depended on the presence of oxygen, very quickly reached a maximum and then faded more slowly. These authors found that other related substances gave a fluorescence, although less pronounced than that of adrenaline. Noradrenaline gave only about 2% of the fluorescence of adrenaline. They also found that a blue fluorescence presumably arising from blood proteins concealed the green fluorescence of adrenaline when they tried to apply the

method to blood plasma or serum. When they precipitated proteins to remove them, they found that adrenaline disappeared simultaneously, and thought that this loss was probably due to adsorption on the precipitated protein. Loewi (106) however, used this method to demonstrate the presence of adrenaline in extracts of frog's heart. Other authors also used it to demonstrate the presence of adrenaline in various extracts from tissues.

Several groups of authors, whose work has been reviewed by Jørgensen (79, 106, a-d), attempted to improve upon this method and tried to separate adrenaline from the blood proteins in various ways. Hueber dialysed blood against distilled water and found that a substance giving a green fluorescence with alkali passed over into the dialysate. The results are uncertain, however, as the fluorescence soon fades. Kalaja and Savolainen modified this method by diluting the blood with a slightly acid solution containing glycine, and then dialysed it against this acid solution with a view to stabilizing the adrenaline in solution. They measured the intensity of fluorescence against a weak eosin solution. Lehmann and Michaelis, using minute amounts of blood, measured its fluorescence after the addition of alkali and elimination of blue by-fluorescences by the use of appropriate filters; they then destroyed the fluorescence and added a known amount of sympatol whose fluorescence is equivalent to that of the adrenaline. They also demonstrated that the formation of the fluorescent substance reaches a maximum very quickly, a matter of seconds, that it is dependent on the presence of oxygen and can be inhibited by traces of heavy metals, especially iron, but also copper. Jørgensen (79) in a study of the fluorescent properties

of adrenaline and the application of the fluorescence method to blood, used a small double dialysing vessel, similar to that used by Loewi (106). On one side of the membrane was placed diluted blood and on the other side, a slightly acid recipient fluid containing aluminum hydroxide suspension (for removal of interfering substances, since at pH 4 it will not adsorb adrenaline); the recipient fluid on treatment with alkali was compared to a standard solution of eosin with regard to fluorescence. All of these methods are of interest purely from the point of view of studying the properties of the fluorescent substance, which is extremely unstable and the specificity of which is questionable.

Lund (82, 101) was the first to attempt specifically to remove the catecholamines from blood through their specific adsorption on aluminum oxide and then measure their fluorescent products in a stablized form. He showed again the gradual development of yellow-green fluorescence when strong alkali was added to an adrenaline solution, oxidation proceeding by means of the oxygen dissolved in the solution; the fading proceeded through further oxidation. In this way two simultaneous processes were occuring, the production and breakdown of adrenolutine (see Fig. 2), the fluorescent product; the result of which was that only one quarter of the adrenaline was present as adrenolutine at maximum fluorescence intensity. Adrenochrome was shown to be converted to adrenolutine in an oxygen-free atmosphere with a strong base; if oxygen was added

the resulting

fluorescence was reduced, thus showing that the fluorescent substance has the same degree of oxidation as adrenochrome. Lund then oxidized adrenaline in acid solution to adrenochrome, and by addition of ascorbic acid with alkali allowed the adrenochrome to rearrange itself without oxidative decomposition taking place. In the initial step of oxidizing adrenaline to adrenochrome, Lund found that iodine, potassium persulphate, potassium ferricyanide, etc. interfere with the fluorescence reaction by possessing a fair amount of fluorescence themselves. He therefore tried several insoluble oxidizing agents such as manganese dioxide, silver oxide, and lead oxide and found the first the most suitable. In use, the insoluble oxidizing agent is filtered off and the filtrate containing the chromes is suitable for alkalization to the lutines. Lund employed his technique with plasma, serum and haemolysed blood. A column of aluminum oxide is used to adsorb blood catechols at pH 8.4; the catechols are eluted with acetic acid and washed through the column with water; the eluate is divided into three parts for estimation: i) the first aliquot is treated with manganese dioxide, filtered, and the filtrate made alkaline with NaOH; the fluorescence is allowed to fade; ii) The second aliquot is adjusted to pH 3.0 before adding manganese dioxide (at this pH only about 5% of the noradrenaline is oxidized to noradenochrome), after oxidation, it is filtered

and a mixture of NaOH-ascorbic acid is added for rearrangement to adrenolutine; iii) The third aliquot is adjusted to pH 6.5 where both adrenaline and noradrenaline are oxidized. The filtrate is again treated with the NaOH-ascorbic acid mixture and the fluorescence read against a standard quinine sulphate solution. Lund used primary filters with maximum transmission at 365 mμ. or 400 mμ. and found that the sensitivity to adrenolutine fluorescence increased 6-7 times and that of noradrenolutine increased 3-4 times with the latter filter. At the same time the value of the blank also increased with this filter. He used a secondary filter which passed all emitted light above 500 mμ. As the fluorescent derivative from noradrenaline amounts to no more than 64% of that of the adrenaline derivative at pH 6.5, the fluorescence due to noradrenaline at pH 3 is negligible and is omitted in the calculations. A factor of 1.25 is employed to account for a 20% loss in washing the column of alumina with sodium acetate buffer. The method permits measurement of adrenaline and noradrenaline in plasma down to 1 μg. % and 1-2 μg. % respectively with an accuracy of ± 5-10% and ± 10% respectively. Lund evaluated his method by recovering added catecholamines from plasma or serum. He was unable to demonstrate measurable amounts of either adrenaline or noradrenaline in normal human

venous plasma, probably because he used too small a volume of blood for the estimation. He found that haemolysed blood frequently produces a quenching effect. With respect to specificity, this method requires for the fluorescent product: i) the catechol nucleus; ii) indole ring closure, achieved most readily by the presence of a secondary amino group (the basis for the different rates of oxidation); iii) intramolecular autoreduction, which is responsible for the formation of the fluorescent product, requires that a hydrogen atom be attached to the α and β carbon atoms of the side chain. According to von Euler (107), DOPAmine does not interfere with these estimations, its fluorescent intensity being only 0.64% of that of adrenaline in equimolar solutions.

Goldenberg et al. (108) adapted Lund's procedure for screening purposes in cases of pheochromocytoma. In this method, hydrolysed urine is subjected to adsorption on aluminum hydroxide formed in the solution; the catecholamines are extracted into a solution of acetone-ethanol which is then concentrated in vacuo. Aliquots are removed for evaluation. The authors were not able to obtain satisfactory results by the differential oxidation at pH's 3 and 6.5 and so they measured the total fluorescence at pH 6. Interestingly enough, this paper questions implicitly the specificity in regard to the fluorescence measured. Thus, Goldenberg's technique does

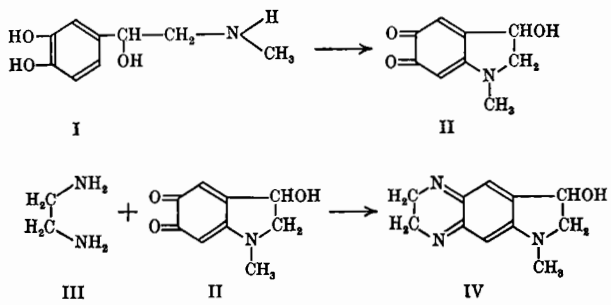
not attempt to differentiate adrenaline from noradrenaline; he has chosen rather, to evaluate his readings by exciting with light of different peak wavelengths. The emitted light is measured at 530 mμ. and up. Thus by exciting with a light predominantly of 405 mμ. the highest specific readings for adrenaline or noradrenaline are obtained. Adrenaline gives a value of 0.7 when the ratio of emitted light is measured at exciting lights 365 and 436 mμ; noradrenaline gives a value of 2.0. Thus with urine samples the value ranges from 0.7 to 8.0. They suggest that urine samples with high fluorescence when irradiated with light of 405 mμ., and with a value over 2.0 for the ratio of readings of exciting lights 365 and 436 mμ., owe their fluorescence values not to significant quantities of adrenaline and noradrenaline but to non-specific fluorescence. To quantify the amounts of adrenaline and noradrenaline present in the urine extract, this group used paper chromotography and then ran the eluted spots through the fluorimetric procedure.

Recently von Euler and Floding (109) published another sensitive fluorimetric method for the differential estimations of adrenaline and noradrenaline based on their different rates of oxidation at acid pH's. This method is a modification of Ehrlén's (110) in which potassium ferricyanide is the oxidant used to bring the catecholamines to the chrome stage; the stabilized lutines are then formed as before by the

addition of alkali and ascorbic acid. The filter system recommended to measure the intensity of fluorescence gives a ratio of 1.6 for adrenaline:noradrenaline. Von Euler and Floding have modified this technique by affording an oxidation of adrenaline to adrenochrome at pH 3.5 where less than 5% of the noradrenaline is oxidized. Zinc sulphate is used to catalyse this oxidation. Both catechols are oxidized at pH 6. The lutines are formed by addition of NaOH and ascorbic acid, and reagent and extract blanks are prepared. This is more convenient than Lund's procedure or the iodine colourimetric method. The fluorescent samples are stable for about an hour as in Lund's and Ehrlén's methods. This method is suitable for urine extracts (55, 111) purified by adsorption on aluminum oxide. The recovery of catecholamines added to urine, tested by bioassay, is about 80%. 0.001 µg. of adrenaline per ml. can be estimated in a sensitive fluorimeter. It is concluded on theoretical grounds by Bülock and Harley-Mason (97) that only those catecholamines which possess a β-OH group and a hydrogen at the α-carbon in the side chain are able to form fluorescent compounds from the corresponding chromes. Thus isopropylnoradrenaline gives a strong fluorescence while DOPAmine gives only a very weak fluorescence. It should be noted that the urine contains relatively large amounts of DOPAmine (112).

The method with which we are most concerned is that

of Weil-Malherbe and Bone (63, 84). It is not based on the formation of adrenolutine or noradrenolutine but on the condensation of ethylene diamine (see Fig. 3. III) with adrenochrome (II) to form a fairly stable fluorescent product (IV). The following reaction is formulated by Weil-Malherbe and Bone;



I II III II IV

(I) Adrenaline. (II) Adrenochrome. (III) Ethylene diamine. (IV) Condensation product (hypothetical).

FIGURE 3: A proposed scheme for the formation of the fluorescent derivative of adrenaline in the Weil-Malherbe and Bone method (84). The structure of the condensation product is hypothetical and has not been determined.

The condensation takes place in alkaline solution and requires oxygen; with adrenochrome, in the presence or absence of oxygen, a fluorescence intensity curve is obtained which is identical with that produced by equimolar amounts of

adrenaline in the presence of oxygen. Natelson, et al. (1949, 113) were the first to employ ethylene diamine condensation to effect an extractable fluorescent produce of adrenaline. Working with protein hydrolysates, they observed that aged tryptophan and tyrosine fluoresced in a similar manner to adrenaline when they were subjected to alkaline oxidation; furthermore, the fluorescence was not completely extractable with organic solvents. Tryptophan and tyrosine were found to fluoresce in alkaline solution when excited by light of 365 mu., while they do not fluoresce when excited by light of 435 mu. The ethylene diamine condensate could be extracted with an aliphatic alcohol and the fluorescence intensity read at the longer wavelength, thereby avoiding interference from many substances. Weil-Malherbe and Bone have modified this technique to provide a highly sensitive measurement of the catechols in blood. They collect the blood in a solution of $NaF-Na_2S_2O_3$ (114) which combines anticoagulant with anti-oxidant properties; the catechols are eluted from a column of alumina (acid-washed) which is prepared with a buffer freed of traces of metals by passing it through a cation-exchange resin. The buffer is adjusted to pH 8.4 with a glass electrode for maximum adsorption of the catecholamines. In this way, quantitative recovery from the alumina column is achieved as compared with Lund's losses sustained during adsorption. The catechols are eluted, similarly, with acetic acid. Adrenaline and noradrenaline are estimated simultaneously

in the mixture by taking advantage of their different fluorescence spectra. This is achieved by determining the fluorescent intensities with two different secondary filters (63). The condensation of the eluate is achieved by heating the solution under controlled conditions with a mixture of ethylene diamine and ethylene diamine dihydrochloride. The free base produces the required degree of alkalinity while the ionized form of ethylene diamine is the reactive agent. The condensates are extracted quantitatively into isobutanol. The adrenaline derivative has been found to have its strongest peak in the orange region (about 580 mμ.) while the noradrenaline derivative is strongest in the blue region (about 450 mμ.). The ratios of their fluorescence intensities with a blue-green filter are almost identical while with a yellow filter the fluorescence of the adrenaline derivative is about four and a half times that of the noradrenaline derivative. By running standards, each time, through the condensation procedure the fluorescence ratios may be calculated at each of the wavelengths and test samples read against them. The fluorescences have been shown to be additive, and a linearity is observed when a calibration curve plotting fluorescence intensity against concentration is prepared.

The sensitivity is such that less than 1 μg. per litre of adrenaline and 1 to 7 μg. of noradrenaline per litre

may be measured. Recovery experiments (84), with horse serum, were quantitative with a standard deviation of about 5%.

The specificity of this method has been questioned by several authors (109, 86). However, Weil-Malherbe maintains that the specificity for adrenaline and noradrenaline is high, based upon a variety of evidence. Thus among possible interfering substances: DOPA forms a derivative which is extracted only to a slight extent, its fluorescence amounting to only 0.2% of that of the adrenaline derivative; catechol, adrenochrome and 5-OH tryptamine are not adsorbed on the alumina column (71). He has, further, extracted plasma and subjected the extract to paper chromatography. This revealed two spots corresponding in position to adrenaline and noradrenaline, eluted, these areas gave their characteristic spectral properties in the fluorescence reaction. (63, 115, 71). Moreover, he has compared samples from the same blood by the method of Lund and the ethylene diamine condensation and found essentially identical results (116, 115, 71).

Modifications of this method have been reported by several groups of authors. Manger et al. (117) used sodium thiosulphate to reduce the intensity of fluorescence of the noradrenaline derivative; however, they have not applied this method to blood. What advantage there is to this procedure is questionable as the amount of noradrenaline measureable in blood yields only a small fluorescence and to increase the fluorescence would be more advantageous. Persky and Roston (118) determined the different fluorescence spectra and

proposed the use of an exciting wavelength of 436 mμ., and secondary filters which pass a narrow band peaking at 510 mμ., and another, which cuts off below 600 mμ . These combinations yield maximum differentiation between adrenaline and noradrenaline derivatives and minimum blanks.

Aronow and Howard (119) have published a shortened procedure of the Weil-Malherbe and Bone method, whereby they shake plasma with alumina, without buffering to the pH of maximum adsorption, and carry out the condensation in a dark room to shield the solutions from blue light. They claim recoveries of 97 ± 5% of adrenaline and 85 ± 5% of noradrenaline.

Erne and Canbäck (120) while working out the fluorescence procedure to estimate noradrenaline in solutions stabilized with pyrosulphite, which interferes with the cyclization of noradrenaline and ethylene diamine, studied many of the properties of this condensation. This is discussed further in the experimental section of this thesis.

II

EXPERIMENTAL

A. METHODOLOGICAL STUDIES

1) Materials and Preparation.

a) Stock solutions of adrenaline and noradrenaline.

L-epinephrine bitartrate and dl-arterenol hydrochloride (both from Winthrop-Stearns Inc.) were used for the preparation of standard solutions. Stock solutions were prepared by dissolving 10 mg. of adrenaline (A) or noradrenaline (N) in 100 ml. of 0.2 N acetic acid. In a later preparation, the diluent used was 0.001 N hydrochloric acid. In use, the stored stock solutions were diluted twice; the first dilution, 100 times, the second, 50 times, resulting in a standard working solution of 0.2 µg. of adrenaline or noradrenaline per 10 ml.

The earlier stock solutions were kept in the refrigerator at 3-4° C.; later stock solutions were stored in small test-tubes held in the freezer cabinet.

b) Stability of stock solutions on storage.

Standard solutions were carried through the condensation procedure of each experiment, and the relative intensities of fluorescence of equivalent concentrations of adrenaline and noradrenaline were measured at two wavelengths. According to Weil-Malherbe and Bone (84), a linear concentration curve is obtained when a standard solution of 0.2 µg. of adrenaline is used to adjust the sensitivity setting to 100. In the differential estimation of the two amines (63), a standard noradrenaline solution of 0.2 µg. is estimated at the two wavelengths on the basis of this arbitrary adrenaline setting.

The following table may serve as an illustration of the variability incurred under different conditions of storage when the ratios of fluorescence are estimated after condensation has proceeded for twenty minutes at 50°C.

TABLE I: Stability of stock solutions of adrenaline and noradrenaline as determined by their fluorescence ratios.

Stock solutions		Condition of Storage	n*	m**
Span of days in use	No. of detns.		Mean ± S.E.	Mean ± S.E.
2-26	6	Refriger-ated at 2° C.	1.59 ± 0.07	3.59 ± 0.31
33-85 (A)				
1-53 (NA)	6		1.49 ± 0.08	3.64 ± 0.40
0-15	4		0.83 ± 0.06	2.71 ± 0.09
1-49	4	frozen	1.99 ± 0.34	5.33 ± 0.58
3-17	4	frozen	0.98 ± 0.12	2.72 ± 0.20

* ratio of fluorescence; adrenaline/noradrenaline at 510 mμ.
** ratio of fluorescence; adrenaline/noradrenaline at 600 mμ.

It can be seen from the values of the standard error in Table I, that the values of 'n' and 'm', i.e, the ratios of fluorescence, tend to fluctuate more when solutions stored frozen are used as standards, than when these solutions are simply refrigerated.

A factorial experiment was carried out to analyse the variation due to instability of the solutions on storage. Four storage conditions were as follows:

1) Stock solutions kept in the refrigerator; the adrenaline solution was stored 201 days and the noradrenaline solution was stored 175 days.

2) Stock solutions kept deep-frozen for 117 days.

3) Stock solutions kept deep-frozen for 17 days.

4) Fresh stock solutions prepared the day of the experiment.

Standard solutions, at a concentration of 0.16 µg. of adrenaline or noradrenaline were run through the condensation procedure with ethylene diamine and its salt at 50°C. for twenty minutes and the fluorescence was read. Each solution was run in triplicate. This provided 48 data (4 storages of two catecholamines read with two filters and in triplicate), which were subjected to analysis of variance according to the method described by Snedecor (121). All statistical methods in this thesis are based upon Snedecor's directions.

The following abbreviations are used in statistical tables:

DF - Degrees of Freedom

F ratio -mean square for an effect/mean square for error
HS -highly significant at the 1% level, P less than 1% that the effect is due to chance
S -Significant at the 5% level, P less than 5%

NS -not significant

On examination of Table II, the differences between the two catecholamines ("C") and the differential filter system ("F") used are readily observable and are to be expected.

TABLE II: Analysis of Variance of prominent factors in the fluorimetric determination of adrenaline and noradrenaline.

Sources of variation	DF	Sum of Squares	Mean Square	F. Ratio	
Total	47	23,062.5450	-		
Storage Conditions (4) "S"	3	3,779.8324	1,259.9441	24.989	HS
Catecholamines (2) "E"	1	1,664.0430	1,664.0430	33,004	HS
Secondary filters (2) "F"	1	4,358.2597	4,358.2597	86.440	HS
S x C	3	276.4836	92.1612	1.828	NS
C x F	1	11,266.3280	11,266.3280	223.452	HS
S x F	3	37.6090	12.5363	0.249	NS
S x F x C	3	66.5687	22.1896	0.440	NS
Remainder(for error)	32	1,613.4206	50.4194	(1.0)	

Storage Conditions - Frozen and Liquid
Catecholamines - adrenaline and noradrenaline
Filters - secondary filters transmitting in region of 510 mu.
- secondary filters transmitting in region of 600 mu.

The storage conditions ("S") also present a significant picture, namely, that samples of adrenaline and noradrenaline held under different storage conditions apparently undergo chemical changes which are reflected in their reactivity with ethylene diamine. The interactions, S x C and S x F x C, are not statistically significant; this indicates that the relative fluorescence of the derivatives of adrenaline and noradrenaline is independent of storage condition; or, put in another way, that adrenaline and noradrenaline undergo the same type of chemical change, whatever that may be, under all 4 storage conditions. The interaction, C x F, is highly significant; as is to be expected the two catecholamines react in different ways to the filter system employed.

When the degrees of freedom for storage conditions are broken down to their component states (Table III) highly significant variations may be observed between the two frozen stock solutions at the 1% level, as well as a highly significant difference between the frozen and the liquid solutions.

TABLE III: Analysis of Variance: Conditions of storage of Stock solutions (cf. Table II).

Sources of Variation	D F	Sum of Squares	mean Square	F Ratio
Storage Conditions				
Between frozen and liquid	1	959.6197	959.6197	19.033 HS
Between 2 frozen	1	2,562.6667	2,562.6667	50.827 HS
Between 2 liquid	1	257.5460	257.5460	5.108 S at 5% NS at 1%
Remainder (for error as in Table II)	32	1,613.4206	50.4194	(1.0)

The two liquid solutions show a significant difference at the 5% level as might be expected considering that one of these storage conditions was of a length of five to seven months, while the other preparation was

freshly made up; this difference is not significant at the 1% level. This is, however, not a crucial point of the method because solutions can be conveniently made up frequently, e.g, once in a fortnight, and in this way the problem of stability can be minimized as illustrated in Table I.

2) Chromatography
   a) Preparation of Plasma

Blood is drawn from an arm vein into a syringe containing 5 ml. of fluoride-thiosulphate solution; about 15 ml. of blood is drawn (84). The preservative is prepared at a concentration of 2% sodium fluoride and 3% sodium thiosulphate, and it possesses both anti-oxidant and anti-coagulant properties. The solution is stored in 10 ml. ampoules which are autoclaved for 15 minutes at 15 pounds pressure in a pressure cooker. This method of blood collection leads to disintegration of the platelets. Weil-Malherbe and Bone (122) were able to demonstrate that 70 to 80% of the adrenaline and noradrenaline normally estimated by their method was unaccounted for when special precautions were taken to preserve the platelets. Blood was collected in silicone-coated glassware containing heparin in an isotonic medium. After centrifugation to throw down the platelets, it was found that considerable portion of the catecholamines could be recovered in the platelet residue.

The usefulness of metal-chelators and anti-oxidants has been considered during the course of this study. Versene (ethylenediaminetetraacetate) was added to the preservative solution in the syringe for blood collection in one experiment. Several samples in the one experiment showed no improvement in the variation of duplicate samples. However, Weil-Malherbe and Bone have reported that they are now using it in their preservative solution to replace sodium thiosulphate; they report that identical results

are obtained using either preservative solutions (73). Solutions of Versene and ascorbic acid as diluents for the preparation of standard solutions of noradrenaline were tested in one experiment and no striking improvement was observed. The possible usefulness of Versene (EDTA) has not been followed up.

The blood is prepared for chromatography by centrifuging at 4600 r.p.m. (increasing the r.p.m. gradually over a period of 25 minutes). The plasma is combined with an equal volumn of 0.2 M sodium acetate, buffered at pH 8.4. The buffer is prepared in glass-distilled water, as are all solutions and glassware; it is further treated by passing over a column of Amberlite IR-120* (H) to remove all traces of heavy metals. This column is prepared by repeated washings of the resin with 2 N HCl, glass-distilled water, and 4% sodium chloride (84). Excess NaCl is flushed out with glass-distilled water. The column measures 2.5 cm. x 24.5 cm. and filtration proceeds at approximately 20 drops per minute.

A model G Beckman pH meter with outside shielded electrodes, is employed for titration of the buffer and buffer-plasma mixtures. 0.5 N Na_2CO_3 is used for the adjustment. The solution of plasma and buffer is adjusted to pH 8.4 just prior to chromatography on the alumina column. A glass stirring rod is fixed on the stand holding the glass electrodes to facilitate adjustments and speed the process, as the time period at this pH is critical (82).

b) Adsorption of the catecholamines.

Adsorption of the catecholamines in plasma is followed (84). Merck alumina, acid-washed, "for chromatographic analysis" is used in the preparation of the alumina column. A comparison of different types of alumina was made

employing the Merck alumina, the same acid-washed according to the directions of Weil-Malherbe and Bone (84), Woelm acid alumina (pH 4) "anionotropic, activity grade 1 for chromatographic analysis", and Woelm "non-alkaline (pH 7.4), activity grade 1 for chromatographic analysis" alumina. The average level for triplicate determinations in two experiments using aliquots of a sample of pooled plasma is given in Table IV.

TABLE IV: Apparent plasma concentrations of adrenaline and noradrenaline eluted from variously treated aluminas (2 experiments with pooled plasma).

	Adrenaline µg./litre blood		Noradrenaline µg./ litre blood	
Alumina	Series 1	Series 2	Series 1	Series 2
Acid-washed Merck	3.0	2.4	1.4	1.4
Acid-washed Merck, further acid-washed	2.4	2.0	1.1	1.3
Acid-washed Woelm	1.8	2.4	1.3	1.6
"Non-alkaline" Woelm	0.9	0.7	1.0	2.8

It would seem that considerable loss of adrenaline may be incurred on the column prepared with non-acid-washed alumina. The mesh of the Merck alumina used throughout this work is between 100 and 200. Acid-washing was carried out by stirring 100 grams of alumina with 500 ml. boiling 2 N HCl for 20 minutes, filtering over suction and washing with 500 ml. of hot 2N HCl; it was then repeatedly washed with glass-distilled water over suction and dried 3 hours at 300°C. (84).

The apparatus for chromatography was suggested by a diagram of Bone (116); previous attempts employing a thistle funnel mounted on a filter flask containing a test-tube trap proved arduous (84). A wooden stand was set up,

to which were attached by means of terry clips, six 50 ml. standard test-tubes having a 26.5 cm. glass stem, with a 5 mm. bore, sealed to the base of each test-tube. A constriction approximately 18 cm. from the base of the test-tube holds a plug of glass wool and a column of alumina. In operation, a nitrogen tank is connected by means of pressure rubing to a manifold , with lead-offs to a sintered glass filter for each column. The diameter of the lead-off opening is controlled by means of a Hoffman screw clamp. A layer of mercury over the sintered glass plate serves as a non-return valve. The tube from the sintered glass filter, in turn, is connected with a T-tube having two outlets, one through a rubber stopper filling the top of the test-tube and column, the other to a test-tube containing a column of mercury. The latter can be adjusted vertically for regulation of the gas pressure.

The chromatographic column is prepared by washing into it 0.7 g. of alumina with 0.2 M sodium acetate buffer, pH 8.4, over the glass wool plug. A fine glass rod which fits into the bore of the column assists in removing air bubbles. The plasma-buffer mixture is added to the top of the column, the rubber stopper fitted on and the gas pressure applied. This allows the filtration to proceed at about 20-30 drops per minute. The plasma is washed through the column with 5 ml. of buffer, followed by 5 ml. of glass-distilled water. The washings are discarded. A 40 ml. glass-stoppered centrifuge tube is used to collect the eluate which passes through when 5 ml. of 0.2 N acetic acid is added to the column, followed by a wash with 5 ml. of glass-distilled water. By means of this apparatus, six plasmas can be chromatographed simultaneously; the rate of filtration can be regulated and any columns not in use can be clamped off. Clogging of the column occurs occasionally, generally when plasmas which have been stored in the

refrigerator are chromatographed; this seems to be due to a separation of fibrin (the latter can often be removed by poking at the top of the column with a glass rod).

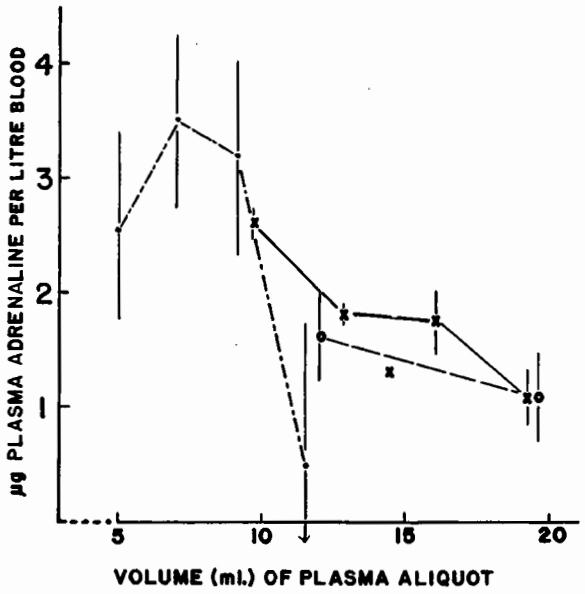
In this work the volume of the sample of plasma chromatographed appears to be critical, as evidenced by experiments i) with different volumes of pooled plasma, and ii) with plasma divided into two unequal aliquots for duplicate determinations. The results of such experiments are shown in Table V.

TABLE V: Variations observed due to Differences in Volume of Plasma taken for Estimation of Adrenaline and Noradrenaline.

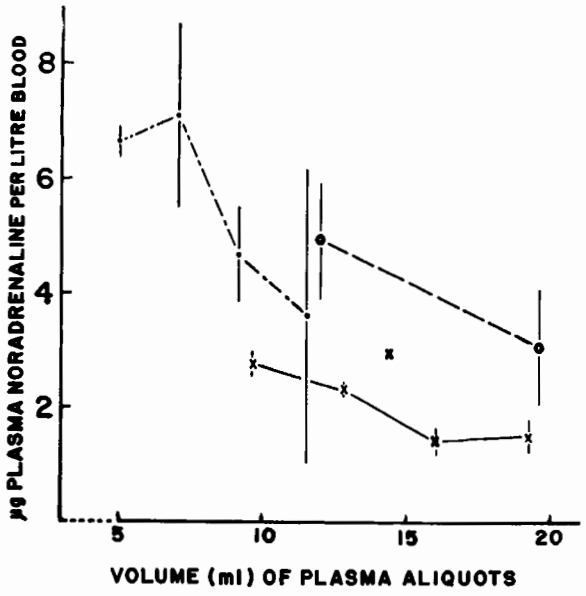
Source of Sampling	Blood Vol. (ml.)	n*	Plasma Adrenaline µg./l Whole blood Mean ± S.E.**	Plasma Adrenaline Relative Concn. %	Plasma Noradrenaline µg./l whole blood Mean ± S.E.**	Plasma Noradrenaline Relative Concn. %
Series I Disproportionate division of samples drawn during	12.0 ± 1.3	21	1.62 ± 0.38	100	4.93 ± 0.99	100
Mecholyl testing	19.6 ± 3.2	21	1.09 ± 0.38	67	3.08 ± 0.99	62
Series 2 Pooled plasma divided for estimation of variation due to volume taken for estimation	9.63	3	2.63 ± 0.09	100	2.77 ± 0.20	100
	12.83	3	1.82 ± 0.07	69	2.33 ± 0.09	84
	14.44	1	1.32	50	2.98	108
	16.05	3	1.77 ± 0.31	67	1.44 ± 0.23	52
	19.25	3	1.09 ± 0.24	41	1.53 ± 0.27	55
Series 3 Pooled plasma divided for estimation of variation due to volume taken for estimation	5.03	3	2.58 ± 0.80	100	6.63 ± 0.24	100
	8.04	3	3.50 ± 0.76	136	7.10 ± 1.59	107
	9.15	3	3.20 ± 0.84	124	4.67 ± 0.81	70
	11.53	3	0.49 ± 1.24	19	3.61 ± 2.55	54

* n - number of samples taken for estimation of mean
** Standard Error.

On examining a series of values for the two catecholamines where plasma or pooled plasma has been divided disproportionately for their estimation, a trend is observed whereby the lower plasma volumes consistently yield a higher value; this is illustrated in Table V, Series 1, where the mean values show that the larger volumes of plasma yield lower apparent adrenaline and noradrenaline concentrations. Regarding the percentage concentrations of the two catecholamines estimated in Series 1, the higher volumes are consistent in that 67% and 62% for adrenaline and noradrenaline respectively are recovered of the concentrations estimated when the lower volume was determined. This is borne out by the more complete Series 2; here however, the percentage concentrations of the higher volumes with respect to the lowest volume taken for the determination are not consistent throughout where adrenaline and noradrenaline are regarded in parallel. Thus, 50% and 108% for adrenaline and noradrenaline respectively, are not equivalent recoveries of the concentrations estimated as 100%. In Series 3, there is a lower mean concentration of adrenaline and noradrenaline estimated with the smallest volume, whereas the intermediate volumes, 7 and 9 ml., in the case of adrenaline and 7 ml. in the case of noradrenaline yield the higher concentration values. Again the percentage concentrations of the higher volume estimations do not run parallel with regard to the estimations of the smallest volume taken as 100%. The standard error is very large in Series 3 and these differences may be only accidental and not real.



4A: Fluorimetric determinations of adrenaline in pooled plasma. (cf. Table V)



4B: Fluorimetric determinations of noradrenaline in pooled plasma. (cf. Table V)

FIGURE 4: Effect of plasma volume on the apparent concentration of adrenaline (upper fig. 4A) and noradrenaline (lower fig. 4B) measured in blood.

Legend:
- - - Mean of 21 determinations at two mean volumes: Series 1, Table V
-•-•-•Mean of triplicate determinations at pooled plasma volumes of Series 2, Table V
_____Mean of triplicate determinations at four pooled plasma volumes of Series 3, Table V

The data of Table V are illustrated in Figures 4A and 4B. A reasonable continuity in the data of the three Series is observed in the relation of volume used for the estimation of the apparent concentration of adrenaline and noradrenaline. A plateau may occur in the region of 6 to 10 ml. plasma volumes since all three series show the same trend of the highest values in this region and the high standard errors of Series 3 overlap in this region.

The data of Series 2 and 3 were obtained as sets of homogeneous data and therefore lend themselves to further statistical analysis. For these Series, blood was obtained from donors in the hospital; the blood was centrifuged and the plasma was pooled. Adrenaline and noradrenaline were then determined by the Weil-Malherbe and Bone method (63) at four levels of plasma volume, each volume replicated three times providing 12 data. The variance between replicates (that is, variance not attributable to differences in volumes of plasmas, the "within-volume" variance, or mean square for error) was estimated as shown in Tables VI and VII, and was used to calculate the difference between different plasma volumes required to reach a statistically significant level ($P = 0.05$). This calculation was not performed for Series 3, since the analysis of variance (Table VII) indicated that the means were not significantly different.

TABLE VI: Analysis of Variance of Data of Series 2-pooled plasma of Table V.

Sources of Variation	DF	ADRENALINE Sum of Squares	Mean Square	F Ratio	NORADRENALINE Sum of Squares	Mean Square	F Ratio
Total	11	4.5644	-		4.7703	-	
Volumes (4)	3	3.5605	1.1868	9.45	3.6970	1.2323	9.18
Remainder (for error)	8	1.0039	0.1254	(1.0)	1.0733	0.1342	(1.0)
Variance of a mean of 3 replicates = Mean Square / 3			0.0418			0.0447	
Variance of a difference between 2 means, each of three items			0.0837			0.0894	
Standard Error of this difference = $\sqrt{d}$			±0.29			±0.30	
Necessary difference = t x $\sqrt{d}$ (t = 2.776 for P = 5% and for 4 degrees of freedom)			0.81			0.83	

Means (From Table V)

Volume of Blood represented (ml.)	Mean adrenaline ug./ litre blood	Mean noradrenaline ug./litre blood
9.63	2.63	2.77
12.83	1.82	2.33
16.05	1.77	1.44
19.25	1.09	1.53

TABLE VII: Analysis of Variance of Data of Series 3 - pooled plasma of Table V.

Sources of Variation	Deg. of Free.	ADRENALINE Sum of Squares	Mean Square	F Ratio	NORADRENALINE Sum of Squares	Mean Square	F Ratio
Total	11	37.3296	-		47.5165	-	
Volumes (4)	3	16.5396	5.5132	2.12	24.3365	8.1122	2.79
Remainder (for error)	8	20.7900	2.5988	(1.0)	23.1800	2.8975	(1.0)

The calculation of necessary differences in Table VI is formally justified, but may involve an error: if the variances of the means in triplicates calculated separately (as in Table V) are compared, it is seen that they are quite different, tending to be larger with the larger blood volumes. Hence, the variances may not be normally distributed. No test of normality of the variance distribution has been performed.

The volume taken for estimation of adrenaline and noradrenaline is highly significant for the larger volumes of Series 2, as is indicated by the results in Table VI. Hence the apparent concentration of adrenaline and noradrenaline depends upon the volume of blood chosen for the estimation. This was not demonstrated with the lower volumes taken in the second pooled plasma experiment, Series 3, as shown in Tables V and VII.

In Table VIII from the first pooled plasma experiment, Series 2, the observed differences between adrenaline and noradrenaline concentrations due to the plasma volumes taken for estimation, are compared to those necessary to attain statistical significance at the 5% level of probability.

TABLE VIII: Statistical Significance of Observed Differences in Concentrations of Adrenaline and Noradrenaline measured in Different Volumes from Pooled Plasma.

Volumes being Compared	Differences in Estimated Concentrations					
	Adrenaline			Noradrenaline		
	Observed a	Necessary b	Signif.	Observed a	Necessary b	Signif.
9.63 - 12.83	0.81	0.81	+	0.44	0.83	-
9.63 - 16.05	0.86	0.81	+	1.33	0.83	+
9.63 - 19.25	1.54	0.81	+	1.24	0.83	+
12.83 - 16.05	0.05	0.81	-	0.89	0.83	+
12.83 - 19.25	0.73	0.81	-	0.80	0.83	-
16.05 - 19225	0.68	0.81	-	0.09	0.83	-

a - calculated by subtracting the arithmetic means of the two concentrations as given in Table V and in Figures 4A and 4B.
b - calculated from the pooled sampling error as in Table VI.

The smallest volume yields a significantly higher value than the three larger volumes in the case of adrenaline estimation. The latter three volumes are not significantly different from one another. In the case of noradrenaline, the two smaller volumes are not statistically significantly different in their yield of noradrenaline. The same holds true of the two larger volumes. However, both smaller volumes yield a higher concentration of noradrenaline than do both larger volumes.

In the second pooled plasma experiment, Series 3, as shown in Table VII, the adrenaline and noradrenaline estimations do not show any significant difference due to the different volumes taken. These volumes are in a lower range and the standard error is high so that the means overlap throughout (as seen in Figures 4A and 4B).

3) The Condensation Reaction
   a) Initial Work

The basis of this method lies in the formation of a fairly stable fluorescent condensation product (84). It is postulated that the catechol amine is oxidized to a quinone in alkaline solution, and that the quinone condenses with an aliphatic primary amine. Ethylene diamine is used as the primary organic amine as it yields the least fluorescent reagent blank of the aliphatic diamines examined. This condensation takes place in alkaline solution in the presence of oxygen; the adrenaline may be replaced by adrenochrome, in the absence or presence of oxygen, in which case an identical fluorescence intensity curve results when equi-molar amounts of adrenaline and adrenochrome are used. Weil-Malherbe and Bone claim the fluorescence of the final product remains constant for at least 24 hours.

The fluorescent products are extractable with aliphatic alcohols such as butyl and amyl alcohols, complete extraction being achieved by

saturating the aqueous phase with sodium chloride. The fluorescence of the condensation products of adrenaline and noradrenaline behaves additively.

Condensation takes place by heating a solution containing the catechols with ethylene diamine and an aqueous solution of ethylene diamine dihydrochloride at 50°C. for 20 minutes. Ethylene diamine in the ionized form is the reactive agent while the free base provides the desired alkalinity; the pH of the mixture is 10.4 (84).

Early experiments were carried out using ethylene diamine dihydrochloride obtained from the Eastman Chemical Co.; this reagent contained an impurity yielding blank values of 75-80 units on the fluorescence scale of 100 units, where 0.2 µg. of adrenaline was employed to set the instrument to 100. In other experiments, the fluorescence of the reagent blank was higher than that of the standard solutions of adrenaline when fluorescence of 510 mµ. was measured, and slightly lower, when fluorescence in the area of 600 mµ. (the peak of adrenaline fluorescence) was measured. Re-crystallization of of the salt lowered the fluorescence value of the reagent blank considerably, the fluorescence amounting to only about 50% of the adrenaline standard at 510 mµ. and 41% at 600 mµ; a second lot of crystals yielded an even lower fluorescence value. Ethylene diamine dihydrochloride was then prepared from freshly distilled ethylene diamine, by dissolving the base in freshly distilled ethanol and precipitating the salt crystals in a solution of ethanol-hydrochloric acid (115). The crystals were washed and dried over concentrated sulphuric acid. Re-crystallization of the salt twice yields a product with a fairly low value of fluorescence. The base, ethylene diamine, is also responsible for a large part of the blank fluorescence. Where its fluorescence gave a value of 91 units at 510 mµ. and 76 units at 600 mµ. against an adrenaline standard of 0.16 µg., after distillation (B.P. = 117°C.) the fluorescence only amounted to 17 units at 510 mµ. and 5 units at 600 mµ. against a standard of

0.2 µg. of adrenaline set to 100 units on the fluorescence scale. The procedure was followed of distilling the ethylene diamine, obtained from Brickman Chemicals and Eastman Chemicals, (95-100% pure), just before use in every experiment, as the fluorescence of the base increases rapidly on standing. The fraction distilling between 116° and 118° C. is used. The dihydrochloride of ethylene diamine does not appear to increase in fluorescence when stored in the crystalline state.

The effect of cigarette smoke (a spurious source of fluorescent contaminants) or rather, lack of it (no smoking in the laboratory) did not appear to make any difference on the reagent blank values. Charcoal when shaken up with ethylene diamine did not remove any impurities which might be contributing to the fluorescence. Oxygen has a quenching effect on some fluorescences but when bubbled through either reagent did not have any action on these.

Adrenaline, 0.12 µg, was used as standard to adjust the instrument to 50 units of fluorescence in the following experiment, the standard solution condensed with the treated reagents as following in each case: heating time being one hour at 50° C.; 0.7 ml.of ethylene diamine and/or 0.5 ml. ethylene diamine dihydrochloride treated as follows, heated in solution and extracted with iso-butanol as the standard in each case:

Treatment of reagents	Reagents	Fluorescent Units 510 mµ.	600 mµ.
Shaken with charcoal	ED	16	16
Shaken with charcoal and oxygenated	ED	17	14
Both rgs. oxygenated ED shaken with charcoal	ED and EDD	23.5	18
Rgs. untreated	ED	15.5	17.5
	EDD	14	18
	ED and EDD	21.5	20.5
Oxygenation of both reagents	ED	17	18
	EDD	17.5	21
	ED and EDD	21.5	21

ED - ethylene diamine.
EDD - ethylene diamine dihydrochloride.

None of these conditions, of treating the reagents, reveals any remarkable variation. It may be observed that the respective fluorescences of the reagents are not additive in each case (in the case where the reagents were untreated and where both reagents were oxygenated); an explanation of this phenomenon is not at hand; however, it may be considered that the pH of the solution extracted with iso-butanol after heating is different in each case, that of the mixture, intermediate between the two reagents, and since the ionized form is considered the active one for the condensation reaction it may be that they are extracted differently and/or react differently to the excitant light, in combination.

In practice, 0.5 ml. of 2M ethylene diamine dihydrochloride and 0.7 ml. of ethylene diamine (both oxygenated just before use - see section following, 4) e)) are added to 10 ml. of the solutions being tested. (84). In a recent paper (120), it was concluded that for noradrenaline, a reagent composition of about equal volumes of base and of a 2M solution of the dihydrochloride, the fluorescence is not affected markedly by moderate changes in reagent composition.

Occasionally the reagent blank is so high as to prevent adjustment of the standard to 100 units and the reagent blank to 0 on the fluorometer scale. In such cases the remaining blank value is taken into account in the calculations, after the standard has been set to 100. This reduces the scale of standard values in slope determinations. The value of the reagent blank may range from 25-60 units on the galvanometer scale where 0.16 or 0.2 µg. of adrenaline is used as standard to set the scale to 100.

b) Effect of Temperature.

The effect of temperature on the development of the condensation product has been studied by Bone (116), and Erne and Canbäck (120). Much the same kind of effect was found for adrenaline by the former author, as for noradrenaline, by the latter. That is, that the maximum intensity is reached more quickly, the higher the temperature, but the highest intensity is reached by the lower temperatures acting for a longer time. Thus, for noradrenaline these authors found that heating for 20 minutes at 50°C. utilizes the steep slope of the curve whose plateau is not reached until 2 hours at 50°C. Bone demonstrated that the plateau for adrenaline fluorescence is achieved in 20 minutes at 50°C. where maximum fluorescence is achieved at a lower heating time. The present experiments have been carried out employing a temperature of 50°C., while the effect of varying the heating time has been studied.

c) Heating Time.

Lengthening of the heating time for the condensation reaction was studied as a means of improving the calibration curve of noradrenaline and of increasing the noradrenaline slope. That adrenaline reaches its plateau at 50°C. in 20 minutes is illustrated by the following extract from a table in Bone's paper. (116).

1 µg. adrenaline in 1 ml. N HCl incubated with 0.5 ml. 2:1 mixture of 6 N NH_4OH and redist. ethylene diamine. Extraction with 6 ml. isobutanol, 5 ml. taken for estimation:

Temperature (°C.)	Time (min.)	Fluorescence (cm.)
50	5	12.3
50	10	14.4
50	20	15.1
50	30	14.9
50	60	15.0

The paper of Erne and Canbäck (120) illustrates that with noradrenaline at 50°C. the fluorescence increases with heating time, the greatest rise in intensity being achieved in one and a half hours, while stability of the plateau occurs in about two hours. These authors suggest that the condensation product is stable, whereas the precursor presumably noradrenochrome is subject to a synthesizing as well as a degrading reaction.

Several experiments were carried out to determine the effect of altering the heating time on the fluorescence of the condensation products.

One experiment was carried out in which adrenaline and noradrenaline at a concentration of 0.16 µg. were incubated respectively with the reagents, ethylene diamine and its salt, at 50°C. for varying periods of time; 20,30, 40,60,90 and 120 minutes. One sample each of adrenaline and noradrenaline was taken in the estimation of slop value for each time period. A fluorescein solution of 32.8 µg./litre and 66.6 µg./litre was employed as standard at 510 mµ. and 600 mµ. respectively, matching approximately the fluorescence of the adrenaline condensation product which produced the maximum fluorescence, that is the condensation product of adrenaline which had been incubated for 120 minutes at 50°C. The effect of increasing the time of incubation at 50°C. is seen in the following table.

TABLE IX: Fluorescence Intensity of Adrenaline and Noradrenaline Derivatives as a Function of Time of Incubation at 50° C.

Time Period (minutes)	SLOPE[a] 510 mμ. A	510 mμ. NA	600 mμ. A	600 mμ. NA	n	m	Absolute fluorescence intensity per unit concentration of fluorescein[b] 510 mμ. A	510 mμ. NA	600 mμ. A	600 mμ. NA
20	431	333	426	156	1.29	2.73	13.1	10.1	6.4	2.3
30	382	312	375	125	1.22	3.0	11.7	9.5	5.63	1.88
40	433	370	437	172	1.17	2.54	13.2	11.3	6.6	2.6
60	487	487	448	155	1.00	2.89	14.9	14.9	6.74	2.33
90	493	497	445	170	0.99	2.62	15.0	15.2	6.69	2.55
120	596	459	503	161	1.30	3.12	18.2	14.0	7.6	2.4

a Slope = mm. on galvanometer scale per μg. A or NA

b Slope/μg. fluorescein per litre

A = 0.16 μg. adrenaline used

NA = 0.16 μg. noradrenaline used

n = is the ratio A/NA at 510 mμ.

m = is the ratio A/NA at 600 mμ.

In two experiments, slopes were calculated or determined "by eye" from values in a concentration scale.

TABLE X: Effect of heating time on ratios of adrenaline/noradrenaline Fluorescence at two wavelengths.

No. of samples taken for estimation of slope		Heating Time		
A	NA	(Minutes)	n*	m**
5	4	20	2.07	6.54
5	4	120	1.17	4.14
3	3	20	1.61	5.88
3	3	30	1.43	4.55
3	3	40	0.93	3.16

* n - ratio of adrenaline/noradrenaline fluorescence at 510 mμ.
** m - ratio of adrenaline/noradrenaline fluorescence at 600 mμ.
A - adrenaline.
NA - noradrenaline.

In Table IX it will be observed that where a single concentration of fluorescein sodium was used as standard to adjust the instrument to 100 at each of the two wavelengths, the fluorescence of both adrenaline and noradrenaline increases with the increased heating period; this is observable in the increase in the slopes and the increase in fluorescence per gamma fluorescein. The noradrenaline slope suffers the greatest change as can be observed by the decrease in the 'n' value, the 'm' value shows no particular trend perhaps due to the unreliability of the low fluorescence readings at the longer wavelength. In Table X the decrease in both 'n' and 'm' values with the longer heating periods may be again observed, indicating the rise in slope of noradrenaline with the longer heating time. Fluorescein was not employed as a standard in these experiments and therefore an absolute measure of the increase in both slopes was not available. Solutions of adrenaline and noradrenaline approximating the concentrations found in the plasma of a 15 ml.

blood sample were run through the various heating periods of Table IX and the observed values compared with those estimated from the determined slopes, as follows:

Fluorescence Values (mm.) for 0.02 µg. A and 0.08 µg. N.A. run through the standard condensation procedure at 50°C. for various lengths of time.

Heat. Time (mins.)	510 mµ. calc. from slope of standards	Observed fluor. reading	610 mµ. calc. from slope of standards	observed fluor. reading
20	35.5	39	22	26
30	33	36.25	18	22.8
40	39	40	23	26.4
60	50	47.8	22	24
90	50	40.6	23	20.75
120	49	51.75	23	27.5

No appreciable, greater reliability was observed in the longer periods of heating of the condensation mixtures, and so the shorter (20 minute period) was generally adopted.

d) The Stability of the Condensation Products.

The stability of the condensation products of adrenaline and noradrenaline with ethylene diamine is generally accepted as the greatest advantage of this method over the methods where measurement of the unstable 'lutins' is the principle. The 'lutins' are stable only over a half hour and even so this is not without doubt. The fluorescence of the extracted condensation product is claimed to be stable for at least 24 hours (84) on the other hand.

The following table illustrates the stability of the extracts over a period of 24 hours:

Replicate readings of the fluorescent products of adrenaline and noradrenaline as observed in their fluorescent ratios at two wavelengths, and their individual fluorescence per gamma of fluorescein;

Heating Time (mins.)	Approx. no. of hours after first reading	n	m	mm. fluorescence/µg. A or NA µg. fluorescein/litre 510 mµ. A	510 mµ. NA	600 mµ. A	600 mµ. NA
20	-	2.91	5.00	25.6	8.8	11.4	2.3
	29 hours	3.44	5.21	22.8	6.6	12.5	2.4
20	-	0.78	2.97	9.7	12.5	8.6	2.9
	22.5 hrs.	1.59	2.93	17.8	11.2	9.6	3.3
35	-	0.80	2.88	18.4	22.8	11.5	4.0
	19 hrs.	0.95	2.33	22.0	23.2	22.4	9.6
60	-	0.54	2.12	11.6	21.6	?	?
	19.5 hrs.	0.83	2.67	26.4	31.6	?	?
120	-	0.80	3.35	16.6	20.7	12.5	3.7
	14.5 hrs.	0.90	3.09	18.3	20.4	?	?
120	-	0.90	3.77	9.3	10.3	7.4	2.0
	13 hrs.	1.16	3.37	14.6	12.6	7.6	2.3

n - ratio of adrenaline/noradrenaline at 510 mµ.
m - ratio of adrenaline/noradrenaline at 600 mµ.

Examining the data from this table, it is observed that the general trend is for the intensity of fluorescence of both the adrenaline and the noradrenaline derivatives to increase slightly, since more fluorescein is required to match their respective fluorescences in the second reading; that the adrenaline derivative increases in intensity at a greater rate than the noradrenaline derivative may be concluded by the general trend of the 'n' and 'm' values to increase rather than decrease. No precautions were taken to guard the test-tubes containing the adrenaline and noradrenaline derivative solutions from light of any particular source between the first and second readings.

e) Oxygenation of Reagents

Oxygenation of the reagents, ethylene diamine and ethylene diamine dihydrochloride, was tested as a possible means of quenching the fluorescence of the reagent blank. Quenching did not follow; however, advantageously the calibration curve for the noradrenaline derivative improved considerably. The actual slope value of noradrenaline does not appear to be affected, but the improved linearity of the values is a great asset in assessing the value of the slope. Oxygenation of the reagents is carried out by bubbling 100% O_2 through them for 10-20 minutes just prior to adding the reagents to the test solutions. Mylon and Roston (123) oxygenated their alkaline reagent employed in the oxidation of adrenaline but they do not mention whether this is critical or not.

4) Instrumentation.
a) Fluorometer and Filter System.

A Farrand Photoelectric Fluorometer, Model A, is employed in the measurement of fluorescence concerned in this work. Primary filters; Corning filters, numbers 3389 and 5113 are used to pass the exciting light of 436 mμ (approximately 12.5% transmission - range approximately 400-475 mμ). These monochromatic filters are used to isolate the desired emission line of the mercury spectrum which issues from the mercury vapor lamp employed. As suggested by Persky and Roston (118), secondary filters, Corning filters numbers 5433 and 3384, in combination, which pass a narrow band peaking in the region of 510 mμ (probably about 15% transmission), and Corning filter, number 2418, which cuts off all wavelengths below 600 mμ (having its greatest transmission above 600 mμ), are employed. The former combination of filters yields approximately equivalent fluorescence for adrenaline and noradrenaline in the present experimentation. The latter filter yields roughly 3 to 6 times as much fluorescence from the adrenaline derivative as

from the noradrenaline derivative. This is illustrated in the 'm' values of Tables I, IX, and X, and in the text table, page 68. To estimate differentially the fluorescent derivatives simultaneous linear equations are set up. Attempts were made to use Corning filter number 3486 which transmits light (80% efficient) of wavelengths from 557 to 750 mμ, however blank fluorescence prevented the establishment of the adrenaline standard to 100% while subtracting the reagent blank instrumentally, and so the use of this filter was dropped. The emitted light of the fluorescent substances is screened through the secondary filters to the desired wavelength and focussed on a phototube whose excitation is recorded on a Rubicon galvanometer.

b) Intensity of Incident Light

The diaphragm which passes the exciting light from the mercury lamp has six apertures of varying size. It is observed when adrenaline is employed as standard that fluctuations occur not only in the standard setting of approximately plus or minus 5 units on the scale of 100, but also in the reagent blank which is adjusted to 0 instrumentally. Repeated readings for this reason tend to vary in themselves, usually in the range of plus or minus 5 units especially when the shutter for the exciting light is held open for periods of 10 to 30 seconds in an attempt to set the machine with the fluctuating standard or reagent blank. It is also observable that the noradrenaline standard solutions rapidly lose their fluorescence value on the galvanometer if they are exposed too long to the excitant light. It is considered that the phototube may become fatigued on prolonged irradiation from the fluorescent solutions while the galvanometer does not respond quickly enough to the excitation of the phototube. It has been found that when the smallest aperture is reduced even more by the use of a piece of cardboard with a slit cut in it (placed in the filter carrier with the primary

filters) a comparative stability is achieved not only of the standard and reagent blank adjustments but of the standard solutions and samples. This reduced sensitivity slows the response of the galvanometer but may delay fatigue of the phototube. The use of the cardboard filter was kindly suggested by Mr. L. Rubinstein of this Institute.

c) Cuvettes and their carrier

It was frequently observed, in adjusting the fluorometer to 100 units on the galvanometer scale with a standard solution of adrenaline, that lower concentrations did not always fall into the linear scale expected, with respect to the standard. These lower concentrations followed a calibration curve which formed a plateau in respect to the standard. The cuvette carrier has three positions, one each, marked for the standard, reagent blank and sample, so that the machine is adjusted before each reading of the sample with the standard and blank. Standard 4 ml. pyrex test-tubes serve as cuvettes. These are screened by reading them filled with a standard fluorescein solution of about 0.02 µg. % (this dilution being in the region of plasma sample readings).

An arbitrarily selected tube filled with the same solution is used to set the galvanometer to 50 units. It was observed that every other test-tube gives a reading above 50; when another test-tube is selected arbitrarily and placed in the standard position in the cuvette carrier, it is observed that the former standard produces the greater deflection. On this basis, it is assumed that the three positions in the cuvette carrier of our fluorometer are not equivalent. This may also explain in part the lack of linearity of calibration curves of adrenaline with the concentration employed in the standard position.

5) Readings and Calculations
a) Calibration curves.

Aside from the experimental error incurred in the preliminery setting up of experiments, i.e, those involved in blood collection, chromotograph and condensation procedures, it appears necessary to assess those resulting from subjective and mechanical measurement. Assessment of slope values for calibration curves by the method of least squares is not always satisfactory. This method frequently yields calibration curves which cut the y-axis above or below zero, with a different intercept for adrenaline and noradrenaline. Considering that the reagent blank fluorescence has been cancelled instrumentally, these intercepts must be artefacts and represent a false blank fluorescence. Generally the plotted adrenaline slope quite obviously passes through the reagent blank estimation of 0 concentration of adrenaline when it has been subtracted instrumentally; the noradrenaline slope on the other hand is fairly unpredictable; sometimes it, also, is obviously linear running through 0 concentration although the various concentrations may yield scattered points, in which case, the intermediate slope is used in calculation. In some cases, the lower concentrations of noradrenaline appear linear, while the higher concentrations fall off considerably. The reason for this phenomenon is difficult to assess; it may be due to incomplete condensation to the fluorescent products, or to extraneous fluorescence which to be accounted for graphically would result in a very low slope for noradrenaline while an "extra blank fluorescence", due to incomplete conversion of noradrenaline to its fluorescent product or due to the production of some unknown fluorescent compounds, would cut across the y-axis and would have to be accounted for mathematically. This latter method did not improve the results of duplicate blood samples but led to greater variation; it was discarded.

b) Standard Substances.

In view of the comparative instability of the recorded readings for adrenaline and noradrenaline on the galvanometer, and of the frequent discrepancies between the adrenaline standard and the remainder of the adrenaline concentrations employed in the measurement of the adrenaline slope, the use of a standard solution of fluorescein sodium was investigated, as a replacement for adrenaline in the arbitrary adjustment of the galvanometer to 100. In this way, the discrepancy involved in the different positions of the cuvette carrier, as mentioned in 4) c), is overcome. Fluorescein is suitably diluted from a stock solution, to match approximately the fluorescence of the adrenaline standard or in the case where noradrenaline fluoresces maximumly at 510 mμ, the noradrenaline standard; this is the case for each wavelength. In the latter part of this reported experimentation, graded concentrations of both adrenaline and noradrenaline were replaced by the running of triplicate concentrations of each of the two amines, the averages of which were used in the estimation of slope values and ratios of fluorescence.

c) Variations in readings.

In the earlier part of this work the tendency was to take readings of samples very slowly; that is the galvanometer was adjusted to 100 and 0 with the standard and reagent blank respectively, this involving several manipulations at either end of the scale since a fluctuation of one resulted in a corresponding change in the other; when both were satisfactorily adjusted the sample was read, and the standard and blank re-checked, if they had shifted, the whole procedure was repeated until a reading was made that seemed satisfactory on all accounts. The shifting of the standard and blank positions

may well be due to changes in line voltage although a voltage stabilizer is in line. This procedure may also take in the area of ten to fifteen minutes per reading, and hence may result in fatiguing not only of the phototube, but also of the standard solutions. The average of duplicate readings was introduced as a means of improving on the above procedure, and has proved satisfactory, the variation between readings being generally not greater than plus or minus 5 units. The whole series of readings are made by adjusting the standard and blank instrumentally as before, before each reading, but the manipulations are performed much more rapidly so as to avoid undue fatigue, and then, second readings are made in the same order. In the earlier experiments, mentioned above, the standard employed was for the most part the adrenaline product. Frequently when the fluorescent products were determined directly after the condensation procedure, the fluorescence of the standards and samples behaved erratically. In such cases when the adrenaline standard was adjusted to 100% instrumentally, lower concentrations on the calibration curve demonstrated greater fluorescence and on re-checking the standard setting it was usually aberrant. These determinations were left overnight and read the next day. These vagaries have not been observed when fluorescein is employed as standard.

d) Importance of Accuracy in Fluorometer Readings

Weil-Malherbe and Bone (84) who employ the mean of duplicate readings in their calculation, report that their standard deviation is close to one half a division on the scale of 100 divisions, and that their single readings are accurate to approximately plus or minus one division; they also find that the error does not vary in different ranges of dial readings.

In the present work the standard deviation of single readings was calculated:

	Number of paired readings	S.D. of a single reading*
readings at 510 mμ.	146	± 6.20 mm.
readings at 600 mμ.	146	± 2.82 mm.
combined readings at both wavelengths.	292	± 4.81 mm.

* figures refer to divisions on the galvanometer scale of 100; readings are taken to 1/4 of a division.

It is of interest to know whether the accuracy of the readings is dependent upon the catecholamine in question since in actual practice noradrenaline appears to be the more unstable when it is excited with monochromatic light from the mercury lamp. The following table indicates that at 510 mμ, the noradrenaline derivative does indeed give a broader variation in duplicate readings, thus illustrating the instability of the fluorescence evoked;

	Number of paired readings	S.D. of a single reading*
Adrenaline readings at 510 mμ.	30	± 4.24 mm.
Adrenaline readings at 600 mμ.	30	± 3.52 mm.
Noradrenaline readings at 510 mμ.	30	± 12.12 mm.
Noradrenaline readings at 600 mμ.	30	± 1.93 mm.

* Figures refer to divisions on the galvanometer scale of 100; readings are taken to 1/4 of a division.

At 600 mμ, both fluorescent compounds reflect a higher degree of stability than at 510 mμ, in that the spread of duplicate readings is less and hence, a greater degree of confidence is accorded in the mean of the two readings.

6) Summary of Final Procedure Followed

12-20 ml. of blood is drawn from the arm vein into syringe containing 5 ml. of an anti-coagulant preservative solution - $Na_2S_2O_3$:NaFl (3%:2%). The volume is noted, and the plasma separated by centrifugation, after which it is divided into two portions for a mean determination of the content.

The plasma is combined with an equal volume of 0.2 M sodium acetate buffer previously de-mineralized by passing through a cationic exchange resin column.

The plasma - buffer mixture is adjusted to pH 8.4 by the aid of a glass electrode and employing 0.5 N sodium carbonate for the adjustment. It is immediately passed through a column of alumina, previously prepared with 0.2 M sodium acetate buffer pH 8.4. Filtration is allowed to proceed at a rate of 20-30 drops per minute and is regulated by the use of positive pressure (see section 2b). The plasma - acetate mixture is washed through the column with 5 ml. of the buffer and 5 ml. of glass distilled water; a glass-stoppered centrifuge tube is then placed under the column to collect the eluate from the column which is eluted with 5 ml. 0.2 N acetic acid and 5 ml. of glass distilled water.

The eluates are now ready for the condensation procedure. At this point, standard solutions of adrenaline and noradrenaline are prepared in glass distilled water, a concentration of 0.2 µg. of adrenaline or noradrenaline per 10 ml; these standards are run in triplicate along with the eluates. 0.5 ml. of 2 M ethylene diamine dihydrochloride and 0.7 ml. of freshly distilled ethylene diamine, both saturated with oxygen for 10 to 15 minutes, are added to the solutions, and the tubes are shaken briefly to ensure homogeneity. The tubes are then placed in a rack and the whole incubated in a water bath at

50°C. for twenty minutes. At the end of this period they are cooled to room temperature by placing them in a cold water bath.

About 4 grams of solid sodium chloride is added to each test-tube, followed by 6 ml. of isobutanol from a burette. The test tubes are then stoppered, and shaken in a mechanical shaker for four or five minutes to ensure extraction. The emulsion formed (especially in the plasma eluates) is broken by centrifuging at 2500 r.p.m. for 15 minutes.

About four ml. of the isobutanol layer is removed by means of an automatic pipette and transferred to the test-tube cuvette. A standard solution of fluorescein sodium is diluted from a stock solution containing 10 mg. per 100 ml.; the first dilution is 1:100, giving a concentration of 0.1 mg./100 ml.

The resulting solution is diluted to about 2.5 µg.% in order to match approximately the fluorescence of the standard adrenaline or noradrenaline derivative at 510 mµ. The fluorometer is turned on during this period and left 10 to 20 minutes to warm up, as fluctuations may occur shortly after the lamp is turned on. The standards and samples are read twice at the one wavelength and then a new fluorescein standard is prepared to match the fluorescence of the standard adrenaline derivative at 600 mµ. This fluorescein solution is approximately 4 µg.%. The standards and samples are then read twice at this wavelength.

The mean slopes for adrenaline and noradrenaline, estimated from standards run in triplicate and read twice, at each wavelength, and the fluorescence ratios of adrenaline/noradrenaline are calculated. The equations are set up after Weil-Malherbe and Bone (63,115) as following:

m = ratio of adrenaline/noradrenaline fluorescence at 600 mµ.
n = ratio of adrenaline/ noradrenaline fluorescence at 510 mµ.

A = amount of adrenaline present in the mixture
N = amount of noradrenaline present in the mixture

y - amount of adrenaline corresponding to the fluorescence measured at 600 mμ.
b - amount of adrenaline corresponding to the fluorescence measured at 510 mμ.

The two following equations may be set up;

1) $A + \frac{N}{m} = y$

2) $A + \frac{N}{n} = b$

and therefore,

3) $N = mn \frac{(b-y)}{(m-n)}$

4) $A = y - \frac{N}{n} = b - \frac{N}{m}$

These calculations give the concentrations found in the sample of plasma taken for the estimation. This is corrected for the volume of blood taken and then modified to give the concentration in μg. in plasma/litre of whole blood.

A copy of the data sheet which was employed in these determinations will be found in appendix I.

B. Clinical Studies

1) Determinations on normal and clinical plasmas

Recovery experiments were attempted with blood samples obtained from volunteers working in the hospital. The plasmas from these samples were divided fairly evenly (see table below) for duplicate determination of their content of catecholamines. Adrenaline and noradrenaline were added, in anounts approximately equivalent to the content of i.e. a 15 ml. blood sample of plasma, to one aliquot of the divided plasma sample. A volume of isotonic sodium chloride equivalent to the volume of added adrenaline and noradrenaline, was added to the second aliquot of the divided plasma sample. In one experiment, adrenaline was recovered in two cases out of three, however, noradrenaline was not recovered quantitatively:

Volume of plasma sample (divided per case)	µg. A/sample	µg.NA/sample	Recovery of added adrenaline and noradrenaline µg.A/sample	µg.NA/sample
Case I				
16.1 ml.*	0.014	0.049	-	-
16.5 ml.**	0.027	0.100	0.013	0.051
Case II				
17.2 ml.*	0.011	0.065	-	-
16.3 ml.**	0.037	0.088	0.026	0.023
Case III				
14.1 ml*	0.024	0.057	-	-
13.8 ml**	0.040	0.064	0.016	0.007

* To these sample of plasma were added isotonic sodium chloride
** 0.02 µg. adrenaline and 0.08 µg. of noradrenaline were added to these samples.

Other experiments in which adrenaline and noradrenaline were added to one aliquot of a duplicate sample of plasma, resulted in a positive increase of fluorescence but numerically the results were incalculable; in general this was due to the instability of the standard solutions involved in the estimation of slope values. Owing to the variation between duplicate estimations, recovery experiments were dropped until such time that a degree of confidence is attained in these duplicate estimations. In view of the variable factors in the procedure, blood samples are estimated in duplicate and the mean taken as the estimated value.

The concentration of adrenaline and noradrenaline has been estimated in the blood plasma of 34 males and 17 females. These subjects include normal healthy persons working in our laboratories and in the hospital, suspected phaeochromocytoma patients who were not actually suffering from that disorder, and a few psychiatric patients not on medication. The results are shown in Table XI.

TABLE XI: Determination of adrenaline and noradrenaline in human blood plasma (mean values ± standard error)

Subjects	n*	Adrenaline µg./litre blood	Noradrenaline µg./litre blood
Normal males	17	1.70 ± 0.28	4.22 ± 0.47
Normal females	7	1.74 ± 0.54	4.78 ± 1.33
Male patients	17	1.57 ± 0.17	3.54 ± 0.48
Female patients	10	1.37 ± 0.53	5.38 ± 1.81
All males	34	1.63 ± 0.16	3.88 ± 0.33
All females	17	1.56 ± 0.30	5.08 ± 0.88

n* - number of determinations

2) The Measurement of Adrenaline and Noradrenaline during the Course of Mecholyl Tests

Mecholyl tests were carried out on a group of psychiatric patients with various diagnoses. These tests were performed by Dr. R.B. Sloane, who, during the course of this testing, drew blood samples for the estimation of adrenaline and noradrenaline by the method of Weil-Malherbe and Bone (63).

Patients subjected to this test had fasted for 12 hours and were in a resting state for at least one half hour preceding the test. The blood pressure was recorded every minute for a minimum of fifteen minutes after the rest period until a satisfactory resting blood pressure, of three consecutive readings varying within a range of 6 mm. of mercury, was recorded. At this resting point, the first sample of blood was withdrawn and 10 mg. of methacholine chloride were injected intramuscularly. Blood pressure was recorded continuously for 25 minutes. Blood samples were drawn 2 to 3 minutes after the injection, during the hypotensive reaction to the drug, and 10 to 25 minutes after the injection, during which time the blood pressure would be expected to have returned to normal.

The following table gives the values of adrenaline and noradrenaline estimated in the plasma from samples of blood drawn during the course of a number of these tests. The first six sets of values were made on single samples of plasma. The later sets were estimated from single samples of plasma which were divided for duplicate estimation. The division of the sample of plasma was disproportionate, and hence, the values at two different plasma concentrations vary as shown in Table XII. For this reason, both estimated values are given in the following table under the headings Low and High, to indicate that the higher values are, as to be expected from the experiments of Table V, associated with the lower plasma volumes.

TABLE XII: Adrenaline and Noradrenaline Levels estimated in the plasma from blood samples drawn during the course of Mecholyl tests:- 10 mg. methacholine chloride injected intramuscularly; blood drawn from an arm vein at the times indicated, subjected to estimation according to the method of Weil-Malherbe and Bone.

Subject	Sampling Time. (Mins.)	µg. A./L. blood		µg.NA/L. Blood	
		Low [a]	High [b]	Low [a]	High [b]
1	0		0.11		4.05
	7		0.57		2.81
	20		0.39		1.84
1	0		0.30		1.05
	7		0.89		3.23
	20		0.89		2.76
2	0		0.78		2.21
	3		0.06		2.92
	15		0.94		0.36
2	0		0.53		2.74
	3		1.04		0.01
	15		1.07		1.41
3	0		2.70		2.01
	2		1.23		4.06
	15		2.30		5.15
4	0		1.42		6.05
	2.5		1.57		5.36
	11		0.88		4.67
5	0	-0.84	0.88	15.30	5.21
	2.5	0.40	0.55	14.48	4.73
	16	1.81	1.21*	5.16	3.22*
6	0	1.34	1.58	5.59	3.86
	2-3	3.11	0.78	3.84	5.61
	15	1.05	1.12	4.65	2.70
7	0	2.18	0.23	1.63	2.01
	2	1.59	0.97	2.14	1.30
	13	1.58	0.98	2.63	1.74
8	0	1.40	0.93*	8.20	5.11*
	2	2.08	1.08	3.60	3.87
	10	1.43	1.21	4.89	3.27
9	0	1.97	1.37	3.90	2.47
	2	2.09	1.51	4.40	4.09
	22	1.49	0.68	4.93	3.17
10	0	2.60	1.09	1.83	1.83
	2	2.65	1.43	2.32	1.97
	20	0.73	0.75	2.43	1.71

Table XII cont'd.:

Subject	Sampling Time (Mins.)	µg. A./L. Blood		µg. NA/L. Blood	
		Low [a]	High [b]	Low [a]	High [b]
11	0	1.47	1.11	5.18	3.06
	2	2.49	1.82	3.13	2.34
	25	1.52	1.51	3.41	2.36
12	0	1.52	0.89	1.68	1.99
	2	1.58	1.13	1.01	1.37
	22	2.13	0.81	1.64	1.39

a - refers to the lower volumes of plasma taken for the estimation
b - refers to the higher volumes of plasma taken for the estimation of adrenaline and noradrenaline.
* - these are estimated values of a concentration expected if the plasma had been divided disproportionately as in the other samples of the same set. The actual sample was lost during the procedure.

Examination of Table XII shows that there is a variety of patterns of sympathetic response to Mecholyl in these patients, based on these estimations. It is of interest therefore to analyse some of the preceding data statistically, to see if any significant differences exist between the subjects and their reactions to Mecholyl during the test.

Seven sets of data from Table XII were taken for analysis of variance. The plasmas, determined in duplicate, from seven subjects were determined three times during the course of a Mecholyl test for their content of adrenaline and noradrenaline. This provided 42 data which are analysed statistically in Table XIII.

TABLE XIII: Analysis of Variance of Adrenaline and Noradrenaline Estimations During Mecholyl Testing.

Sources of Variation	DF	Adrenaline Sum of Squares	Mean Square	F Ratio	Noradrenaline Sum of Squares	Mean Square	F Ratio	
Total	41	20.6980			331.9818			
Subjects (7) "S"	6	3.9126	0.6521		147.3214	24.5536	8.358	HS
Times (3) - Effect of Mecholyl "T" 2	2	1.3701	0.6851		11.7767	5.8884	2.004	HS
Volumes of blood used (2) "V"	1	3.0671	3.0671	7.09	36,2329	36.2329	12.333	HS
S x T	12	5.2411	0.4308		44.0179	3.6682	1.249	HS
S x V	6	2.0969	0.3495		59.2986	9.8831	3.364	S
T x V	2	0.6843	0.3422		3.9565	1.9783	6.734	S
Missing Values	2	-	-		-	-		
S x T x V, for error determination	10	4.3259	0.4326	(1.0)	29.3778	2.9378	(1.0)	

Assessing the data in Table XIII it is noticed that the difference between the subjects ("S") chosen are highly significant for the estimation of noradrenaline, but not in the case of adrenaline estimation. This might be expected if the response to Mecholyl was primarily a post-ganglionic sympathetic response. The difference between the times ("T") at which the samples were drawn, i.e., the effect of Mecholyl, is not significant in either case. The volume ("V") of blood chosen for the estimation, as is expected, is highly significant in the case of noradrenaline and significant in adrenaline estimation. The interaction of subjects and the effect of Mecholyl (S x T) is not significantly different in either estimation, which might be expected if these subjects reacted similarly.

The interactions, of subjects and the volumes taken for the estimation (S x V), and times (or the effect of Mecholyl) and the volumes taken (T x V) are not significant in the case of adrenaline estimation. In the estimation of noradrenaline, however, both interactions are significant. This means that in the interaction, subjects and plasma volumes taken for the estimation (S x V), that is, the effect of the volumes taken (large or small) is not qualitatively or quantitatively consistent through all the subjects. Moreover, in the interaction, time of sampling on the volume chosen for the estimation of noradrenaline (T x V), it may be concluded that the effect of Mecholyl observed is dependent upon the volume taken in the estimation. The times (or the effect of Mecholyl) are not significant; judging from the following table it is possible that a drop in the noradrenaline level, due to Mecholyl, during the test may be detectable when lower volumes of plasma are taken for estimation:

Times of Sampling (minutes)	Mean (7) noradren. estim. with low vols.	Mean (7) noradren. estim. with high vols.
0	5.95	3.22
2-3	4.70	3.42
15-25	4.01	2.60

Further experiments are necessary to ascertain this.

III

D I S C U S S I O N

The fluorimetric procedure for the estimation of adrenaline and noradrenaline in the plasma of human blood has been given in a preceding section of this thesis. The mean level for males has been found to be 1.63 µg. of adrenaline and 3.88 µg of noradrenaline per litre of blood; while for females, the mean level has been found as 1.56 µg. of adrenaline and 5.08 µg. of noradrenaline per litre of blood. These levels are similar to those found by other authors. The mean levels in our procedure are in the same range as the earlier levels reported by Weil-Malherbe and Bone, although the standard error is larger probably due to certain incongruities encountered in the procedure. The levels reported by Weil-Malherbe and Bone (63), and illustrated in the text table of page 16, were subsequently reported to be lower (115). In the latter publication, the authors found that from a larger series of analyses the mean plasma noradrenaline concentration is approximately 4.5 µg. per litre of blood. These authors, on the basis of a plasma volume of 55% of blood, give the concentrations of adrenaline and noradrenaline in plasma as 0.015 µM. and 0.048 µM.

respectively. Certain subtle changes in the method over a large series of determinations are no doubt reflected in the mean levels attained.

In a recent communication from Weil-Malherbe (64), he reported levels of 0.5 to 1.0 µg. of adrenaline and 2.5 to 3.5 µg. of noradrenaline per litre of blood. These latter values were obtained after a series of determinations had been made employing an isotonic solution of ethylenediaminetetraacetate and sodium thiosulphate as the preservative solution for blood collection. In a preceding paper (73), the authors claimed that the use of this preservative solution led to identical estimations of the catecholamines as were estimated when the fluoride-thiosulphate solution was employed as preservative. However, it may have been that when a larger series of estimations was performed, the mean levels were noticeably lower. The levels reported in this thesis have all been estimated employing the fluoride-thiosulphate solution as preservative. The usefulness of the metal chelator bears further consideration however.

The original intention in the setting up of this study was to observe whether any changes in the levels of catecholamines in the blood plasm could be observed during the course of treatment of psychiatric patients in this Institute. The procedure for estimation, however, is beset with deviations which for analysis of

subtle changes in the circulating levels, must be refined further.

Methodological studies have been performed in order to determine what factors play significant roles in the variations incurred in the measurement of the catecholamines in blood plasma. It has been found that stock solutions of adrenaline and noradrenaline, stored in acid solution and frozen, deteriorate in a matter of weeks in regard to the condensation product formed and used as the standard for the fluorimetric estimation of the catecholamines in plasma. Refrigeration of the stock adrenaline and noradrenaline in acid solution has been found in the present work to be the more satisfactory condition; the error incurred by the deterioration of the stock solutions can be minimized by preparing fresh stock solutions in acid about once in a fortnight and storing them in a refrigerator.

A salient point of the procedure was found to be the oxygenation of the reagents, ethylene diamine and ethylene diamine dihydrochloride, employed in the condensation of adrenaline and noradrenaline to their fluorescent products. This treatment was found to improve the linearity of calibration curves for noradrenaline considerably. It is assumed that in other laboratories (84,113) the reagents are sufficiently saturated with atmospheric oxygen to cause complete oxidation of the standard solutions under the experimental

conditions used, and that the linearity of the noradrenaline calibration curves is satisfactory enough to assure confidence in the complete oxidation of noradrenaline in the sample determinations.

Another aspect of the method was examined; that of increasing the heating time of the condensation procedure at 50° C. from 20 minutes to 2 hours, as recommended by Erne and Canbäck (120) to bring the formation of the noradrenaline derivative to its peak plateau. However, this did not increase the reproducibility of duplicate determinations and so the 20 minute heating period was retained. The stability of the condensation products is assumed to be constant for a period of at least 24 hours (84); this has been confirmed in that the products do not lose their ability to fluoresce but increase slightly in their absolute fluorescence (i.e. their fluorescence in relation to fluorescein) when left overnight.

It has been found that the measurement of fluorescence is an undulating phenomenon; initial experiments were improved in confidence by the use of a fluorescein standard which maintained a fairly constant degree of fluorescence intensity in comparison with the use of a standard adrenaline derivative solution for the repetitive adjustments of the standard position on the galvanometer scale. Part of this instability is attributed to fatiguing of the phototube when it is bombarded with light of high intensity and the slow response of the galvanometer to record this energy. This is eliminated in part by reducing the slit width for the excitant light. Weil-Malherbe, in a personal communication (64), mentioned that fluctuations of light due

to flicker of the mercury arc rather than voltage changes troubled their readings at one time. This was minimized by running the mercury lamp somewhat below its intended maximum brightness. The mercury arc, instead of being very thin and constricted, then burns with a rather diffuse and stable arc. Mr.L. Rubinstein, of the A.M.I., has suggested that the latter effect may be achieved by the insertion of a frosted glass plate in the primary filter case. A Variac may be installed in the line to reduce the mercury lamp output. Experiments have not as yet been performed employing the latter conditions.

When plasma determinations are made, it has been found that the volume taken for the estimation is an important factor on the apparent level determined. Thus it has been found that volumes above 10 ml. of plasma give lower values than a duplicate sample of 6 ml. It is assumed that a plateau may occur in the region of 6 to 10 ml. where the highest values for the plasma content may be determined. Further experimentation is necessary to ascertain this. Weil-Malherbe (64), on the other hand, has not experienced this effect. He found that concentrations of catecholamines extracted from increasing volumes of plasma on identical alumina columns showed a perfectly linear increase up to a total volume of 400 ml. of the plasma-buffer mixture. Earlier experiments from this laboratory (116, 64) have shown that a column of 0.5 g. of alumina adsorbs

up to 80 µg. of adrenaline completely, and that over 90% can be recovered on elution; the usual size column (presumably containing 0.7 g. of alumina (84))can absorb several hundred µg. of catecholamines.

No complete explanation seems to account for the large standard errors incurred in the present procedure. Several possibilities can be explored.

1) Merck alumina employed in the present procedure may differ in adsorption properties from that of the British Drug Houses Ltd. (84); however, if these differences do exist it is unlikely that they are very large. At the time experiments on comparison of aluminas were done, B.D.H. alumina was not available. This point will be investigated.

2) Incomplete condensation of adrenaline and noradrenaline may occur as a result of hydration of the alumina during the adsorption and washing procedure on the basis of the following interpretation. Von Euler and Orwén (55) state:

> "The adsorption on aluminum oxide in alkaline medium (pH 8.5) probably depends on the formation of a coating of aluminum hydroxide on the surface of the aluminum oxide grains. During elution this layer is dissolved in acid together with the catechol compounds adsorbed, leaving the "carrier" grains behind, thus ensuring easy filtration."

It is conceivable that this small amount of alumina, varying in amount from one determination to another, may readsorb the catecholamines in the alkaline solution of the condensation reaction. This could account for the anomalous results of duplicate

volume estimations, and may be related as well to the following point.

3) Differences in particle size of the aluminas used may account for the different adsorption properties. The size of the Merck alumina particles varies from about 100 to 200 mesh. Weil-Malherbe and Bone (84) after acid-washing of the alumina, employ repeated washing of the acid-treated alumina with distilled water by decantation in their procedure. A substantial amount of the 'fines' would be lost in this step and the larger particles would be retained. Merck alumina "acid-washed" which was further acid-washed in our laboratory (see Table IV) does not give evidence of this 'possible interpretation' as the acid-washed alumina was centrifuged, throwing down the 'fines' as well as the heavier particles. The significance of the alumina particle size on its adsorptive properties warrants further investigation. It was observed that when plasma was stored frozen or refrigerated, a separation of some unidentified material, probably fibrin or a mucoprotein, occurs on thawing. It is possible that this protein may adsorb some of the catecholamines. None of these interpretations serves to explain the gross discrepancies observed however, and until a satisfactory explanation is achieved, plasma volumes in the range of 6 to 10 ml., as mentioned previously, yield maximum recovery of the plasma content. The use of the higher volumes is to be considered nevertheless, as the concentrations contained lie in a more discriminate range for their fluorimetric determination.

The matter of specificity of the method has not been dealt with in this laboratory as yet. This specifity has been questioned by a number of investigators, but these objections have been rebutted on several occasions by Weil-Malherbe.

Mecholyl tests, which have been described in the preceding section, showed no consistent pattern in the changes of catecholamines with time. However, statistical treatment of the data showed that although apparent adrenaline levels were not affected by Mecholyl, certain treatment combinations affect the circulating levels of noradrenaline. Thus, the effect of the blood volumes on the "Apparent noradrenaline" levels was not consistent for all the subjects. Although the effect of Mecholyl is not significant in the case of noradrenaline, the interaction of "T x V" (as seen in Table XIII) is significant at the 5% level. Hence, the effect of Mecholyl could be assessed differently depending upon whether small or large blood sample volumes were taken for the estimation. These suggestive results warrant further determinations of Mecholyl tests, especially in view of the effect of volume size on the estimation, and in consideration of the large standard error involved.

IV

SUMMARY

The fluorimetric procedure of Weil-Malherbe and Bone (84,63), for the estimation of adrenaline and noradrenaline in blood plasma, based upon the condensation of the catecholamines with ethylene diamine to yield stable fluorescent derivatives, has been studied methodologically.

The stability of stock solutions of adrenaline and noradrenaline has been studied with respect to the fluorescent derivatives formed in the procedure. The use of fresh stock solutions, i.e. made up fresh fortnightly, has been obviated. Saturation of the reagents, employed in the condensation procedure, with oxygen has been found to assure satisfactory oxidation of noradrenaline to its fluorescent derivative. The use of a stable fluorescing standard, i.e. fluorescein, has been found expedient for repetitive adjustments of the galvanometer scale on the fluorometer to a standard setting. Comparatively stable readings of the fluorescent products are achieved by an empirically determined reduction in the intensity of the incident light. The volume of blood taken for the estimation has been found to be of critical importance in the determination of the apparent content of adrenaline and noradrenaline.

The mean levels of adrenaline and noradrenaline in the blood plasma of 34 males and 17 females have been determined. Thus, for males, the levels are 1.63 µg. of adrenaline and 3.88 µg. of noradrenaline per litre of blood, and for females, 1.56 µg. of adrenaline and 5.08 µg. of noradrenaline per litre of blood. These values have been compared with others in the literature.

The changes in the levels of adrenaline and noradrenaline during the course of Mecholyl tests have been determined in twelve patients. Some

of these results have been analysed statistically. Mecholyl had no significant effect on the adrenaline levels, but the noradrenaline levels showed highly significant differences between subjects; as well, the effect of the blood volume chosen for the estimation of noradrenaline was not consistent for all subjects.

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APPENDIX I

CATECHOLAMINES

Ref.

Condensation Time: Time of Reading: Date:

	SLOPES		RATIOS	CONSTANTS:
	A	N	A/N	
510 mμ				1/m =
				mn =
600 mμ				m - n =
				$\frac{mn}{m-n}$ =

$$N = \frac{mn(b-y)}{m-n}$$

$$A = y - \frac{N}{m}$$

Tube No.	R_{510}	b	R_{600}	y	b - y	N	N/m	A	Blood Volume	μg. per litre	
										A	N
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											

APPENDIX II

Plasma adrenaline and noradrenaline were determined in consecutive subjects. The means of these determinations are given in Table XI.

All concentrations are given in µg. per litre of whole blood.

MALE				FEMALE			
Normals		Patients (A.M.I.)		Normals		Patients (A.M.I.)	
A	NA	A	NA	A	NA	A	NA
0.56	7.44	0.71	1.94	2.97	0.25	1.26	2.60
0.30	5.95	1.46	0.87	0.11	7.52	1.84	4.04
1.18	4.91	1.16	1.90	0.33	5.68	1.42	6.04
1.39	0.33	1.25	5.30	1.06	0.49	0.20	7.23
1.28	3.63	1.42	1.57	4.16	3.86	-0.84	15.30
1.82	2.76	3.21	2.29	1.87	5.80	1.34	5.59
4.09	1.97	2.70	2.01	1.65	9.84	2.18	1.63
2.02	2.70	0.84	5.25			1.23	2.11
0.73	4.11	0.98	4.02			3.57	4.09
0.80	6.94	1.91	6.24			1.47	5.18
0.94	2.88	1.63	3.13				
0.75	3.59	1.40	8.20				
3.41	7.58	1.22	5.30				
1.38	3.92	1.97	3.90				
2.23	4.17	0.85	3.83				
3.92	4.52	1.40	2.68				
2.02	4.40	2.60	1.83				