

THE IDENTIFICATION OF SUBSTANCES RELEASED BY

ADRENERGIC NERVES.

Studies on sympathin and the  
adrenal medullary hormones

by

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Thesis presented for the degree of Ph.D.,  
University of Edinburgh.

November, 1951.



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Part I.

The historical background.

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## 1.

### I. The adrenal glands.

Attention has been paid to these organs for nearly a hundred years. It was found that certain staining reactions were given by the medulla only and not by the cortex with ferric chloride (Vulpian, 1856), potassium bichromate (Henle, 1865) and basic dyes like haematoxylin (Srdinko, 1905). As a result, the reacting cells were designated "chromaffin" or "phaeochrome" cells (Poll, 1903). Vulpian also showed that the colour reaction was given by blood leaving the adrenals but not by blood entering the glands. This was probably the first experimental demonstration of internal secretion.

The earliest reported biological experiments were those of Pellecani (1879) and Foà and Pellecani, (1884), who injected an aqueous extract of six ox adrenals intravenously into a rabbit with fatal results. Oliver and Schäfer, (1895) injected extracts of whole adrenals intravenously into dogs and produced a rise of blood pressure, increased heart rate, constriction of arterioles, temporary shallowing of respiration, contraction of the spleen, dilatation of the iris and inhibition of the intestine and bladder. These effects were obtained also with/

with extracts of the separated medulla but not of the cortex. Independent reports of similar results were made by Cybulski (1895) - Szymonowicz (1895), who showed that blood from the adrenal vein contained the active principle. These effects were striking because no such powerfully pressor substance was known at the time.

Intimate connection was believed to exist between the blood pressure raising principle of the medulla and the colour - reacting cells (i.e., those having an affinity for chromate), (Moore, 1895).

#### Adrenaline.

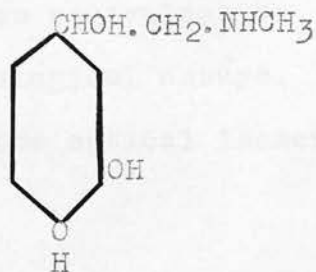
The first hormone to be isolated was from the adrenal medulla. This was achieved by Abel and Crawford (1897), in the form of a benzoyl derivative which they named "epinephrine hydrate". Takamine, (1901a and b) however, isolated the pure base from sheep and ox adrenals as a light, white, micro-crystalline body, stable when dry, sparingly soluble in water and a powerful reducing agent, giving a red colour with iodine and a green with ferric chloride. He named it "adrenalin" and assigned to it the empirical formula  $C_{10}H_{15}NO_3$ .  
Many/

Many other names have been suggested but those given above are still in general use. The true empirical formula of adrenaline is  $C_8H_{13}NO_3$  (Aldrich, 1901).

Determination of the structural formula soon followed. The similarity of a colour reaction given by adrenal extracts and by catechol had been mentioned by Krukenberg, (1885) while Takamine (*loc. cit.*) found that alkaline hydrolysis of adrenaline yielded catechol and catechuic acid. The presence of a catechol nucleus was also suggested by von Fürth (1903), who observed that the molecule contained a methylamine group. The presence of an asymmetric carbon atom was discovered by Pauly (1903) who also suggested possible side chains for the catechol nucleus and showed that the form present in the adrenals was the laevo-isomer. The final elucidation of the formula (Jowett, 1904) was based on the examination of oxidation products. The original base was shown to have the groups



and  $-NH \cdot CH_3$



and the most probable formula was  
Synthesis/

Synthesis and optical isomerism.

Adrenaline was synthesised independently by Stolz (1904), and Dakin (1905a). The synthetic product was shown to be the racemic form and its resolution into the d- and l- optical isomers was accomplished by Flächer (1908), who found too, that the l- form had the same activity as natural adrenaline. These workers also synthesised racemic noradrenaline (Stolz and Flächer, 1906, Dakin, 1905b), but many years were to elapse before this amine was resolved into its optical isomers (Tullar, 1948). The relative biological activity of the optical isomers is of practical importance when comparisons with standard solutions have to be made. Synthetic (dl-) adrenaline was first compared with natural (l-) adrenaline by Cushny (1908, 1909), but many others have determined the ratio of doses for equal action since. The pressor activity of the d- form has been found to be from 1/40 to 1/15 of that of the l- form. The activity of the l- form could therefore be taken as equivalent to twice that of the dl- form for biological assays. Similar relations were found for the optical isomers of/



of noradrenaline (Tainter, Tullar and Ludueña, 1948), when they had been resolved. The belief that a given weight of dl - noradrenaline corresponded to half that weight of the l- isomer was vindicated.

#### Noradrenaline in the adrenal medulla.

Although the parallel problem of nerve sympathin had been pursued more vigorously the finger of suspicion did point to the presence of the non-methylated amine in the adrenal medulla.

Brown, McSwiney and Wadge (1930), observed the effects of stimulation of the thoracic sympathetic trunk in decerebrate cats and dogs on stomach movements. Depending on the nature of the stimulus the result was either contraction or inhibition. The contraction was abolished by ergotoxine. Injected adrenaline however, always caused inhibition. Such experiments were quite inconclusive owing to the wide scope of distribution of the thoracic sympathetic chain, but could be useful for the investigation of nerve sympathin in adrenalectomised animals.

Annau, Huszak, Svirbely and Szent-Györgyi (1932), prepared extracts of ox adrenals and estimated the adrenaline colorimetrically by several tests. They then compared the pressor effects of the extracts with/

with equivalent amounts of adrenaline on decapitated cats. They found that the extracts had greater pressor action than the equivalent amounts of adrenaline. Tested on isolated rabbit's intestine the extracts had a greater inhibitory action as well. They considered that the extracts contained some substance more potent than adrenaline and suggested that it be called "novadrenine".

These experiments were repeated by Schild (1933), and Euler (1933a) but not entirely confirmed. The discrepancies were accounted for by errors in the chemical tests, but Schild did find a small discrepancy which could not be accounted for.

Schümann (1948), injected extracts of pig adrenals into rabbits and produced less hyperglycaemia than equi-pressor amounts of adrenaline. The amount of hyperglycaemia could be equated to that produced by a mixture of adrenaline and noradrenaline in equal proportions. Holtz and Schümann (1948) found that the hyperglycaemia produced by a dialysate of cattle adrenals corresponded to a mixture of three parts of adrenaline with one of noradrenaline. (Schümann (1949), showed that the ratio of equally active doses of l-noradrenaline/l-adrenaline for producing hyperglycaemia in rabbits is about ten.)

Holton (1949<sub>a</sub>), investigated a portion of a human/

human suprarenal tumour. This was a phaeochromocytoma which consists of chromaffin tissue. Acid extracts were tested on the rat's uterus and the frog's perfused heart (both of which are sensitive to adrenaline) and on the isolated rabbit's duodenum and the spinal cat's blood pressure (which are equally sensitive to adrenaline and to noradrenaline).

About twice as much noradrenaline as adrenaline was found. This was confirmed by two other tests, incubation with a polyphenolase (colour reactions for both adrenaline and noradrenaline being given) and by paper chromatography with phenol as solvent. Both these tests had been described by James (1948). The paper method did not show the presence of significant amounts of any other amines.

Bülbring and Burn (1949a) carried out similar experiments on extracts of normal dog's adrenals and found that the percentage methylation varied but could be as low as 50%.

Euler and Hamberg (1949b) tested extracts of cattle adrenals on the cat's blood pressure (with the assistance of the adrenergic blocking agent F933) and chemically by a colorimetric method using the formation of iodochromes and by paper chromatography. The extracts were found to contain both adrenaline and/

and noradrenaline.

Goldenberg, Faber, Alston and Chargaff (1949), applied the paper chromatographic method to extracts of phaeochromocytomata and to U.S.P. "adrenaline" and U.S.P. reference standard, both of which are prepared from cattle adrenals. The method was usable quantitatively because it had been shown (Fisher, Parsons and Morrison, 1948) that under controlled conditions the areas of the spots after development of the chromatogram were linearly related to and depended upon the log. concentrations of the amines applied originally. The "adrenalines" were found to be from 82% to 100% methylated, but one sample had only 64% methylation. The tumours had even lower values, one being only 10% methylated. Similar results were obtained by Auerbach and Angell (1949), for U.S.P. reference standard using a quantitative colorimetric method with sodium  $\beta$ -naphthoquinone-4-sulphonate and benzalkonium chloride.

The isolation of pure l-noradrenaline from these sources was the obvious sequel. It was accomplished by Tullar (1949) who had previously (1948) resolved racemic noradrenaline into its optical isomers by a method depending on the different solubilities of the  
d-/

d- and l- bitartrates in methanol. This difference is also shown by d- and l- adrenaline. The fortunate circumstances that l- adrenaline behaves like d-noradrenaline and that both l- forms occur naturally, enabled Tullar to apply his resolution method to the separation of l-noradrenaline from l-adrenaline in the samples.

Bergström, Euler and Hamberg (1949), 1950), also isolated noradrenaline from cattle adrenals by counter-current extraction using dil. HCl and phenol as the two phases.

#### Liberation of noradrenaline from the adrenals.

The first suggestion that noradrenaline might be liberated from the adrenals was made by Meier and Bein (1948). They showed that adrenaline injected into dogs caused a rise of blood pressure and an increased blood flow through the femoral artery (i.e., that vasodilatation occurred in the hind limbs). After adrenalectomy the same animal responded to adrenaline with a rise of blood pressure but with decreased blood flow through the femoral artery (i.e., vasoconstriction/<sup>now</sup>occurred). The increased/

increased blood flow through the femoral artery could be restored if the animal was infused with noradrenaline in an amount too small to effect the blood pressure before adrenaline was administered. This pointed to the fact of a constant liberation of a small amount of noradrenaline from the adrenals of the dog which was responsible for the usually seen peripheral vascular effect of adrenaline.

Holtz and Schümann (1949) showed that noradrenaline was apparently released with the discharge of adrenal medullary hormones when clamping of the carotid sinus caused a rise of blood pressure. Their evidence was chiefly the contraction of the spleen which occurred simultaneously and was abolished by adrenalectomy. This is probably erroneous because Driver and Vogt (1950), have shown that the adrenal glands are not themselves responsible for the carotid sinus reflex, and that contraction of the spleen is not abolished by adrenalectomy if that operation be carried out without damaging the splenic nerves.

Bülbring and Burn (1949b) stimulated the splanchnic/

splanchnic nerves of the spinal eviscerated cat and estimated the amines in the venous outflow from the adrenals by a method involving the simultaneous recording of the contractions of the denervated and normal nictitating membranes of the cat. The ratio of the heights of contraction varies according to whether pure adrenaline, pure noradrenaline or a mixture of the two amines is injected. The effect of the venous effluent samples was between that of adrenaline and noradrenaline, but could be matched by injecting a mixture of the two. The methylation of the samples varied from 20% to 80%. On prolonged splanchnic nerve stimulation the methylation, high at first, fell steadily to a low value. This change in the percentage methylation did not occur in cats fed for six/<sup>to</sup>ten days previously with methionine.

Bülbring and Burn (1949c) perfused the dog's isolated adrenal with blood. Noradrenaline was added to the perfusing fluid. Estimates of the amines in the outflow were made by assays with the rabbit's intestine and the rat's uterus. There was some correspondence between the gain in adrenaline and the loss in noradrenaline added to the perfusing fluid. The methylation was apparently increased if the glands had been depleted by prolonged (one hour) splanchnic/

splanchnic nerve stimulation. These experiments were in continuation of those of Bülbring, Burn and de Elio (1948), who had shown that adrenaline added similarly to the perfusing fluid modified the medullary hormone in the outflow from the gland. The evidence indicated that the amounts of nor-adrenaline released from the adrenals were related to the amounts they received.

West (1949) tested the adrenal venous blood of a cat by injecting two aliquots of the same sample as nearly together as possible into the arteries supplying the denervated nictitating membrane and the denervated non-pregnant uterus of a second (test) cat. The uterus was a good indicator of adrenaline. With prolonged splanchnic nerve stimulation in the first cat the effect of injections of the outflow from the adrenals on the test uterus became less and less, and after one hour disappeared altogether. West estimated that at this stage the methylation was only 30%. If the stimulus to the splanchnic nerve were now increased a temporary rise in the percentage methylation occurred.

Gaddum and Lembeck (1949), investigated the effects of splanchnic nerve stimulation in cats. They/



They estimated the substances in the adrenal venous blood by assays with the rat's uterus and colon (Gaddum, Peart and Vogt, 1949) in a semi-automatic apparatus and with statistics. They showed that there was conclusive evidence that a mixture of active substances was present in the outflow but that there was not a reliable estimate of their concentrations.

In view of this large body of evidence it is now generally accepted that noradrenaline is both present in and liberated from, the adrenal medulla of several animals. Much less information exists as to the changes in the amounts and in the proportions of the amines under different conditions of rest and activity of the glands. Such studies would be best carried out when reasonably accurate methods of estimating the two amines are available. Among the most useful would be those employing the prior separation of adrenaline and noradrenaline. The development of such a method and investigations along these lines form a considerable part of the present work.

II. Nerve sympathin.

Attention was first drawn to the similarity of action of adrenal extracts to electrical stimulation of smooth muscle by Lewandowsky (1899). This was confirmed by Langley (1901) who tested such extracts on salivary, lachrymal and bile and pancreatic secretion, on the eye, heart, blood vessels, intestine, oesophagus, stomach, rectum, anus, urinary bladder, internal and external generative organs, pilomotor muscles and respiration. He found that the effects varied, some smooth muscles being contracted, others relaxed, while there was little or no effect on still others. He concluded that the effects of the extracts corresponded almost exactly to those of sympathetic nerve stimulation, but that they had little or no action on parasympathetic nerve effectors. Brodie and Dixon (1904), reported similar findings but with adrenaline which was then manufactured commercially. They showed that it did not constrict pulmonary blood vessels and that it dilated vessels in which the initial tone was high.

Early theories about sympathin.

Elliott (1904), also using adrenaline, found that/

that the denervated dilator pupillae muscle was more sensitive to it than the normal one. He thought the site of action of the drug was at the junction of the sympathetic nerve fibre and the muscle cell. He was the first to suggest that adrenaline might be a chemical transmitter, liberated each time there was a nerve impulse. Later Elliott (1905), on the basis of the similarity of action of adrenaline to that of sympathetic nerve stimulation, made a different suggestion. He still thought that the site of action was the junction of nerve and muscle but postulated there a special substance which was acted on by adrenaline. The nature of the response of the tissue or organ, i.e., whether excitatory or inhibitory, depended on the nature of the special substance.

Langley (1905), criticised Elliott's latter hypothesis. He found it difficult to believe that the hypothetical substance could be developed from muscle at the nerve-muscle junction because nerve section did not cause its disappearance. Any such receptive substances would be constituents of the muscle cell itself. He thought that motor and inhibitory substances might be produced in the cell, and/

and the effect of a nerve impulse depend on the relative proportion of the two substances.

Dixon and Hamill (1909), considered that nerve impulses, as well as drugs having a similar effect, acted by causing the release of adrenaline at the site of action.

About this time a fundamental study was made by Barger and Dale (1910) of a large series of amines with a view to determining the relation between their chemical structure and sympathomimetic actions. The term "sympathomimetic" was, in fact, of their coinage. They used several test objects - mainly the arterial blood pressure and the non-pregnant uterus both in situ and isolated, of the cat. The former generally gives an excitatory (pressor) response and the latter an inhibitory one to drugs of this type. They used an important pharmacological technique in determining on the cat's blood pressure ratios of equally active doses of the substances tested. On the other tests, a similar quantitative index of activity was not determined but an order of activity was assigned. They stated the important pharmacological principle that if the ratios of inhibitory activity had been determined these ratios would not have been the same as/

as those for the pressor effects. On this principle is based the method of parallel quantitative assays for estimating substances (Chang and Gaddum, 1933). Another important investigational tool used by them was ergotoxine, which antagonises the excitatory actions of adrenaline (and related substances) but has much less effect on the inhibitory ones (Dale 1905, 1906). They determined the "optimum skeleton" for sympathomimetic activity, from which it appeared that approximation to the structure of adrenaline meant the possession of greatest activity among the many compounds investigated. Of the near-relations of adrenaline (the catechol bases) those possessing a methylamino ( $-NCH_3$ ) group, which included adrenaline itself, had stronger inhibitory than motor effects. The opposite was true of the catechol bases which were primary amines ( $-NH_2$ ) and of these aminoethanol catechol (noradrenaline), had the greatest pressor activity. Methylaminoethanol catechol (adrenaline) was not quite so powerful. On the other hand, whereas adrenaline caused a marked relaxation of the cat's uterus, noradrenaline did not. After ergotoxine the response to adrenaline of the blood pressure was reversed. This was because the excitatory/

excitatory actions, which normally overshadow the inhibitory ones, were antagonised so that the vasodilatation was unmasked. The pressor action of noradrenaline, however, while reduced, showed no such reversal. On those findings they questioned Elliott's suggestion that adrenaline was the substance released on sympathetic nerve stimulation, because there was closer agreement between the effects of nerve stimulation and of certain other amino catechol (i.e., non-methylated) bases (including noradrenaline). The theory of Dixon and Hamill that any sympathomimetic drug whatever acted by releasing the mediator adrenaline also appeared unreasonable when a homologous series was tested. According to this view noradrenaline should act by releasing its less active methylated homologue adrenaline.

#### Early experiments on adrenergic nerves.

##### Sympathin released locally.

The heart. The actual release of sympathin was first demonstrated experimentally by Otto Loewi (1921) and Cannon and Uridil (1921) almost simultaneously. Loewi found that when the cardio-accelerator nerves of a frog's heart were stimulated the Ringer's solution inside the chambers acquired the property of/

of accelerating a second frog's heart. Loewi called the substance released "acceleransstoff" in contradistinction to "vagusstoff" which was released similarly by parasympathetic nerves. Cannon and Uridil's findings are described on page 24.

Similar experiments were performed by others. Brinkman and van Dam (1922) showed that the substance liberated on stimulation of the frog's cardio-accelerator nerves behaved like adrenaline in inhibiting the frog's stomach. Kahn (1926) used a pair of frog's Straub hearts from the same reservoir. The fluid was thoroughly mixed by bubbling. Stimulation of the nerves to one heart caused the release of a substance which mixed in the fluid in the second heart and increased its beat. Lanz (1928) confirmed the inhibitory effect on the stomach of the substance released in the frog's heart and also showed that it caused vasoconstriction in that animal.

On mammalian hearts, Jendrassik (1924) perfused the isolated rabbit's heart through the aorta and the pulmonary artery with Tyrode's solution and stimulated the sympathetic nerves. The outflow inhibited the isolated rabbit's intestine. Rylant and Demoor (1927a) tested rabbit's hearts. They repeated Loewi's/

Loewi's frog heart experiments with similar results. Rylant and Demoor (1927b) also transferred the active substance from one (stimulated) cat's heart to another using blood as perfusate.

Other organs and tissues. Finkleman (1930) in an arrangement in which Locke's solution was made to drip over a piece of rabbit's intestine and off that on to a second piece stimulated the sympathetic nerves to the first piece and produced inhibition in both pieces. Fluid thus running over a test piece of tissue may be called superfusion and has been used by Gaddum, Jang and Kwiatkowski (1939) in the investigation of the properties of the substance liberated by adrenergic nerves in the rabbit's ear. They used the hen's rectal caecum. A modification using the guinea-pig ileum as the test piece of tissue has been developed as a sensitive method for the assay of histamine.

Lehmann (1932), perfused the hind limbs of the frog. On stimulating the lower abdominal sympathetic chains an adrenaline-like substance was released and tested on the frog's heart.

Bain (1933), perfused the dog's tongue. On stimulation of the sympathetic nerves the substance liberated behaved like adrenaline on the rabbit's intestine./



intestine.

Generalised sympathin release.

Cannon and Britten (1927) found that the denervated heart of unanaesthetised cats subjected to excitement e.g., a barking dog, showed a large and sudden increase in the rate. If the cats were adrenalectomised this response was not elicited. There was, none the less, a gradual small increase in the heart rate after about three minutes. This increase was not due to physical factors like raised blood pressure, raised temperature or presence in the blood of products of activity of muscles. It could only have been due to some adrenaline-like substance in the circulation which had originated elsewhere than in the adrenals. Newton, Zwemer and Cannon (1931) found that this gradual increase in heart rate persisted after they had removed the adrenal medullae and accessory chromaffin tissue, cut the hepatic nerve and removed the abdominal sympathetic chains and stellate ganglia. At this stage there were only left strands of the thoracic sympathetic chains. If these were also removed there no longer occurred an increase in the heart rate when the cats were excited.

Partington/

Partington (1936), carried out similar experiments on cats in which both nictitating membranes were denervated, one acutely and the other some days before. The adrenals were tied off. If these animals were excited, effects on the pilomotor muscles, dilatation of the pupils and contraction of the chronically denervated (not the other) nictitating membrane, occurred. If the animals were completely sympathectomised no such effects were seen, showing that the sympathetic chains were concerned in the liberation of an adrenaline-like substance. Exposure to cold (2°C.) caused responses similar to those of excitement, and again not in sympathectomised animals.

Cannon and Bacq (1931) had showed the release of an active substance into the blood stream when they stimulated the lower abdominal sympathetic chain, isolated from the spinal cord. There were pilomotor effects, vasoconstriction in the tail and increased (denervated) heart rate. Because it did not originate in the adrenal glands they called the substance released in this way sympathin.

The nature of sympathin./

The nature of sympathin.Methods of investigation.

Euler (1950a) classified the methods employed in the investigation of sympathin as follows.

1. Stimulation of sympathetic nerves and observation of the remote effects in the same animal.
2. Comparison of the effects of sympathetic nerve stimulation with those of injected sympathomimetic amines.
3. Extraction of nerves and tissues and estimation of the substances therein by chemical and biological tests.
4. Collection of samples of blood and perfusing fluid from organs with sympathetic innervation and estimation of the substances released on nerve stimulation.

This classification is of interest because it presents succinctly the history of this investigation, as will be seen from the following.

Sympathin E and I.

The observations of Cannon and Rosenblueth (in contradistinction/

contradistinction to their theories of sympathin) were the first to show that nerve sympathin was not just adrenaline.

Cannon and Uridil (1921) had found that when the hepatic nerves of the cat were stimulated, though there was a rise of blood pressure and an increase in the rate of the denervated heart, the pupil did not dilate - an action known to be produced by adrenaline.

Cannon and Griffith (1922), showed that the active substance was carried in the blood stream because hepatic venous blood collected during stimulation through a catheter passed down the jugular vein into the inferior vena cava, on re-injection into the vena cava caused cardio-acceleration.

Cannon and Rosenblueth (1933) investigating this further, used the nictitating membrane as their main indicator for excitatory effects and the non-pregnant uterus for inhibitory effects, the adrenals being excluded. They found that the remote effects on these test organs depended on which region of the sympathetic nervous system was stimulated. Most of the regions produced a substance like adrenaline which/

which caused a rise of blood pressure, and dilatation of the pupil together with contraction of the nictitating membrane and relaxation of the uterus, i.e., a mixture of excitatory and inhibitory effects. When the nerves to the liver were stimulated, however, the pupil did not dilate and the uterus did not relax, i.e., the effects were purely excitatory. After ergotoxine, adrenaline gave a fall of blood pressure, but hepatic nerve stimulation and stimulation of the sympathetic chains still had a pressor effect, though somewhat reduced. These results could be explained if nerve sympathin were not identical with adrenaline.

In their theories of sympathin they postulated one mediator (adrenaline) which combined with two different substances in the reacting cells to form an excitatory sympathin (Sympathin E) or an inhibitory sympathin (Sympathin I). The effects on remote organs would depend on whether Sympathin E or a mixture of E and I escaped into the blood stream. Pure Sympathin I without E was presumed not to occur because all organs have blood vessels which must necessarily produce the excitor (vasoconstrictor) sympathin. Liver sympathin on the other hand was Sympathin E.

Other/

Other evidence against the identity of sympathin and adrenaline was that of Rosenblueth and Cannon (1932) who determined the stimulus for the splanchnic nerves of the cat which would just cause a secretion from the adrenals, and a similar threshold stimulus for the lower abdominal sympathetic chains, using the nictitating membrane as indicator in each case. Stimulation of the two regions together provoked a large contraction of the nictitating membrane, much higher than the separate stimulations would have led one to expect.

Rosenblueth and Morison (1934) showed more quantitatively that in cats, when sympathin was released simultaneously from two different sources, the combined effect was greater than that of a dose of adrenaline equivalent to the sum of the doses which matched the separate stimulations.

Meanwhile, the nature of Loewi's frog's heart "acceleransstoff" was being investigated. Loewi and Navratil (1926) found that the substance behaved like adrenaline in its cardio-acceleratory function. They found also that, like adrenaline, it was inactivated when mixed with eosine and exposed to ultra-/  
ultra-

ultra-violet light. Lanz (1928), reported that it was inactivated on standing in air for twenty-four hours or on heating to 100°C. Rosenblueth and Schlossberg (1931) showed that it was like adrenaline in the potentiation of its effects by the previous administration of cocaine.

Gaddum and Schild (1934) described a sensitive physical test for adrenaline. It depended on the development of fluorescence after treatment with strong alkali in the presence of oxygen. It was sensitive to a concentration of  $10^{-8}$  adrenaline. Using this test, Loewi (1936), found good agreement between the active substance released in the frog's heart and adrenaline.

There is no evidence that frog's heart sympathin is anything but adrenaline.

#### Mammalian sympathin.

Sympathin E and noradrenaline. Bacq (1934), first suggested that Sympathin E might be noradrenaline. He later (1935) suggested that Sympathin I might be adrenaline, but at the same time changed his opinion about Sympathin E. He thought that the latter might be a partially oxidised adrenaline in which only excitor properties remained. The main importance/

importance of Bacq's suggestions lies in the idea of two extra-cellular reacting substances with excitatory or inhibitory properties, whereas Cannon and Rosenblueth had postulated two "sympathins" as a result of different intra-cellular changes undergone by one mediator.

Cannon and Rosenblueth (1935, 1937) had criticism to offer of Bacq's views.

1. If Sympathin I were adrenaline they considered that release of a mixture of E and I should have an action equivalent to adrenaline on various structures of the animal. <sup>+++</sup> In fact, stimulation of nerves which did so release a mixture of "sympathins" and having on the nictitating membrane an effect equal to a given dose of adrenaline had a smaller effect than adrenaline on the iris.
2. If Sympathin E were noradrenaline then adrenaline, whether injected or secreted, must be demethylated before it could have excitor effects. There was no evidence that this change did take place. Also small/

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+++

This argument is difficult to follow - A.S.O.



small doses of adrenaline would be demethylated quickly and thus more readily cause excitation. Actually, small doses of adrenaline were inhibitory.

3. Against the suggestion that Sympathin E might be partially oxidised adrenaline was the fact that liver sympathin (purely excitatory) began to act very quickly, in 15-20 seconds, (Cannon and Uridil, 1921) whereas sympathin from the tail region (mixed excitatory and inhibitory) acted after a comparatively long delay (Cannon and Bacq, 1931). This would mean that the substance requiring time for oxidation acted quicker than the one which was already in the form in which it would take effect.
4. Blaschko and Schlossman (1936), had used the cat's blood pressure as a test for excitatory effects and the rabbit's intestine for inhibitory effects. At no stage in the progressive oxidation of adrenaline was there a significant change in it's qualitative effects.

Later still, Bacq (1938), had other hypotheses.

He believed that tissue extracts containing catechol oxidase converted adrenaline into a substance having vaso-dilator effects (adrenoxine). He suggested that the inhibitory effects of adrenaline on certain tissues might be due to the presence of catechol oxidase in them.

Experiments on liver sympathin.

By comparison of the effects of injected adrenaline and noradrenaline with those of nerve stimulation evidence began to accumulate that noradrenaline was a sympathetic mediator. Stehle and Ellsworth (1937), found in cats after ergotoxine in which adrenaline gave a fall of blood pressure while noradrenaline still had a pressor effect that hepatic nerve stimulation released a substance like noradrenaline.

Melville (1937), used two other antagonists, the benzodioxanes F883 and F933. The adrenals were tied off. On stimulation of the (splanchnic) nerves there was a rise of blood pressure which was not reversed by the antagonists. The response to adrenaline was reversed. The pressor effect of noradrenaline was reduced but not reversed.

Greer, Pinkston, Baxter and Brannon (1938) studied/

studied in the cat, before and after ergotoxine, the response of the blood pressure, iris, nictitating membrane, non-pregnant uterus and intestine to adrenaline, noradrenaline and hepatic nerve stimulation. They found that the effects of noradrenaline and nerve stimulation differed from those of adrenaline in essentially the same way. There was, however, occasional slight relaxation of the uterus on nerve stimulation. They suggested that there were at least two adrenergic mediators, one of which was mainly excitatory (Sc) like noradrenaline and the other mainly inhibitory (Sr) like adrenaline although each had the other quality to a certain extent. The two substances differed in degree but not in kind. Possibly Sc was noradrenaline and Sr adrenaline.

Gaddum and Goodwin (1947) studied especially whether differences in the rate at which liver sympathin and adrenaline reach active concentrations in the blood might account for the differences in their effects. This was because, while adrenaline was usually injected quickly, nerves may continue to liberate sympathin for at least as long as they were stimulated. Some of the discrepancies between quick/

quick injections of adrenaline and stimulating hepatic nerves could be matched by similar discrepancies between quick and slow injections of adrenaline. All the effects of liver sympathin were not, however, reproduced with slow injections of adrenaline. On the other hand, the effects of liver sympathin resembled those of noradrenaline more closely than those of adrenaline. Tyramine too, had some of the required properties. They also confirmed that inhibition of the virgin uterus and the intestine was sometimes caused by liver sympathin. They concluded that liver sympathin could be noradrenaline or perhaps tyramine. Tyramine may be excluded on other evidence. Burn and Tainter (1931) found that cocaine abolished the effects of tyramine but potentiated those of liver sympathin.

West (1948), compared hepatic nerve stimulation with intra-portal injection of adrenaline and noradrenaline. Guanidine and cocaine intra-portal did not potentiate the action of noradrenaline or of nerve stimulation, but the action of adrenaline was potentiated. After intra-portal injection of dihydroergotamine (in large doses), nerve stimulation and/

and noradrenaline gave depressor responses but there was no effect on the corresponding doses of adrenaline.

West (1950), using a similar technique to that for testing adrenal venous blood (West 1949), injected samples of hepatic venous blood divided into two portions into the arteries to the denervated nictitating membrane and to the denervated uterus of a second (test) cat. The uterus is a good indicator for adrenaline. Some samples consisted of both adrenaline and noradrenaline while others apparently contained noradrenaline only. The substance methyl priscol (2-benzyl-1-methyl-imidazoline) which potentiates adrenaline when injected intra-portally (Gowdey, 1948), was found to produce no potentiation of noradrenaline and only very slight transient potentiation of hepatic nerve stimulation.

Mann and West (1950), confirmed that liver sympathin was mainly noradrenaline by parallel assays with the nictitating membrane of the cat, the chick's rectum and the rat's uterus.

#### Noradrenaline in extracts of nerves and tissues.

The earlier work was to extract various organs and to estimate the active substances in terms of adrenaline. Euler (1934), found that extracts of the/

the prostate of various species had an adrenaline-like substance which also reacted chemically with iodine (Vulpian's reaction) and was inactivated by oxidation. He also demonstrated the chromaffin reaction histologically. In Loewi's investigations (1936, 1937) into frog's heart sympathin he found that dialysates of frog's and guinea-pig's hearts had cardio-accelerator activity on the isolated frog's heart and fluoresced like adrenaline under the requisite conditions. Shaw (1938) found by his specific colour test that extracts of frog's hearts and rabbit's prostates apparently contained adrenaline. Extracts of many other organs, however, of the rabbit, the intestine, liver, kidney and stomach showed evidence of the colour being due to some other substance. McDowall (1946), prepared heart extracts which had adrenaline-like properties. Euler (1945), found that suitably purified extracts of nearly all organs (with the exception of the nerve-free placenta) had the property of raising the blood pressure of the chloralosed cat, inhibiting the rabbit's intestine and contracting the isolated rabbit's uterus.

A considerable advance was made with more detailed/

detailed investigation of the substances present in certain extracts. Spleen extracts (Euler, 1946a), were compared with adrenaline and corbasil (dihydroxynorephedrine or DNE). This was because noradrenaline itself was not available to him, but corbasil was known to have similar properties (Schaumann, 1931). The main points for the presence of noradrenaline in the extracts were, after doses of ergotoxine which reversed or antagonised equipressor amounts of adrenaline the extracts and DNE had some pressor activity left; the extracts and DNE were less inhibitory on the rabbit's intestine, the non-pregnant cat's uterus and the pupil but had greater stimulating effect on the rabbit's uterus than equipressor amounts of adrenaline; and the fluorescence reaction of Gaddum and Schild (1934), was not given by the extracts and DNE. The active "ergone" was probably noradrenaline.

Similar considerations led Euler (1946b and c) to conclude that noradrenaline was present in extracts of mammalian hearts (cattle, horse and cat) and in adrenergic nerve fibres. Extracts of the thoracic and lumbar sympathetic chain and of splenic peri-arterial nerves had the greatest concentrations. The "ergone" was not confined to nerve endings only but/

but was present in the trunks as well. He also confirmed by these methods that extracts of frog's hearts contained, as Loewi (1936) found, adrenaline only, thus differing from mammalian hearts.

Euler and Schmitterlöw (1947), found similarly that the small amount of sympathomimetic activity in human and bovine blood was compatible with the presence of noradrenaline.

Bacq and Fischer (1947), confirmed Euler's findings with extracts of the spleen, prostate, sympathetic chain, splenic nerves, coronary arteries and coronary nerves of various species, which they tested on the cat's blood pressure, nictitating membrane and non-pregnant uterus, and compared with injected noradrenaline. This amine had by now become more generally available. The extracts of the spleen contained noradrenaline but those of the sympathetic nerves and ganglia a mixture of adrenaline with much more noradrenaline.

Euler (1948a) repeated some of the experiments on spleen and splenic nerve extracts, testing them similarly and on the hen's rectal caecum. He compared the effects with noradrenaline and used the adrenergic blocking agent Dibenamine (Nickerson and Goodman, 1947). The results confirmed the conclusions drawn from the previous experiments using corbasil. The list below summarises the results of/



of these investigations.

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Tissue	Amounts of dl-nor-adrenaline hydrochloride ( $\mu\text{g./g.}$ )
Splenic nerves (freed from sheath)	10-30
Sympathetic chain	3-5
Cutaneous sensory nerves	0.5-1
Phrenic nerves	0.5
Ciliary nerves	0.8
Optic nerves	0.3
Anterior roots	0.5
Posterior roots	0.5
Spinal cord	0.2
Vagus	0.1

---

#### Nerve extracts and released nerve sympathin.

It is generally believed that sympathomimetic substances in extracts of organs and tissues are related to nerve elements. This accounts for extracts of the placenta, which is a nerve free organ, not having any such substances. (Euler, 1945).

The aqueous humour of the eye has been shown to contain a sympathomimetic substance on stimulation of the cervical sympathetic in the rabbit and dog (Bacq, 1931, 1933), in cocainised cats (Luco and Lissak, 1938) and in cattle and horses (Euler, 1950c). The last named was able to identify both adrenaline and noradrenaline.

Experiments on isolated nerves were carried out by/

by Gaddum and Khayyal (1935). On electrical stimulation of sympathetic nerve fibres a substance was released which had adrenaline-like actions on the frog's heart. Other nerves did not release this substance. Gaddum, Khayyal and Rydin (1937) considered these results inconclusive.

Lissak (1939a) made extracts of the superior cervical ganglion, various post-ganglionic fibres, the superior mesenteric plexus and the thoracic sympathetic chain. He found an adrenaline-like substance. He also (1939b) stimulated the isolated nerves. He found that the ganglion extracts after section of the preganglionic fibres contained, and the mesenteric plexus released, a substance which accelerated the frog's heart. The effect was abolished by ergotoxine. Cannon and Lissak (1939) tested these extracts and also extracts of organs to which these particular fibres were distributed, e.g., the heart, and found in all of them a substance which raised the blood pressure, was reversed by ergotoxine, dilated the pupil, contracted the nictitating membrane, relaxed the non-pregnant uterus (all of the cat) and accelerated the frog's heart. The heart extracts (frog and rabbit) also gave Shaw's (1938)/

(1938) specific test for adrenaline. This was suggestive of the presence of adrenaline only, but later Schmitterlöw (1948) showed that all these actions could also have been caused by noradrenaline.

Euler and Anström (1948) collected the substance released by isolated splenic nerves of cattle by having one end dipping in fluid and stimulating the other end. The fluid was then assayed and showed that stimulation released a substance which inhibited the isolated guinea-pig's intestine and was apparently noradrenaline. The liberation of histamine was also indicated. Studies on other nerve extracts by Euler and his co-workers have already been mentioned.

The liberation of noradrenaline from nerve endings was confirmed with cross-circulated perfused hind limbs and the splanchnic region of the cat (Folkow and Uvnäs, 1948), and the hind limbs of the dog (Folkow and Uvnäs, 1949) by comparison with injected adrenaline and noradrenaline and the use of the blocking agent dibenamine. Graham (1949), extracted human lumbar sympathetic chains and found that they contained noradrenaline which may have been mixed with adrenaline.

The effects of adrenergic nerves.

The/

The identification of nerve sympathin depends largely on the comparison of its effects with those of known substances. Light was thrown on the effects of these nerves by the studies of Ahlquist (1948), on adrenotropic receptors. He compared the actions of adrenaline with those of noradrenaline, corbasil, n-methyl corbasil and isoprenaline. All these substances had been studied previously but not together in quantitative comparison. He found that one group of tissues on which the drugs were tested gave mainly excitatory responses (one exception gave an inhibitory response) while another group gave mainly inhibitory responses (again one exception gave an excitatory response). The drugs were placed in the order of activity in each test employed. They gave one order with the tests of the excitatory group and almost exactly the opposite order with the tests of the inhibitory group. He considered that this was due to two different kinds of receptors in the tissues and gave them the name  $\alpha$  and  $\beta$  receptors, respectively. The response of a tissue depended not so much on the drug (for all the drugs tested had both types of action in greater or smaller degree), but on the type of receptors present.

It/

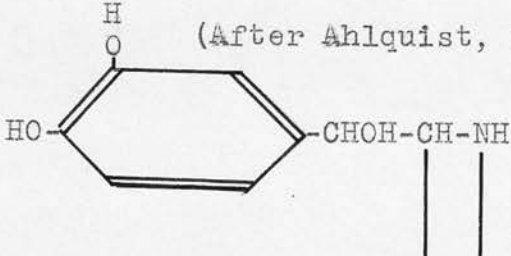
Table I.

Biological preparations for noradrenaline  
and adrenaline assays.

$\alpha$ effects	Equal effect ratio	$\beta$ effects	Equal effect ratio
<u>Excitatory</u>		<u>Inhibitory</u>	
Cat blood pressure	0.5-1	Fowl, rectal caecum	10-60
" pregnant uterus	0.8	Cat, non-pregnant uterus	10
" normal nictitating membrane	2-4	Rat's uterus	75-300
" denervated nictitating membrane	1		
Rabbit's ear	0.75-3		
Cat, spleen	0.5-1		
Rabbit's uterus, pregnant or non-pregnant	4-5		
Guinea-pig's uterus non-pregnant	2.5	Guinea-pig's uterus, pregnant	40
<u>Inhibitory</u>		<u>Excitatory.</u>	
Rat's colon	0.25-1	Frog's heart, Straub	10-20
Rabbit, Cat, Rat, ileum	1-3	Frog's heart, perfused	33

Order of activity of sympathomimetic amines.

(After Ahlquist, 1948)

		<u>Effect</u>	
		$\alpha$	$\beta$
Noradrenaline	H H	2	5
Corbasil	CH <sub>3</sub> H	3	4
Adrenaline	H CH <sub>3</sub>	1	3
N-methyl corbasil	CH <sub>3</sub> CH <sub>3</sub>	4	2
Isoprenaline	H CH(CH <sub>3</sub> ) <sub>2</sub>	5	1

It is generally useful, without going into the question of the receptors themselves, to consider only the effects. Table I has been compiled from several sources (West, 1947; Gaddum, Peart and Vogt, 1949; Bülbring and Burn, 1949b, and some personal observations). It classifies various test objects according to this conception of Ahlquist. The ratios of equally active doses (noradrenaline/adrenaline, both l-isomers) and the orders of activity found by him are also shown.

It will be seen that for  $\alpha$  effects noradrenaline is about as active as, or more active than, adrenaline (ratio 1 or less), while for  $\beta$  effects adrenaline is much more powerful (ratio large). Isoprenaline has powerful  $\beta$  effects, but only weak  $\alpha$  effects.

Ergotoxine and other adrenergic blocking agents affect  $\alpha$  effects only (including the inhibition of the intestine) but not  $\beta$  effects (it is comparatively ineffective against the cardio-accelerator property of adrenaline).

The best way to decide how to classify an effect is to compare the action of noradrenaline and isoprenaline on it. (Gaddum, 1950).

The/

The identification of the active substances in blood or other fluid perfusing sympathetically innervated organs.

"When substances are liberated at an unknown rate into the natural circulation the result is affected by too many variables. Experiments on fluids collected in test tubes provide better evidence, but are difficult owing to the small amount of the active substances which can be collected in this way". (Gaddum, 1950).

Although a considerable amount of work had been done on liver sympathin, blood from this organ had not been removed and tested outside the experimental animal till very recently (Mann and West, 1950). Sympathin from other sources has been tested outside the body.

The tests on the aqueous humour of the eye, (Bacq, 1931 and others) might be considered an ingenious way of doing this.

Gaddum and his colleagues Jang and Kwiatkowski, (1938-1939) perfused rabbits' ears and examined the perfusate pharmacologically. In biological tests on the frog's heart, the fowl's rectal caecum, the ear itself, and chemically by Shaw's (1938) test, the/

the evidence was suggestive of the liberation of adrenaline alone when the sympathetic nerves were stimulated. The tests, however, were not really adequate to exclude noradrenaline. This question has been reinvestigated with newer methods and the results are reported in the present work.

West, (1947), made quantitative studies on adrenaline and noradrenaline on thirteen biological preparations. In continuation Gaddum, Peart and Vogt (1949), examined several new tests for the estimation of these substances in blood. Using these for parallel quantitative assays, Peart, (1949) assayed splenic venous blood (plasma) after nerve stimulation. The substances present contracted the cat's denervated nictitating membrane (and lower lid), caused vaso-constriction in the perfused rabbit's ear and inhibited the rat's colon and sometimes the rat's uterus. The results of these assays showed that the main active substance was not adrenaline, corbasil, epinine, adrenalone or histamine. All the evidence supported the view that the substance was noradrenaline, smaller amounts of adrenaline being sometimes also liberated.

Mann and West (1950), tested splenic venous blood/

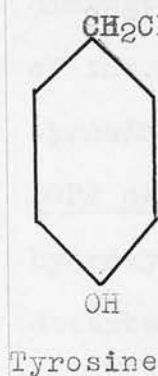


blood on the cat's blood pressure, nictitating membrane and non-pregnant uterus. Their results agreed that the main active substance liberated was noradrenaline.

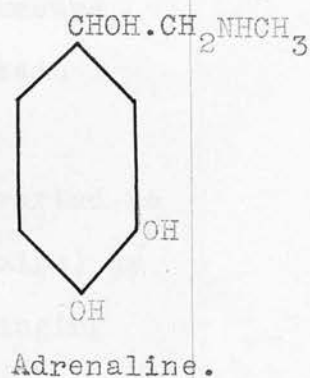
### III. The formation and fate of adrenaline.

#### The formation of adrenaline.

The primary precursors are believed to be tyrosine and/or phenylalanine. At least four chemical processes are necessary for the conversion.



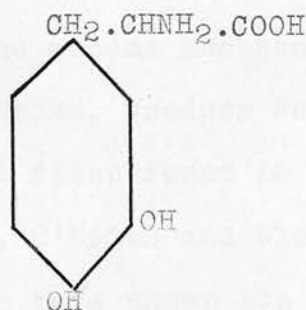
1. Oxidation to introduce a (phenolic)-OH in the m-position.
2. Decarboxylation.
3. Oxidation to introduce an (alcoholic)-OH in the side chain.
4. N-methylation.



There is no evidence that tyrosine is first decarboxylated to tyramine, although a weak tyrosine decarboxylase has been demonstrated in mammalian tissues (Holtz, 1937). It is probably first converted into 3,4-dihydroxyphenylalanine (DOPA)

3,4 dihydroxy-  
-phenylalanine  
(DOPA)

Tyrosinases/

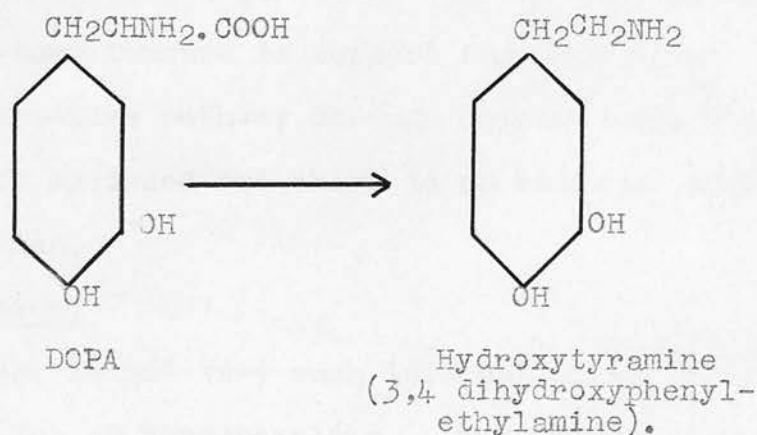


Tyrosinases having such phenoloxidase activity have been demonstrated in invertebrates and plants, but the evidence for their action in normal vertebrates is not conclusive (Neubauer, 1928, Neuberger, Rimington and Wilson, 1947). Melanotic tumours of the skin in mice have been shown to contain tyrosinase (Hogeboom and Adams, 1942).

DOPA decarboxylase. DOPA may then be converted to hydroxytyramine (3,4 dihydroxyphenylethylamine) by decarboxylation. An enzyme capable of bringing about this reaction was discovered in mammalian kidney. (Holtz, Heise and Lüdtkke, 1938). DOPA is the only possible precursor of adrenaline found in the body which it decarboxylates at a reasonable rate. Another possible precursor of adrenaline which it was believed not to act on is 3,4 dihydroxyphenylserine (noradrenaline carboxylic acid) which has recently assumed importance and is referred to later. The enzyme has also been found in mammalian liver (Blaschko, 1939) and in the mucous membrane of guinea-pig small intestine (Holtz, Credner and Reinhold, 1939). It was not at first found in the adrenal gland (Blaschko, Carter, O'Brien and Sloane Stanley, 1948) but later studies have shown its presence there (Langemann, 1950).

In/

In experiments on perfused guinea-pig's and ischaemic cat's kidney, Bing (1941), and Bing and Zucker (1942), produced what was probably hydroxytyramine from the enzyme and substrate. Holtz, Credner and Koepp (1942), found hydroxytyramine in the urine of animals and man after the administration of DOPA orally and subcutaneously. Recently both hydroxytyramine and DOPA have been found in sheep adrenals (Goodall, 1950).



It was considered unlikely that N-methylation preceded decarboxylation because N-methyl DOPA was not decarboxylated under similar conditions (Blaschko 1939, 1942). It was also believed unlikely that introduction of the alcoholic -OH group preceded decarboxylation/

decarboxylation because noradrenaline carboxylic acid was only decarboxylated at a very slow rate by animal tissues, although it could be satisfactorily, by acetone dried preparations of *Streptococcus faecalis* R (Blaschko, Holton and Sloane Stanley, 1948). Newer evidence may modify this view. Presumably noradrenaline is formed from hydroxytyramine as a precursor of adrenaline in the body. The occurrence of noradrenaline and its release from normal adrenal glands as well as from pheochromocytoma tumours is support for this view. The alternative pathway through epinine (cf. Fig.I) cannot be excluded but there is no evidence that it is followed.

#### Methylation.

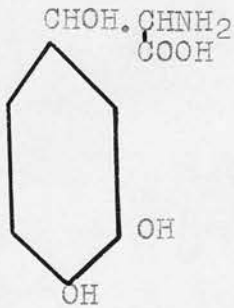
There is not very much information as to the methylation of noradrenaline. Substances like methionine (West, 1947) and choline probably act as methyl donors.

The first experimental evidence was obtained by Bülbring (1949), who showed that suspensions of minced adrenals of dogs and cats when incubated with choline and ATP could methylate noradrenaline to adrenaline. Suspensions of glands which had been subjected to the effects of prolonged splanchnic nerve/

nerve stimulation had an increased methylating power.

Bülbring and Burn (1949) found that in an isolated perfused dog's adrenal gland noradrenaline added to the perfusing fluid was removed and converted quantitatively into adrenaline.

Noradrenaline carboxylic acid.



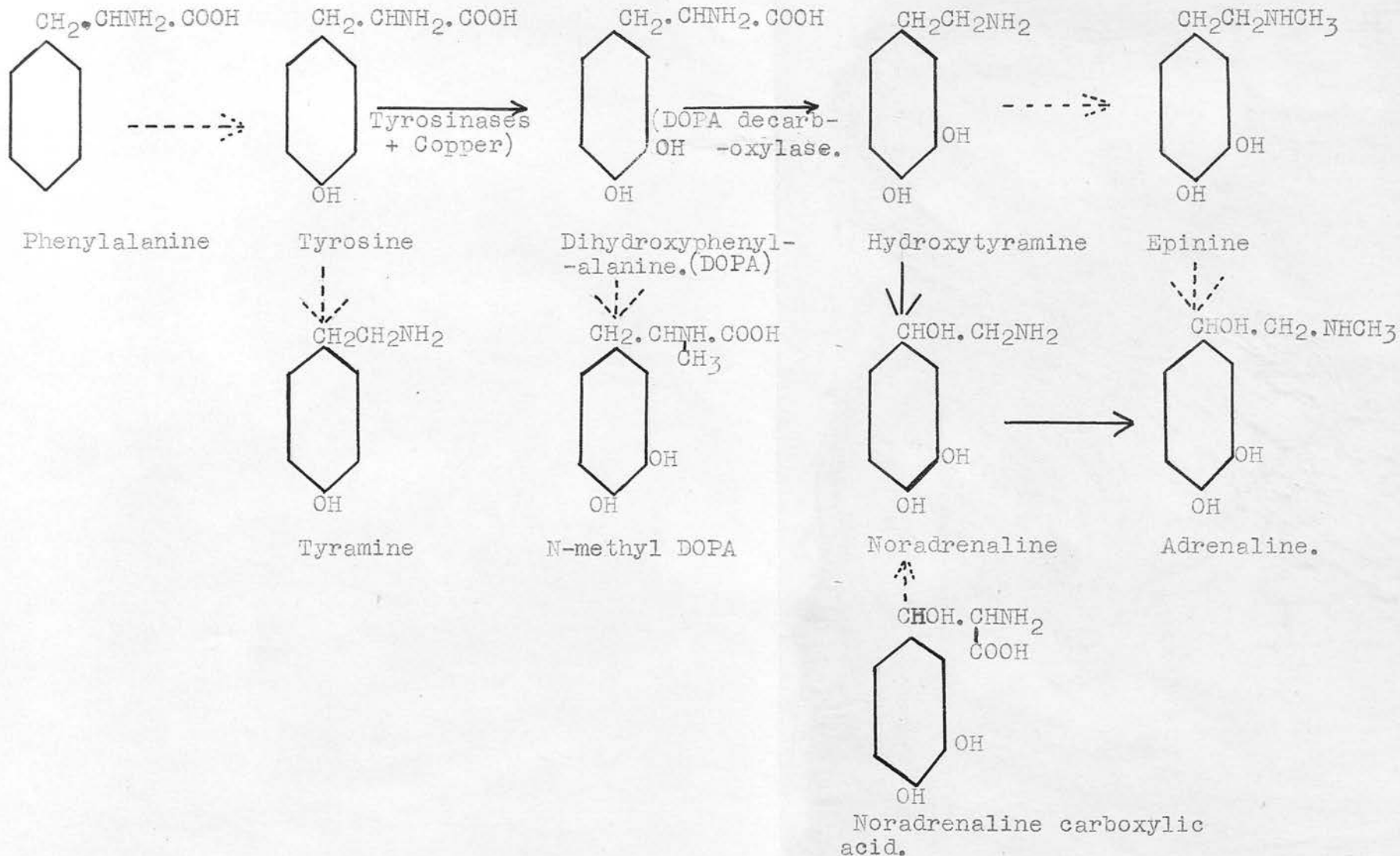
( 3,4 dihydroxy-phenylserine.)

This amino acid was synthesised in 1919 (Rosenmund and Dornsaft, 1919) and again more recently (Dalgliesh and Mann, 1947) and was shown to be decarboxylated, though at a very slow rate, by large amounts of tissue extracts (Blaschko, Burn and Langemann 1950) with the production

of l-noradrenaline. Schmitterlöw (1951) administered noradrenaline carboxylic acid to rabbits (5mg./kg. intravenously) and found noradrenaline, but no detectable adrenaline in the urine. In normal uninjected rabbits no pressor amines were excreted. Administration of the corresponding adrenaline carboxylic acid was not followed by the appearance of a pressor substance in the urine. If noradrenaline carboxylic acid/

Fig. I

DIAGRAM OF FORMATION OF ADRENALINE.



acid could be a possible precursor of noradrenaline there remains the objection that the amino acid has not, as yet, been shown to be present in the animal body. The problem of why the noradrenaline formed was not methylated also requires elucidation.

Phenyl alanine. Indirect evidence for the conversion of this amino acid into adrenaline was obtained by Devine (1940). He found an increase of adrenaline when slices of adrenal medulla were incubated with phenyl alanine.

Gurin and Delluva (1947), injected rats with phenyl alanine labelled with  $C_{14}$  and Tritium ( $H_3$ ) and found that both the catechol group and the side chain of adrenaline showed possible derivation from that precursor.

The most likely pathway of biosynthesis, on the available evidence is shown by the thick arrows in Fig.I.

#### The fate of adrenaline.

It is well-known that injected adrenaline or physiologically released sympathin is inactivated in a few minutes (Trendelenberg, 1929). There are several/



several possible mechanisms for this.

Amine oxidase.

This enzyme is present in fairly large amounts in the liver, intestine and central nervous system. It is not specific for adrenaline, any substrate containing  $\equiv\text{C}-\text{CH}_2-\text{N}<$  being oxidised. It is inhibited by similar compounds in which an -H in the  $\alpha$  position is replaced by  $-\text{CH}_3$ . A compound of this nature is ephedrine (Blaschko, Richter and Schlossmann, 1937a and b, Richter, 1937). Owing to this inhibitory action, Gaddum and Kwiatkowski (1938), were able, by adding ephedrine to the solution perfusing an isolated rabbit's ear, to increase the yield of sympathin released when the nerves were stimulated. Richter and Tingey (1939) were unable to obtain the enzyme by extracting rabbits' ears and thought the experimental rate of oxidation too slow to account for the actual speed of inactivation in the tissues. They thought also, that the concentrations of ephedrine used were too small to inhibit the enzyme. Professor R.H.S. Thomson was successful in obtaining the enzyme by extracting the blood vessels of the rabbit's ear, having failed previously when extracting the whole ear, (quoted by Burn and Robinson, 1951). The objection to the low concentration/

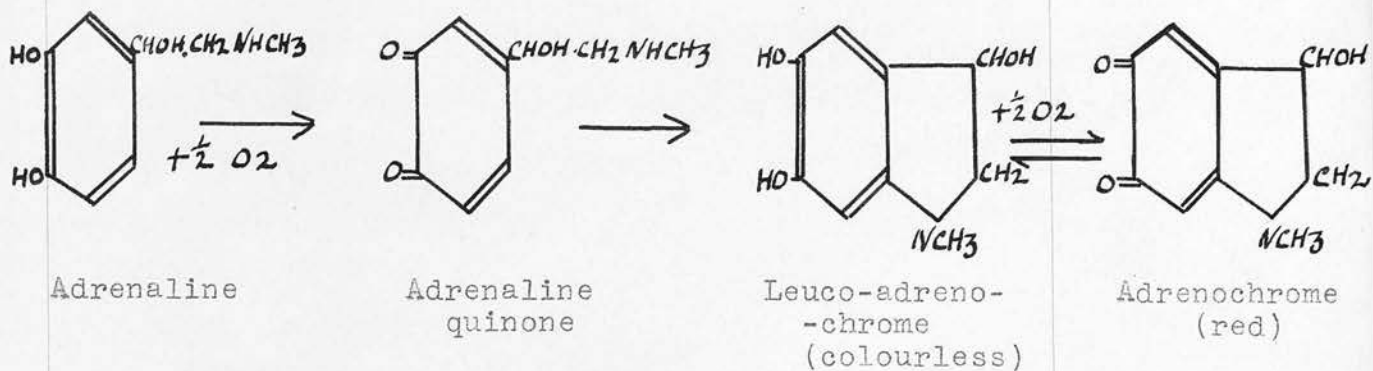


concentration of ephedrine can be met by the possibility of that substance being concentrated in the tissues so that adequate amounts would be available. (Gaddum, 1950).

#### Oxidation to adrenochrome.

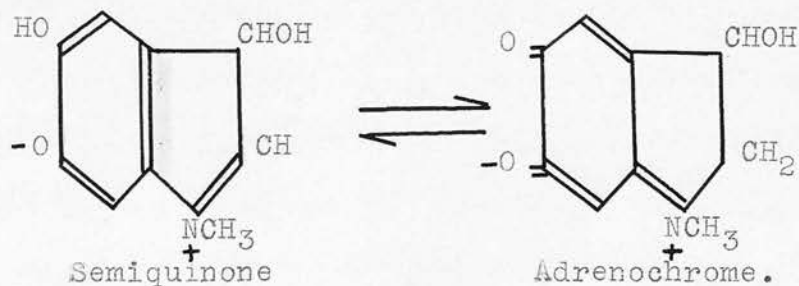
This quinone imparts the red colour to solutions of adrenaline which have undergone oxidation. The process may take place spontaneously (autoxidation) in air, particularly in alkaline solutions. It is accelerated by copper (and certain other heavy metals) but is prevented by reducing substances (e.g., ascorbic acid) present in plasma or introduced intentionally into the solution. Various enzymes promoting this oxidation have been described, e.g., the Pseudophenolases, which are protein complexes containing the active metal copper (Bhagvat and Richter, 1938); the Cytochrome system (Green and Richter, 1937) which Bacq (1948), believes to be the normal oxidising system in the body, and Catechol oxidase, which is thought to catalyse the oxidation of adrenaline to adrenochrome (Bacq, 1938b) after the following scheme:~

scheme: -



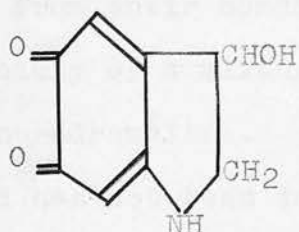
The coloured compound is believed to exist in equilibrium with leuco-adrenochrome forming an oxidation-reduction system (West, 1947).

Harley Mason (1948) believes that adrenochrome has a zwitterion structure and exists in equilibrium with a semiquinone zwitterion involving the transfer of one electron:-



Whichever view is correct, the corresponding nor-adrenochrome/

noradrenochrome could presumably be formed in a similar manner.



Noradrenochrome

Adrenochrome had been isolated by Green and Richter (1937), who prepared it from adrenaline with a purified catechol oxidase concentrate from mushroom (*Agaricus* sp.). The enzyme, however, could not be found by Bhagvat and Richter (1938), in mammalian tissues.

Further oxidation of these compounds results in the formation of various melanins.

#### Other methods of inactivation.

##### Conjugation.

From the products in urine after the administration of adrenaline, Richter (1940), concluded that an important process was the esterification of catechol derivatives and excretion in the urine. This has been confirmed by Beyer and Shapiro (1945) and by/

by Holtz, Credner and Kroneberg (1947) who showed that catechol derivatives in urine are normally present in a conjugated form. The active substances can be released from their bondage by acid hydrolysis and consist probably of a mixture of hydroxytyramine, adrenaline and noradrenaline. The nature of the conjugating acid has not been identified.

#### Storage.

Bain, Gaunt and Suffolk (1937), found that a considerable proportion of adrenaline (about 30% for small initial concentrations) added to oxalated or defibrinated cat's blood enters the red cells until an equilibrium is reached. It can be recovered by laking the blood.

#### Excretion unchanged.

Free adrenaline and noradrenaline have been found in human urine, particularly after muscular work (Euler and Hellner, 1951) and in cases of phaeochromocytoma (Engel and Euler, 1950), while cow's urine contains chiefly noradrenaline (Euler and Hellner, 1951).

#### Demethylation.

There is no evidence that adrenaline is demethylated in the body to form noradrenaline.

IV. Adrenergic blocking agents.I. Natural.Ergot alkaloids.

These were among the earliest reported, (Dale 1906,1913). Ergotoxine was shown to block the excitatory effects of adrenaline leaving the inhibitory effects relatively or absolutely, unaffected. Ergotamine was shown to behave similarly (Dale and Spiro, 1922), while the reduced alkaloids (dihydro-compounds) were found to possess adrenergic blocking action with less side actions than the natural substances (Rothlin, 1947). Certain types of blocking by ergot have been considered exceptional, e.g., the blocking of coronary vaso-dilatation in cattle and of inhibition of the intestine by adrenaline (Rothlin, 1925). In view of Ahlquist's (1948) hypothesis these actions of adrenaline, though inhibitory, are of the  $\alpha$  type. The apparent anomaly is explained by the fact that ergot antagonises  $\alpha$  effects but not  $\beta$  ones.

Recent studies on isoprenaline (a depressor amine) showed that ergot reverses its action to a pressor one (King, 1947). Curiously, the reversed effect (pressor) is antagonised by benzodioxanes and potentiated by cocaine-like pure sympathomimetic effects/

effects (Hazard, Beauvallet and Giudicelli, 1947).

Ergotoxine has been a pioneer investigational tool in the study of sympathomimetic amines and their effects. It now has powerful rivals in some synthetic compounds described below.

### Yohimbine.

This natural alkaloid has been known as an adrenergic blocking agent for a considerable time (Raymond-Hamet, 1925) but it has not proved so useful as ergot in laboratory investigations.

Other natural products with this action are less important still. Cinchona alkaloids, including quinine. It was discovered accidentally that adrenaline was ineffective in raising the blood pressure after intra-venous quinine (McCarrison and Cornwall, 1918). The other members have also been studied (Jackson, 1922). Xanthine alkaloids are known to have this action (Fredericq and Bacq, 1938), and so have Hydrastinine (Raymond-Hamet, 1927) and Ajmalinine, an alkaloid from *Rauwolfia serpentina* (Raymond-Hamet, 1936).

## II. Synthetic.

There are several types of synthetic compounds:- Phenoxyalkylamines, among the first synthetic adrenergic/

adrenergic blocking agents studied (cf. Bovet and Bovet-Nitti, 1948) are related chemically to the dioxanes: the isoquinolines (Fasset and Hjert, 1938) and the heterocyclic alkylamines. The last group includes the benzodioxane derivatives F883 (diethylaminomethyl benzodioxane) (Fourneau and Bovet, 1933) and F933 (piperidylmethyl benzodioxane) (Bovet, 1934, Vleeschouwer, 1934). Vleeschouwer (1933) found that F883 antagonised both adrenaline and sympathetic nerve action. It was found that F933 was a better antagonist of adrenaline than of sympathetic nerve action (Bacq and Fredericq, 1935, Bacq and Bovet, 1935). They stated that it was "adrenolytic but not sympatholytic".

Imidazolines. Priscol (2-benzyl-2-imidazoline) is the member most studied. Its adrenergic blocking action was first reported by Schnetz and Fluch, (1940). It is highly specific (Ahlquist, Huggins and Woodbury, 1947), and its action is powerful but passes off fairly quickly (Chess and Yonkman, 1945), not as quickly as the benzodioxanes but quicker than dibenamine.

$\beta$ -haloalkylamines. The prototype of these is dibenamine (N,N-dibenzyl- $\beta$ -chloroethylamine). The compounds/



compounds appear to be more specific and complete blockers than any others (Nickerson and Goodman, 1945, 1947, 1948). They have been used and are likely to continue increasingly useful as tools in the investigation of sympathetic actions. Dibenamine has the disadvantage of being relatively insoluble and unstable in water but it is fairly soluble in propylene glycol or alcohol. It blocks and often reverses the pressor responses to both adrenaline and sympathetic nerve stimulation, and also other  $\alpha$  effects (Ahlquist, 1948). It is ineffective against  $\beta$  effects.

#### Potentiators of adrenergic effects.

The best known drug having this action is Cocaine, which has been very useful in laboratory investigations. The action was first reported by Fröhlich and Loewi, (1910), and confirmed by many others including Burn and Tainter (1931) and Rosenblueth and Cannon (1936). It has been used routinely to sensitise the denervated nictitating membrane, a test organ in sympathin studies, Rosenblueth, 1932). Cocaine antagonises tyramine (Tainter and Shoemaker, 1925), and ephedrine (de Eds 1927), facts of importance in the identification of/  
of/

of sympathin. This effect was confirmed by Burn and Tainter (1931). Several other local anaesthetics have been found to have a potentiating action like cocaine (Bacq and Lefébvre, 1934). The effects of cocaine on the actions of adrenaline and noradrenaline on the rat's blood pressure are reported in the present work.

## V. Chemical tests for adrenaline and allied substances.

These may be classified to simplify description into the types of reactions used.

A. Oxidation methods. These depended on the formation of red oxidation products with various reagents.

1. Inorganic. Mercuric chloride (Comessatti, 1908), ferric sulphate (Colin, 1856), iodine (Abelous, Soulie and Toujan, 1905), potassium iodate (Fränkel and Allers, 1909), potassium permanganate (Zanfrotnini, 1909), potassium persulphate (Ewins, 1910), potassium ferricyanide (Cevidalli, 1908), copper sulphate, platinum chloride, potassium chlorate, hydrogen peroxide (Fränkel and Allers, 1909), sodium nitrite (Berberg, 1912), gold chloride (Gautier, 1912), manganese dioxide (Seidell, 1913) and nitric acid, sodium persulphate and silver oxide are all stated to have been used. The tests were not very sensitive and could only be used for the amounts found in the adrenal glands but not for those found in body fluids. Barker, Eastland and Evers, (1932), investigated the existing methods and found that the potassium persulphate reaction was the most specific (although given by noradrenaline and epinine as well) and gave the best agreement with biological tests/

tests, if the pH and temperature were properly controlled.

To increase the sensitivity of the potassium iodate reaction sulphanic acid was added (Bayer, 1909), but there was some loss of specificity. For the same purpose Stuber, Russmann and Pröbsting, (1923) added mercuric chloride. Viale (1933), who had found (1930) that compounds chemically similar to adrenaline gave the iodate-sulphanilic acid reaction added formaldehyde to control blood samples to distinguish adrenaline from the substances which gave the reaction. Using this method Viale and Crocetta (1933), estimated adrenaline in dogs blood as 70-250 $\mu$ g. per cent and Dogliotti and Crocetta (1933), as 250-330 $\mu$ g. per cent. Bacq (1933) used it for heart extracts. It is still in use in a modified form (Jackerott, 1941).

The ferric chloride reaction has been applied to the estimation of adrenaline in blood (Battelli, 1902), and it has been used quantitatively and compared with assays with the dog's blood pressure, (Mercier, 1944).

The iodine method has been developed with more accurate spectrophotometric techniques by Euler, (1933b) for tissue extracts. The products of oxidation/

oxidation with this reagent were a number of substances, adrenochrome and its relations. They caused inaccuracies in the test. The addition of potassium ferrocyanide limited the oxidation to the stage of adrenochrome alone (Ehrlén, 1948a, and b). Oxidisation with iodine is the basis of a method of estimating adrenaline and noradrenaline when present together (Euler and Hamberg, 1949a, and b).

2. Organic. Preparations of organic oxidases from potatoes, beetle blood and mushrooms have been reported to give a red colour with adrenaline. The reaction has been used to estimate the amine in adrenals (Bhagvat, 1938). A method using a polyphenolase prepared from *Atropa belladonna* has been developed by James (1948), and has been found useful to identify adrenaline and noradrenaline when present together.

#### B. Reduction methods.

Adrenaline reduces phosphotungstic, arsenotungstic and arsenomolybdic acids with the formation of coloured products. Folin, Cannon and Denis (1912), used phosphotungstic acid for uric acid determination. Maiweg (1922) showed its/

its applicability to solutions of adrenaline treated with Folin's phosphotungstic acid reagent and saturated sodium carbonate to neutralise and develop the colour. It was sensitive to a concentration of  $3 \times 10^{-6}$ , but Barker, Eastland and Evers (1932) found it unspecific owing to the presence of other reducing substances such as ascorbic acid and glutathione (Euler, Burström and Hallström, 1932, Devine, 1937). Its great virtue however, was its convenience (Baker and Marrian, 1927).

The decolourisation of methylene blue by reducing substances in special tubes to prevent autoxidation by air (Thunberg, 1918 system) has been used for adrenaline, chiefly by Euler (1933c) who found it sensitive but inaccurate. Shaw's (1938) method is described in the next section.

#### C. Adsorption methods.

These use chemical or biological tests after prior adsorption of the amines.

Silicic acid. The selective adsorption of adrenaline, separating it from non-basic and strongly basic reducing substances by silicic acid was used by Whitehorn (1925). After subsequent acid elution the adrenaline was estimated by a chemical reduction method/

method (arsenomolybdic acid). Only 70% of the adrenaline was adsorbed and there was further loss owing to incomplete elution.

Aluminium hydroxide. The disadvantages of Whitehorn's method were reduced by Shaw (1938) who replaced the silicic acid with aluminium hydroxide. Interfering substances are adsorbed at pH 4, at which pH adrenaline and related amines are not adsorbed. Adsorption of these amines takes place only at pH 8. Complete recovery was obtained by dissolving the alumina with the adsorbed substances in the reagents used for colorimetric estimation (with arsenomolybdic acid). The method was sensitive, recovering 0.04 $\mu$ g. if present in a concentration of more than  $5 \times 10^{-8}$ , and agreed with estimates with the spinal cat's blood pressure. A specific test for adrenaline was also described. Studies on the method are reported in the present work.

Bloor and Bullen (1941) found adsorption by Shaw's method incomplete and attempted modifications. It has been used extensively by Raab (1941, 1943a and b), and Raab and Humphreys (1947). Recently, Euler (1948, 1950d) obtained better adsorption of small amounts by precipitating alumina in the test solution/

solution itself from aluminium sulphate and sodium hydroxide. The precipitate and adsorbed amines were then filtered off and dissolved in acid. After further treatment a solution for chemical and biological assay was obtained.

Other adsorbents have not been so useful, but the fact that adrenaline and particularly noradrenaline are not adsorbed by fullers' earth has been used in the purification of tissue extracts (Euler, 1946a, b and c) to yield them in a solution free of depressor substances, for testing with the cat's blood pressure and by other methods.

The separation of adrenaline and noradrenaline by adsorption on Amberlite I R C 50 and elution therefrom with 0.1 N. sulphuric acid has been described.

(Bergström and Hansson, 1951)

#### Chemical tests for mixtures.

James (1948) used an enzymatic method by incubating adrenaline with a suspension of a polyphenolase prepared from dried *Atropa belladonna* leaf and root. After ten minutes at 30°C. a bright orange red colour of adrenochrome, stable for several hours, appeared./



appeared. Noradrenaline treated similarly gave a pale brown precipitate at the bottom of the test tube, settling in a dark layer. Methyl adrenaline gave no colour, while 3,4 dihydroxytyramine, DOPA, corbasil, epinine and N-methyl corbasil gave other and transient colours. Mixtures of adrenaline and noradrenaline gave a pale brown colour followed by a stable red colour. This sequence was not obtained for any one substance. The method was insensitive not less than 20µg. adrenaline being required.

It was pointed out by Abelous and Soulie (1922) that the chemical tests depend on the catechol group in the molecule. Quantitative methods of estimation of adrenaline and noradrenaline when present together must therefore depend on the differences in the rates or extent, of chemical reaction which may be determined by the presence or absence of a methyl group attached to the N atom of the side chain. It is possible that chemical tests can be developed which will be given by one substance and not the other, such as a reaction to distinguish between primary and secondary amines. This aspect does not appear to have received much attention.

Auerbach/

Auerbach and Angell (1949), used a method of determining noradrenaline in the presence of adrenaline. This depended on the formation of a red colour with sodium  $\beta$ -naphthoquinone-4-sulphonate and benzalkonium chloride.

Schuler and Heinrich (1949), used 4-nitro-2-chloro-1-diazobenzene- $\beta$ -naphthalene sulphonate (NNCD reagent) which gives a yellow colour with catechols. Adrenaline gives a stronger colour than noradrenaline at a strongly acid reaction, but a weaker colour than noradrenaline at a weakly acid reaction. By working at different pHs it has been possible to estimate noradrenaline in the presence of adrenaline (Heinrich and Schuler, 1950).

Euler and Hamberg (1949a and b) developed a method depending on the formation of adrenochrome and noradrenochrome with iodine. The rates of reaction vary with the pH. At pH 4 the adrenaline is completely converted to adrenochrome in 1.5 min. but only about 10 per cent of the noradrenaline is converted to noradrenochrome. Both amines react completely at pH 6 in 3 minutes. The solution to be tested is buffered (acetate) to pH 4 and treated with iodine for 1.5 minute. The excess iodine is removed/

removed with sodium thiosulphate and the colour reading taken against a blank (without iodine). A second test is then carried out on another portion of the solution but at pH 6 and with iodine for three minutes. The first reading gives a measure of the adrenaline with about 10 per cent of the noradrenaline, and the second the total amines. By mathematical formulae the amounts of each are determined. Noradrenaline was reported in the adrenals of cattle from determinations with this method.

All these tests are relatively insensitive, some 20-40µg. of the combined amines being the minimum amount needed.

#### Separation methods.

Several of these have already been mentioned in the preceding pages.

Fractional crystallisation. This was used by Tullar (1949) to separate adrenaline and noradrenaline occurring naturally. It uses the different solubilities of the tartrates in methanol.

Adsorption. A separation method using adsorption on Amberlite I R C 50 and elution with sulphuric acid has been developed by Bergström and Hansson, (1950)./

(1950).

Counter current distribution. Bergström, Euler and Hamberg (1950), produced partition of a mixture between phenol and 0.02N.HCl and applied the counter current technique through ten separating funnels. Most of the adrenaline was distributed in the phenolic phase and most of the noradrenaline in the aqueous phase. Pure noradrenaline was obtained in this way from extracts of cattle adrenals.

Chromatography. James (1948), developed a method using the one way method of Consden, Gordon and Martin (1944) for the chromatography of adrenaline and noradrenaline on paper. The solvent was phenol. After development of the chromatogram and removal of the solvent the positions of the amines were detected colorimetrically by spraying the paper with alkaline potassium ferricyanide. Adrenaline gave a bright red spot and noradrenaline a purplish red spot, owing to the formation of their respective "chrome" derivatives. Adrenaline moved more rapidly on the paper than noradrenaline. Related amines also gave similar colour reactions but generally travelled at different rates except in the case of DOPA which may have been similar to noradrenaline./

noradrenaline. Doubt was resolvable with James' enzyme test. Goldenberg, Faber, Alston and Chargaff (1949) used James' method quantitatively to determine noradrenaline in U.S.P. adrenaline and reference standard which were prepared from adrenal glands. Comfort and d'Silva (1950) increased the sensitivity of James' test by developing the chromatogram, drying the paper and examining it in ultraviolet light with a Wood's glass filter. Adrenochrome was decolourised to a yellow fluorescent substance. Up to 2 ug. of adrenaline or noradrenaline per sq cm. were detectable.

A quantitative method of separation of adrenaline and noradrenaline using the principle of James' (1948) chromatographic method but in which the amines are assayed biologically, has been developed (Crawford and Outschoorn, 1951) and is described in the present work.

Fluorescence tests. It was shown that a weak solution of adrenaline gave a yellowish green fluorescence with alkali (Loew 1918) and that this was specific for adrenaline (Paget, 1930). Barker, Eastland and Evers (1932), found the reaction unsuitable for quantitative work. Gaddum and Schild (1934)/

(1934) developed it to a sensitive test for adrenaline, detecting  $10^{-8}$  of that amine. They showed that the fluorescence of DOPA, noradrenaline, epinine and catechol was less than 3 per cent of that of adrenaline. Tyramine and ephedrine gave no fluorescence. Serum gave a blue colour which obscured the green fluorescence. Deproteinising with trichloroacetic acid removed the interfering blue but at the same time removed some of the adrenaline as well.

Many attempts were made to improve this test and to make it suitable for use with serum or plasma: dialysis and testing of the dialysate (Kalaja and Savolainen, 1941), and use of a suitable filter in a Pulfrich photometer (Lehmann and Michaelis, 1942) without dialysis. Jørgensen (1945), examined the rates of appearance and disappearance of the fluorescence and found that they depended on the pH, and presence of heavy metals. He also studied the specificity. West (1947), modified some of Jørgensen's improvements and with the use of the method and of Shaw's (1938) and biological tests, showed that adrenaline only (not noradrenaline) was present/

present in normal rabbit's blood. The nature of the fluorescing compound has received much attention. Lund (1949), believes that it is derived from adrenochrome by intramolecular re-arrangement at alkaline reaction. His method of estimation by fluorescence had more of the conditions standardised than those of previous workers. He adsorbed the adrenaline on a column of alumina, eluted it with acetic acid, oxidised it with manganese dioxide to adrenochrome and converted the last to the fluorescing compound (adrenolutine) with alkali. Only adrenaline, propynaline and noradrenaline gave fluorescence under these conditions. At pH 3 only 5 per cent of noradrenaline was converted to the adrenochrome, while at pH 6.5, 100 per cent conversion took place. Adrenaline was completely converted at pH 3-7. This is the basis of a method which Lund (1950), developed for the simultaneous fluorimetric determination of adrenaline and noradrenaline in blood.

PART II.EXPERIMENTAL.

- A. Studies on the methods used in the identification and estimation of sympathin and the adrenal medullary hormones.

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### I. Chemical.

The only chemical test used in the present work was that of Shaw (1938). It was applied almost exactly as in the original description.

The principle of the test is to carry out two adsorptions with alumina. The first is at pH 4 and the adsorbed material is discarded. The second is at pH 8 and the substances adsorbed include adrenaline and related amines. The whole precipitate after the second adsorption, is dissolved in, and subjected to the action of alkali.

In these experiments, soon after collection, a suitable volume of the solution to be tested (about 5 ml.) was added to an equal volume of trichloroacetic acid and subjected to the procedure of the test. Usually, in addition to the test solutions 5 ml. of distilled water (blank) and 5 ml. each of solutions of adrenaline containing 10µg./ml. and 25µg./ml. (standards) were treated in the same way.

The alkali treatment after the second adsorption was timed exactly with a stop watch. Up to eight tubes could be treated at a time by adding the alkali in/

in succession every 15 seconds. Two minutes after beginning the alkalinisation the first tube was acidified and this process continued every 15 seconds so that the alkali had acted in each case for exactly 2 minutes. Eight tubes of the colour reagent were kept ready in a boiling water bath and by rapid manipulation the contents of the eight sample tubes were added to them as quickly as possible.

The colour readings were taken on the Spekker absorptiometer by comparison with the blank. Calculations of the amounts of adrenaline were made by comparison with whichever of the standard adrenaline tubes gave the nearer reading to the test solution. Shaw's specific test for adrenaline.

In the method used, all the samples, the blank and the standards, were subjected to the action of alkali in order to obtain the maximum intensity of colour. Shaw had, however, described a test in which the solution to be tested was divided into two. One half was treated as described. For the other half, instead of using alkali to dissolve the alumina after the second adsorption, slightly acidulated water was added. Other treatment was the same.

Shaw/

Shaw found that the ratio of the colour given by the half which was alkali treated after adsorption to that given by the acid treated half, was 2-3.5. This increase in colour with alkali treatment was not given by noradrenaline. If solutions were tested directly without adsorption the ratio of the colours with and without alkali, was five for adrenaline and one for noradrenaline. The ratio of amounts of noradrenaline and adrenaline giving equivalent colour after alkali treatment was sixteen.

West (1947), also found no increase in colour with noradrenaline but obtained the slightly smaller noradrenaline/adrenaline ratio of 13.5. Verly (1948), however, obtained a 2-4 fold colour increase for noradrenaline and an even greater increase (4-8 fold) after alkali treatment of adrenaline. The noradrenaline/adrenaline ratio for equivalent colour was five if acid treated and sixteen if alkali treated.

An attempt was made to re-determine some of these figures.

#### Experiment./

Experiment.

Tubes containing 5 ml. each of standard adrenaline 10 $\mu$ g./ml. and 25 $\mu$ g./ml. and standard noradrenaline 150 $\mu$ g./ml. were alkali treated; while 5 ml. each of adrenaline (10 $\mu$ g./ml.) and noradrenaline (150 $\mu$ g./ml.) were acid treated. In addition a blank was tested which contained 5 ml. of water.

The table shows the results obtained compared with those of the workers quoted above.

<u>Ratio</u>		<u>Shaw</u>	<u>West</u>	<u>Verly</u>	<u>Present expt.</u>
Colours of alk. treated/ acid treated portions:-					
After adsorption	Adr.	2-3.5	-	4-8	6-10
	Nor.	1	1	2-4	~4
Directly	Adr.	5	-	-	-
	Nor.	1	-	-	-
Nor./Adr. for equivalent colour:-					
<u>Alkali treated:</u>					
After adsorption		-	13.5	16	6-15
Directly		16	-	-	-
<u>Acid treated:</u>					
After adsorption		-	-	5	<15

The results indicate that the ratios are liable to vary under different conditions and that estimates made/

made with the specific test must be checked against other tests. The specific test was not used in the present work, but use was made of Shaw's ordinary test in which all samples were alkali treated.

II. Biological.A. Methods of assay.

These tests have been described by Gaddum, Peart and Vogt (1949).

The rat's uterus. About 3-4cm. of the ovarian end of the uterus of an adult female rat which was either virgin or had not been pregnant for eight weeks, were suspended in a 2ml. bath (about 6cm. long) and kept at 30°C. The solution was Locke's (modified) containing NaCl 9, KCl. 0.42, CaCl<sub>2</sub>.0.06, NaHCO<sub>3</sub> 0.2 and Glucose 0.5g./litre. Doses of acetylcholine (0.5-2µg., usually 1µg.) were added at intervals of 2 minutes and allowed to act for 30-40 seconds. The bath was then emptied and refilled twice. The addition of a suitable dose of adrenaline one minute before a particular dose of acetylcholine inhibited that member of the series of regular contractions produced. The amount of inhibition was a measure of the amount of adrenaline added and this was estimated by comparison with standard adrenaline. Generally, the contraction next to an inhibited one showed slight inhibition as well but usually recovery was complete by the third contraction.

The/

The rat's colon. About 3-4cm. of the ascending part were set up similarly. The temperature of the bath was lower - 25°C. and sometimes the calcium content of the solution had to be reduced still further ( $\text{CaCl}_2$  0.03g./litre) to abolish spontaneous movements. The assay procedure was the same, but the dose of acetylcholine was much smaller (0.01-0.1  $\mu\text{g.}$ , usually 0.03 $\mu\text{g.}$ ).

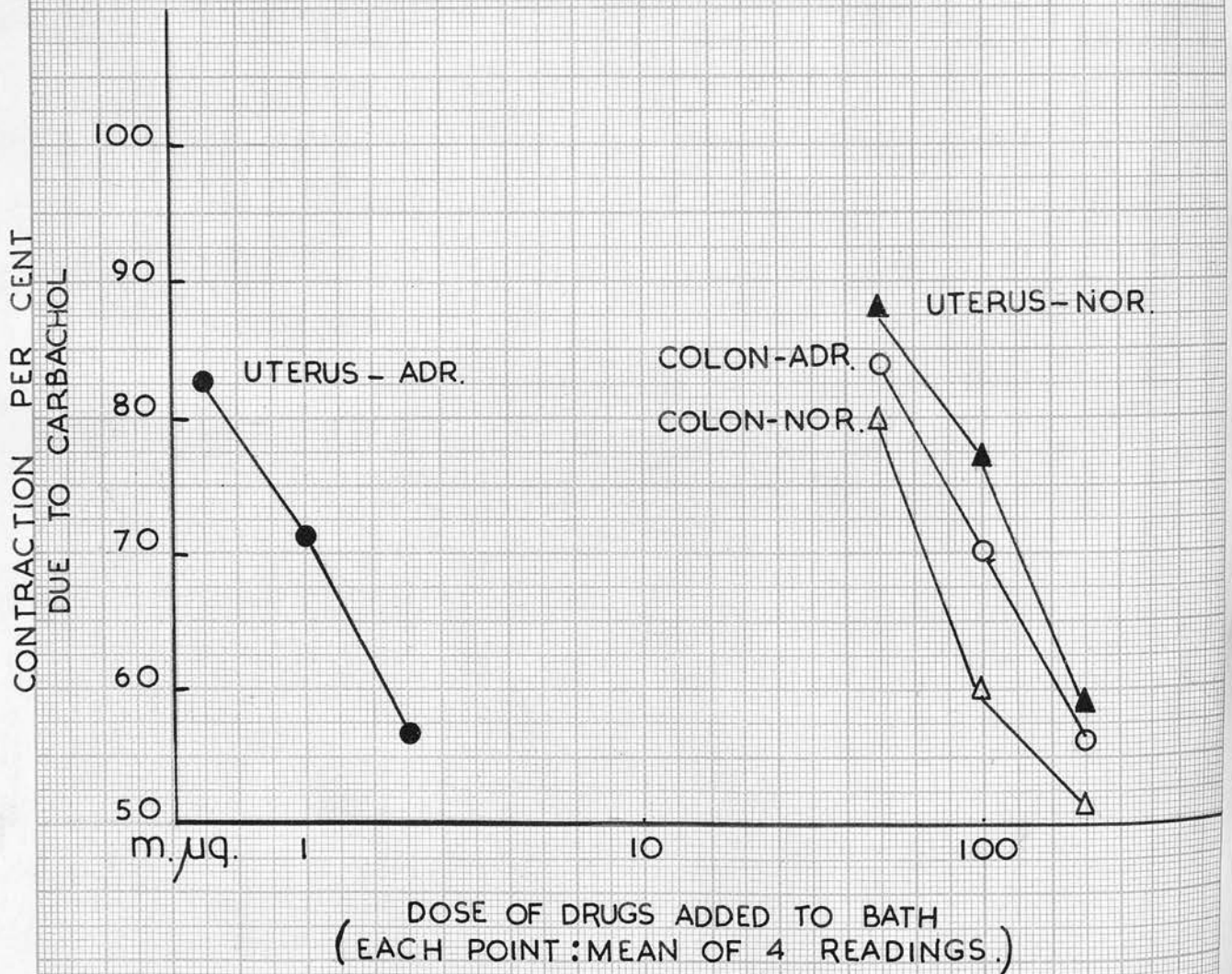
Semi-automatic apparatus. This has been described by Gaddum and Lembeck (1949). Here the organ bath was never emptied but the solution was changed by running in fresh Locke from below. The old solution was removed by overflow, the level of the liquid being kept constant by an overflow tube connected to a suction pump. Instead of acetylcholine, carbachol was used in the same dosage (1mg./litre for the uterus and 10 $\mu\text{g.}$ /litre for the colon) dissolved in the Locke's solution in one reservoir, while the washing out solution (plain Locke's solution) was in another. The flow was controlled by electric relays which compressed pieces of rubber tubing leading from the reservoirs.

Tracings/

FIG. 11.

RATS UTERUS AND COLON

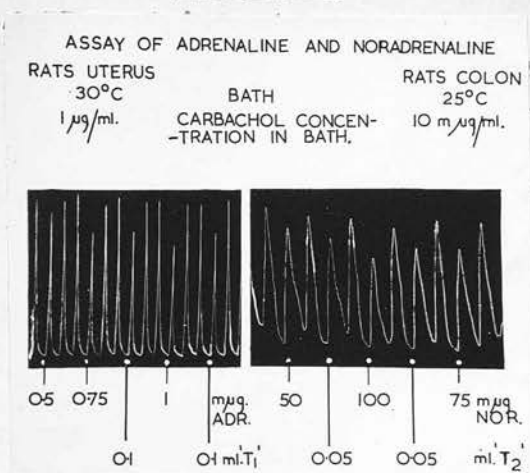
RELATION BETWEEN LOG DOSE AND EFFECT  
IN USUAL ASSAY RANGE.





Tracings of assays with these tissues are reproduced below. Least time was needed for assays when the doses of test and standard adrenaline (or noradrenaline) produced inhibitions less than 50 per cent of the normal contractions. Dose-response curves in this range are shown in the graphs alongside.

Fig. III.



Rat's uterus:

0.1ml. of solution T<sub>1</sub> gave an inhibition lying between that of 0.5µg. and 1µg. and equal to that of 0.75µg. of standard adrenaline.

Rat's colon:

0.05ml. of solution T<sub>2</sub> gave an inhibition lying between that of 50µg. and 100µg. and equal to that of 75µg. of standard noradrenaline.

In/

In the semi-automatic apparatus the added adrenaline was washed out of the bath as soon as the carbachol came in, whereas in the hand operated system the inhibitory amine acted throughout the contraction and was washed out with the choline ester. This did not appear to affect the sensitivity of the test.

Using this apparatus assays could be carried out on both the uterus and the colon at the same time, the baths being placed conveniently alongside on the laboratory bench shortening the time required to carry out the assays.

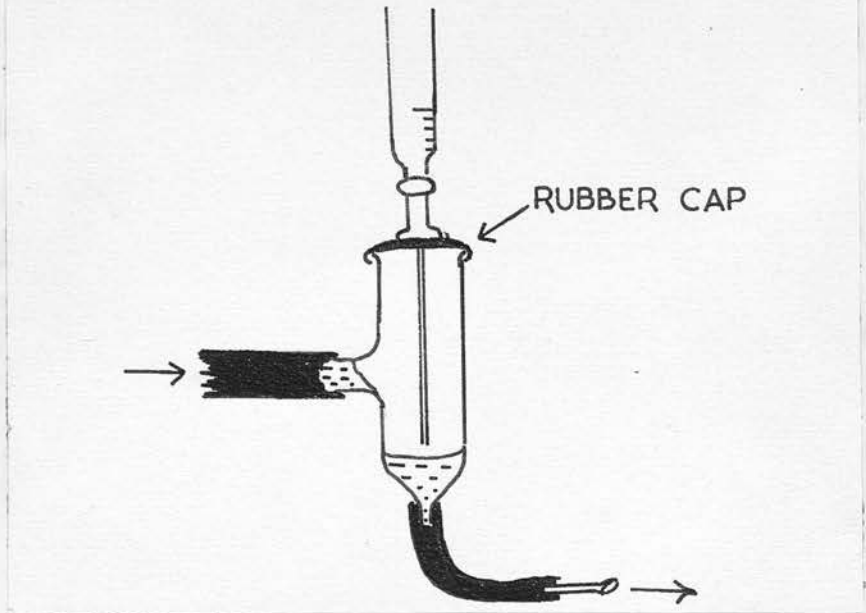
#### Storage of tissues.

Both the uterus and the colon could be used satisfactorily after 24 hours storage in the refrigerator. The colon was often more sensitive and less liable to spontaneous movements when so used.

#### The rabbit's ear.

Method. When this preparation was set up for assays only, the complete procedure for nerve stimulation experiments ( vide pp. 153-156 on rabbit's ear sympathin) was not followed. Instead, the animal was killed by stunning and immediately decapitated. The soft tissues were divided with a scalpel and the vertebral column with bone forceps. A special/

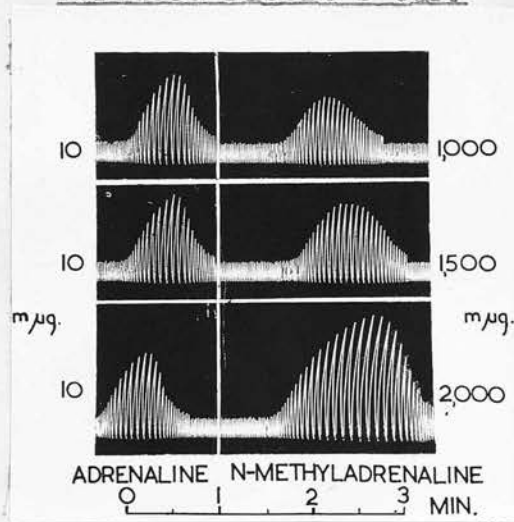
PERFUSED RABBITS EAR.



Special arterial cannula for injection of drugs.

Fig.V

Perfused rabbit's ear.



Comparison of adrenaline and N-methyladrenaline.  
Height of record measures time interval between drops.  
10 mμg. of adrenaline had an effect roughly equal to that of 1,500 mμg of N-methyladrenaline.

special cannula (described below) was tied into one common carotid artery and perfusion started with Locke's solution from a reservoir about 3 feet above the head, so that the blood in the vessels was washed out before clotting occurred.

At this stage the head was placed on a tray into which the perfusate flowed. While the perfusing fluid was passing through the vessels the carotid artery was followed upwards and all its branches except the one to the ear, tied off. The opposite carotid was also ligatured. The perfusing fluid then flowed only through the ipsilateral ear.

The great auricular vein was next identified and a fine straight cannula tied in. The outflow was led from this into the silver tubing which forms one of the contacts of Gaddum's drop recorder (Gaddum and Kwiatkowski, 1938).

Gaddum and Kwiatkowski's special arterial cannula is illustrated in Figure IV. It enabled drugs to be injected through the rubber capped top so that they did not mix back into the perfusing fluid.

The record of the outflow was a series of vertical lines the height of which represented the intervals between drops. When adrenaline or other vasoconstrictor substances were injected, the heights of/  
of/

of the lines increased owing to lengthening of the intervals between drops of the effluent (see Figure V).

Injections were usually made every five minutes and when given had to be at a slow rate so that the level of the meniscus in the arterial cannula did not change.

The sensitivity of this preparation increased considerably (often tenfold) when it had been perfused slowly overnight. Owing to this, fresh ears which had been used for nerve stimulation experiments were often kept till the next day for assay purposes.

The perfusion was carried out at room temperature throughout.

Method of estimation of adrenaline and noradrenaline in mixtures.

This may be effected by carrying out parallel assays on two or more tissues which vary in their sensitivity to the two amines independently. For the three biological tests described figures obtained by Gaddum, Peart and Vogt (1949), are shown: /

shown:

Test	Ratio of equally active doses: 1 ' Nor./1'Adr.	Adrenaline Mean dose for a definite effect (m $\mu$ g)
Rat's uterus.	100 (75-300)	0.25
Rat's colon	0.75 (0.2-1)	25
Rabbit's ear (24-hrs old)	2 (0.75-4)	2

Calculation of the amounts of the two substances in the mixture may be made from the results of the assays by means of suitable formulae. These have been devised by Gaddum and Lembeck(1949) and applied to assays on the uterus and colon. As pointed out by them, Euler (1950b) and Bülbring (1949) independently devised similar formulae.

Calculation.

Notation:	Equivalentents by assay:-		Actual Conc.
	Uterus	Colon	
Adrenaline.	Au	Ac	A
Noradrenaline.	Nu	Nc	N
Ratio of doses of noradrenaline- to equivalent doses of adrenaline	Ru (=Nu/Au )	Rc (=Nc/Ac )	

All figures substituted for these symbols should be in/

in the same units ( $\mu\text{g.}$  are convenient;  $1\mu\text{g.}=0.001\mu\text{g.}$ )

Formulae:

$$A = A_u - A_c R_c / R_u \dots \dots \dots (1)$$

$$N = N_c - R_c A_u \dots \dots \dots (2)$$

Statistical analysis. Using a suitable experimental design, a method of assay which obviates the necessity for a complete analysis of variance has been described by Noel (1945) and this has been applied by Gaddum and Lembeck to assays on the rat's uterus and colon. It has been used in the present work with minor modifications. An example which compares the activities of adrenaline and noradrenaline on two tissues is shown in Table II. Two doses of a standard solution of adrenaline ( $S_1$  and  $S_2$ ) and two of the unknown solution ( $U_1$  and  $U_2$ ) so chosen that  $S_2/S_1 = U_2/U_1 = 2$  and that  $U_1$  and  $U_2$  give approximately the same effects as  $S_1$  and  $S_2$  respectively, are administered in the test. The four doses constitute a set and are randomised within it. The sets are repeated four times, the doses within each set being in a different order to that of the other sets. A Latin square is the most convenient way of accompli-

shing this, e.g.,

ABCD
CDAB
BADC
DCBA

the dose assigned to each letter originally being determined by drawing slips from a hat or by using a

table /

Table II.

Ratio of equally active doses.

Calculations for uterus.

Standard : Adrenaline  
0.5 and 1.0 µg.

"Unknown" : Noradren-  
aline.  
50 and 100µg.

Calculations for colon.

Standard : Adrenaline  
50 and 100µg.

"Unknown" : Noradren-  
aline.  
50 and 100µg.

N= 4 sets of results		Calculations for uterus.				Calculations for colon.			
		1	2	3	4	1	2	3	4
Mean of contractions before U or S (in mm.)		59.6	58.9	57.1	54.6	43.2	42.2	38.6	37.6
S <sub>1</sub> } S <sub>2</sub> }	Effects of Standard	Contractions after							
		U or S (as % of mean).							
U <sub>1</sub> } U <sub>2</sub> }	Effects of "Unknown"	91.4	75.6	84.2	81.5	76.8	81.5	84.8	92.7
		77.2	75.5	68.3	65.9	61.8	72.0	73.4	76.1
		90.6	90.8	87.0	84.4	79.8	76.1	78.9	84.5
		79.4	77.1	73.6	76.0	63.3	43.3	64.1	70.4
D <sub>1</sub>	U <sub>2</sub> - S <sub>2</sub>	2.2	1.6	5.3	10.1	1.5	-28.7	-9.3	-5.7
D <sub>2</sub>	U <sub>1</sub> - S <sub>1</sub>	-0.8	15.2	2.8	2.9	3.0	-5.4	-5.9	-8.2
D <sub>3</sub>	U <sub>2</sub> - U <sub>1</sub>	-11.2	-13.7	-13.4	-8.4	-16.5	-32.8	-14.8	-14.1
D <sub>4</sub>	S <sub>2</sub> - S <sub>1</sub>	-14.2	-0.1	-15.9	-15.6	-15.0	-9.5	-11.4	-16.6
y <sub>1</sub>	D <sub>1</sub> + D <sub>2</sub>	1.4	16.8	8.1	13.0	4.5	-34.1	-15.2	-13.9
y <sub>2</sub>	D <sub>3</sub> + D <sub>4</sub>	-25.4	-13.8	-29.3	-24.0	-31.5	-42.3	-26.2	-30.7
y <sub>3</sub>	D <sub>1</sub> - D <sub>2</sub>	3.0	-13.6	2.5	7.2	-1.5	-23.3	-3.4	2.5
T <sub>1</sub>	S(y <sub>1</sub> )	Sample difference		39.3		-58.7			
T <sub>2</sub>	S(y <sub>2</sub> )	Dose difference		-92.5		-130.7			
T <sub>3</sub>	S(y <sub>3</sub> )	Slope difference		-0.9		-25.7			
I	Log. ratio of doses	Log. 2		0.3010		0.3010			
M/									



Table II. (Contd.)

Ratio of equally active doses.

Calculations for uterus.

Calculation for colon.

Standard : Adrenaline  
0.5 and 1.0µg

Standard : Adrenaline  
50 and 100µg.

"Unknown" : Noradrenaline  
50 and 100µg.

"Unknown" : Noradrenaline  
50 and 100µg.

M	$T_1 I / T_2$	Log potency of U/S	0.1279	0.1352
R	100 antilog M	Potency of U (% of S)	134.3	136.6
b	$T_2 / 2IN$	Slope	-38.41	-54.28
A	$S(y_1)^2 + S(y_2)^2 + S(y_3)^2$	-	3,041	6,581
B	$S(T_1^2 + T_2^2 + T_3^2)$	-	10,102	21,189
s	$\sqrt{(A - B/N) / 12 (N - 1)}$	S.D. of effect	3.7840	5.9722
$\lambda$	s/b	S.D. of log. dose	0.09852	0.11002
$\lambda_M$	$\lambda \sqrt{(1 + T_1^2 / T_2^2) / N}$	S.D. of M	0.05352	0.06030
-	$(100 \text{ antilog. } \lambda_M) - 100$	Coefficient of variation (%)	13.1	14.9
-	$(1 - \text{antilog. } \lambda_M) R \text{ or } 2.303 \lambda_M R$	S.D. of R	16.6	19.0
t	$T_3 / 2s \sqrt{N}$	-	0.059	1.076
r	$\frac{\text{low dose unkn.}}{\text{low dose std.}} = \frac{\text{high dose unkn.}}{\text{high dose std.}}$	Ratio of amounts compared	100	1
-	$(100/R)r$	Ratio of equally active doses (Nor./Adr.)	74.5	0.73

spontaneous movements and relaxed the organ, but that adrenaline was decidedly more effective in this respect.

As the organ is very liable to show excessive spontaneous activity de Jalon, Bayo and de Jalon (1945) recommended the procedure of lowering the temperature and the calcium content of the Locke's solution in which it was suspended. This reduced the organ to quiescence and it was possible by causing contractions with acetylcholine to show that those contractions could be inhibited by adrenaline. Their method was, however, only qualitative.

West (1947) found that of many test objects studied, the rat's uterus (non-quiescent) was the most sensitive to adrenaline, 0.1 $\mu$ g. causing a just submaximal inhibition. It was much less sensitive to dl'noradrenaline, 30-100 times a particular amount of adrenaline being needed for equal inhibition.

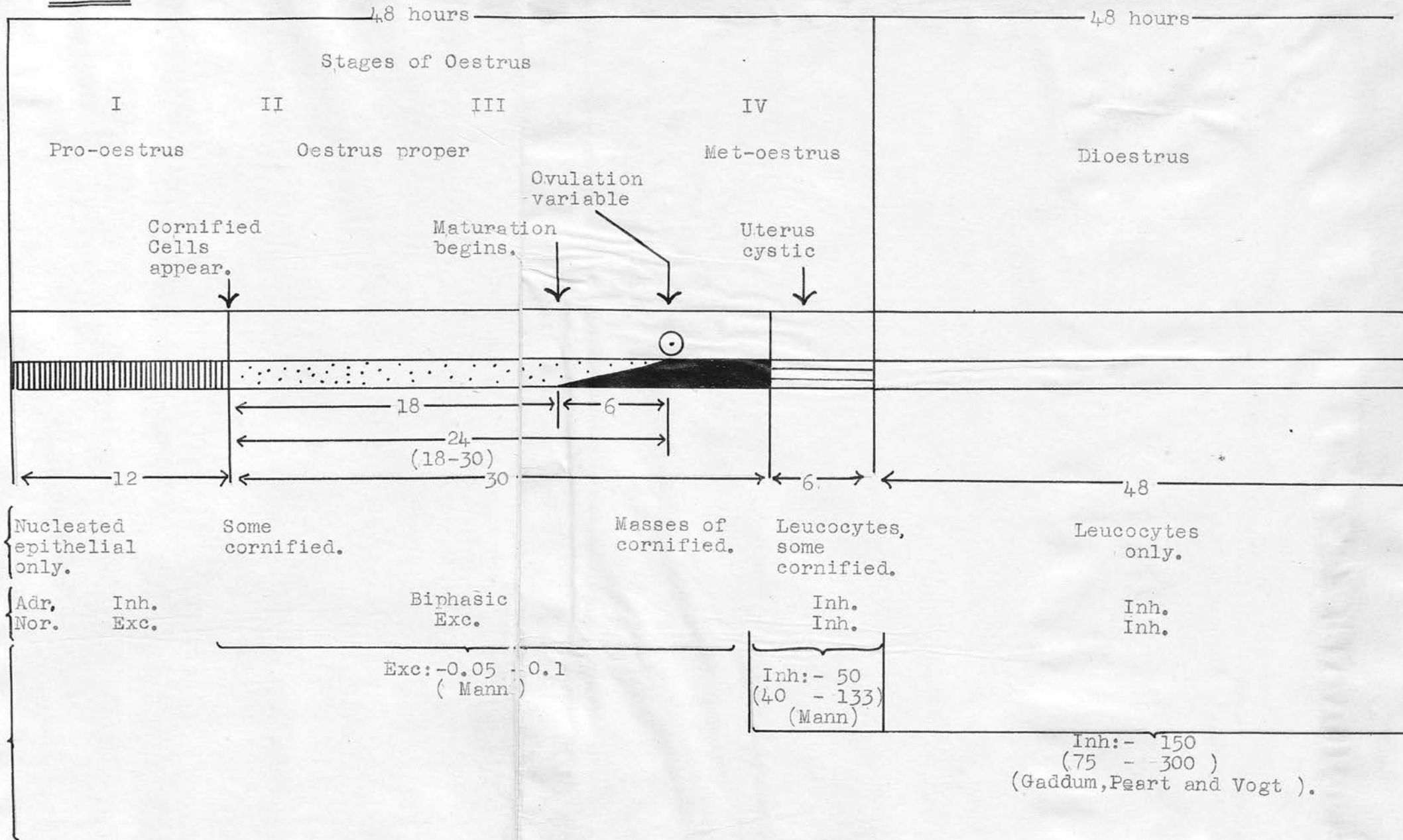
Gaddum, Peart and Vogt (1949) made use of this organ for quantitative estimation of adrenaline and allied substances as described in the preceding section.

#### Relation of response to sexual state of the rat.

The experiments mentioned so far, have all been on the non-pregnant uterus and without relation to the oestrus cycle of the animals. In fact, under the/

FIG. VI

OESTRUS CYCLE OF THE RAT.



the conditions of Gaddum, Peart and Vogt's test the state of the cycle did not affect the assays.

The relation of the responses to adrenaline and noradrenaline of the rat's uterus under different conditions and at different stages of the cycle and in pregnancy was reported by Mann (1949).

Oestrus cycle of the rat. One part of the accompanying diagram (Figure VI) of the rat's oestrus cycle which has been constructed from the description of Long and Evans (1922) shows the changes in the vaginal smear by which the different phases of oestrus may be identified.

In Mann's experiments, the uterus was suspended in a 15ml. bath in oxygenated Tyrode's solution.

The main event occurring in oestrus was the development of a state of contractility of the organ in response to adrenaline and noradrenaline. For adrenaline, this effect was confined to stages II and III of oestrus (when cornified cells were present in the vaginal smear) and to the majority of the uteri, some being inhibited with small doses. The excitatory response required a mean dose of 20 $\mu$ g. adrenaline added to the bath. For noradrenaline the uterus invariably contracted from the onset of oestrus on the addition of a mean dose of 0.5 $\mu$ g. noradrenaline to/

to the bath.

In late oestrus and throughout dioestrus both adrenaline (mean dose of  $0.03\mu\text{g.}$ ) and noradrenaline (mean dose of  $1.5\mu\text{g.}$ ) inhibited the spontaneous movements and relaxed the uterus. These inhibitory amounts and ratios are similar to those found by Gaddum, Peart and Vogt, (1949).

The changes in the responses are shown in the lower part of the accompanying diagram. (Fig. VI).

That the changes were due to hormonal differences was shown by the fact that uteri of immature and ovariectomised rats behaved like those of rats in dioestrus, but if oestrus were artificially induced by injected oestrogens the responses were identical with those of rats in natural oestrus. If however, immature rats were given oestrogens but the onset of oestrus was prevented by the simultaneous administration of progesterone, the responses were like those of adults in dioestrus.

In view of the development of the great sensitivity to noradrenaline during oestrus, it was decided to investigate these phenomena further.

#### Excitatory effects.

Method. Rats were selected in which the vaginal smears contained large amounts of cornified cells./

cells. If there were both cornified cells and leucocytes in the smear it meant that it was doubtful whether the rat was in late oestrus or in dioestrus. Such animals in which a definite decision could not be made were not included.

As soon as a rat was identified as in oestrus proper it was killed and the uterus suspended in the 2ml. bath used routinely for assays.

At first, the solution at 30°C. and containing one quarter the amount of calcium (NaCl 9, KCl. 0.42, CaCl<sub>2</sub> 0.06, NaHCO<sub>3</sub> 0.2, Glucose 0.5g./litre) was tried. In this, there were no spontaneous movements but there were no excitatory responses to added adrenaline and noradrenaline either. When the calcium content was increased to the usual amount (CaCl<sub>2</sub> 0.24g./litre) and the temperature raised to 32°-34°C the uterus contracted when suitable doses of adrenaline and noradrenaline were added. Spontaneous movements were however, troublesome and Tyrode's solution at 32°-34°C was tried, (containing NaCl 8, KCl 0.2, CaCl<sub>2</sub> 0.2, NaHCO<sub>3</sub> 1, MgCl<sub>2</sub> 0.1, NaH<sub>2</sub>PO<sub>4</sub> 0.05, Glucose 1g./litre). This reduced both the spontaneous movements as well as the responses to the amines tested.

The most suitable solution was found to be Locke's/

Locke's containing the full amount of calcium to which had been added magnesium chloride in a concentration of 0.1, 0.05, or 0.025g./litre, whichever was just sufficient to abolish the spontaneous movements. The temperature of 32°-34°C was selected as the highest just not causing spontaneous contractions. Occasionally, an uterus would be quiescent without any magnesium at all.

Solutions of adrenaline and noradrenaline were added to the bath with a tuberculin syringe in a volume not exceeding 0.2ml. The amines were made up in saline containing ascorbic acid  $10^{-5}$ . Instead of saline, bicarbonate-free Locke's solution was tried with the same result. The same solutions, containing ascorbic acid but no amines, had no effect on the tissue.

The added drug was allowed to act for 30-45 seconds and then washed out by flushing the bath three times without emptying it.

Doses were added not oftener than every four minutes. If the schedule was shortened to every two minutes after two or three responses by the uterus one would be missed, as though the muscle were "refractory". This did not occur with a four minute schedule.

Results/

Results. The addition of 10-50 $\mu$ g. noradrenaline usually caused the uterus to contract. Occasionally, as little as 0.5-2 $\mu$ g. was sufficient. Very much larger doses of adrenaline were required (0.5-1 $\mu$ g., occasionally 0.1 $\mu$ g.). The ratio of doses (noradrenaline/adrenaline) which caused contraction was thus 0.1-0.01 and occasionally, even smaller.

The contractions could be elicited for a variable time after setting up the uterus, from two hours to more than five hours. Towards the end of the period, larger doses were required till eventually the tissue became quite insensitive and did not give an excitatory response.

Storage of the tissue. One horn of the uterus of a rat in oestrus was tested soon after killing the animal, whilst the other horn was stored in the refrigerator and tested twenty-four hours later. The stored horn was still stimulated by noradrenaline after that time but required a larger amount of drug. The responses ceased to be elicited more quickly than in the fresh tissue.

Control experiments were carried out in which rats definitely in dioestrus (only leucocytes in the vaginal smear) were selected for testing the uterus. In no case did the uterus contract with noradrenaline (up/

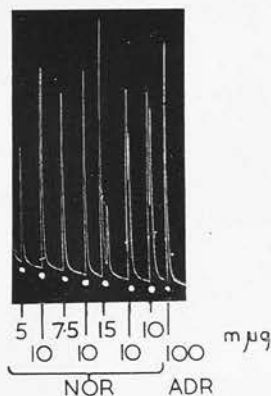


Excitatory effects of adrenaline and noradrenaline.

Isolated uterus of rat in oestrus.

Figure VII

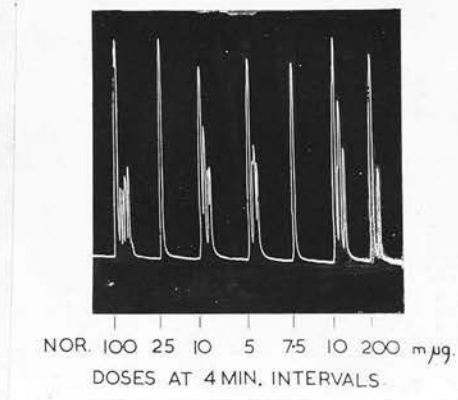
RAT'S UTERUS  
(OESTRUS)  
NORADRENALINE & ADRENALINE EFFECTS.



Contractions caused by the addition of different amounts of noradrenaline and of one of adrenaline.

Figure VIII

RAT'S UTERUS  
(OESTRUS)  
EFFECT OF NORADRENALINE



Contractions caused by noradrenaline - showing that the height of contraction was not related to the amount added (provided an amount sufficient to cause a contraction at all was exceeded).

(up to 200µg.) or adrenaline (up to 1µg.), both of which inhibited the slight spontaneous movements of the tissue.

Use of the excitatory response for quantitative purposes.

Figure VII shows the responses of the uterus (contractions) to different doses of noradrenaline and to one of adrenaline. It would appear at first sight that the responses are graded according to the dose and the ratio of equally active doses (noradrenaline/adrenaline) would be between 0.1 and 0.15. The use of the uterus of the rat in oestrus for assays is based on this and it has been so used by Mann (1949), and Mann and West (1950).

More detailed investigation however, showed that such assays could be misleading. The same uterus which gave the tracing (Figure VII) given a further series of doses of noradrenaline, did not respond at all regularly. Provided a threshold dose of noradrenaline was exceeded the uterus contracted. The height of contraction varied and was not related to the amount of noradrenaline administered. Adrenaline produced similar contractions, but generally, larger amounts were required than of noradrenaline.

The/

The tracing in Figure VIII shows part of an experiment with noradrenaline on another uterus and illustrates this point.

The excitor actions of these amines under the conditions described appeared to depend on some kind of "trigger" mechanism. The only way in which they could be used for a fairly reasonable assay of these substances would be to determine the least amount of standard adrenaline or noradrenaline and of unknown solutions which would produce a contraction at all, but without reference to its height.

Adrenaline-noradrenaline interaction. When a series of contractions produced at intervals of 1.5-2 minutes by doses of noradrenaline was followed by a dose of adrenaline, the amount of adrenaline necessary to excite the uterus was generally smaller than the threshold amount of adrenaline if noradrenaline had not been given previously. On the other hand, if a series of doses of adrenaline which caused contractions of the uterus at the same intervals was followed by a dose of noradrenaline, relatively larger amounts of noradrenaline were required to excite the uterus than were necessary if adrenaline had not been administered previously. That is to say, in a series of regular administrations noradrenaline tended to make the uterus more sensitive to/

to one or two doses of adrenaline but adrenaline tended to reduce the sensitivity to one or two doses of noradrenaline. In either case, the status quo was re-established if administration of the second amine was continued for three or four doses.

Effects of other drugs. Attempts were made to determine whether contractions of the oestrus uterus produced at regular intervals by a fixed dose of carbachol or a fixed electrical stimulus (described below) would be increased by the presence of small amounts of adrenaline and noradrenaline. Addition of increasing amounts of these amines to the bath before one of the stimuli at first, (small doses) did not affect the heights of contraction, but later (large doses) inhibited them. This showed that once the uterus began contracting the only influence to which it was susceptible was an inhibitory one.

The presence in the bath of small amounts of carbachol, insufficient to cause contraction of the uterus, did not affect the power of adrenaline and noradrenaline to produce an excitatory response.

Atropine sulphate (2.5µg./ml.) continuously in/

in the bath did not affect the contraction caused by the sympathomimetic amines, or by electrical stimulation. Carbachol contraction was almost abolished.

Histamine. The rat uterus has been known for decades as the one example of smooth muscle which is relaxed by this drug. The addition of amounts of histamine acid phosphate up to 0.2 $\mu$ g. (as base) to the bath did not cause the quiescent organ to contract.

Inhibitory effects and oestrus. The previous experiments showed that whether a rat was in oestrus or not, contractions of the uterus were inhibited by adrenaline and noradrenaline. Such contractions were suitably produced by a fixed dose of a choline ester or an electrical stimulus. If the rat was in oestrus the amines inhibited the contractions they themselves produced, and this accounts for the briefness of the stimulatory responses.

Ahlquist (1948) points out that the well-known variations in the responses of uteri to adrenaline (and related amines) might be accounted for by the presence of two types of adrenotropic receptors in these organs, one excitatory and the other inhibitory. These he termed  $\alpha$  and  $\beta$  receptors respectively. The/

The particular response which occurs could be determined by which type of receptor predominates.

If so, it is probable that the rat uterus always has  $\beta$  receptors, but that during oestrus  $\alpha$  receptors appear as well. If the uterus were in suitably "excitable" conditions, the  $\alpha$  receptors would combine quickly with the added amine and produce a contraction. The slower reacting  $\beta$  mechanism would then inhibit the organ. If the uterus were in conditions tending to keep it quiet only the inhibitory effects would be likely to be seen.

Support is afforded for this view by the fact that noradrenaline is more effective than adrenaline in causing the uterus to contract. Also Mann (1949) found that the uterus of rats in oestrus no longer contracted with noradrenaline and adrenaline after the administration of dibenamine and ergotoxine, but showed a purely inhibitory response.

The conditions under which Gaddum, Peart and Vogt (1949) kept the uterus would admit only of inhibitory effects being seen and that is probably why the state of the oestrus cycle of the rat did not affect the tissue significantly when used for assays.

Differences/

Differences in the inhibitory effects of adrenaline and noradrenaline on uteri kept in an excitable state and their relation to different phases of the cycle.

I. Method. The uterus was set up in the same solution which was optimal for production of excitor effects but at a lower temperature (as described below). In a few experiments oxygen was bubbled through the bath instead of air.

For electrical stimulation the tissue was impaled at the bottom of the bath on a platinum hook which led out through the rubber bung and formed one terminal. The other terminal was a piece of platinum wire the tip of which dipped just below the surface of the liquid in the bath. Care was taken that the uterus, even when relaxed, was entirely submerged in the bath solution so that the same current would flow across it from the commencement to the end of contraction.

In some experiments stimulation was with an alternating current (60 cycles/second) usually every two minutes. At the commencement of an experiment about/

about 5-10 volts for 15 seconds was sufficient to produce a good contraction but as time went on this had sometimes to be increased to 15 volts. In other experiments an induction coil was used for stimulation, the secondary coil being 7-8.5cm. from the primary, the latter being supplied from a 4 volt accumulator. The bath was washed out with Locke's solution after each contraction.

A series of equal contractions was produced either by electrical stimulation or with carbachol and a dose of adrenaline or noradrenaline was given about 1 minute before a particular contraction was due. The excitor effects of these amines which the previous experiments had shown were related to certain stages of oestrus, were prevented by lowering the temperature of the bath. Generally, a temperature of 25°-28°C. was necessary to obtain this effect whereby the uterus contracted only to the stimulus given electrically or with carbachol.

The alternative method of keeping the uterus at a higher temperature, but using one quarter the calcium content of Locke's solution, was also tried but proved unsuitable. Although this too eliminated the excitatory effects of adrenaline and noradrenaline the/



the responses to electrical stimulation were poor. The voltage had to be increased more and more over a short period to obtain the same height of contraction and this reduced the sensitivity to the inhibitory amines.

In order correctly to assign any changes in the inhibitory actions of the amines to the events of the oestrus cycle of the rat, information, in addition to that afforded by the vaginal smear, was sought from the ovaries and tubes. As soon as a rat was considered in oestrus, as diagnosed by the vaginal smear, its uterus was suspended in the bath and its ovaries and tubes stored in the refrigerator. When the particular response of that uterus had been established, the ovaries and tubes were examined by the method of Sawyer, Everett and Markee (1949):-

The ovaries were examined under the dissecting microscope for evidence of hypertrophy, hyperaemia or ruptured follicles.

The tubes were unravelled carefully in saline with dissecting needles and flattened on a glass slide with a thick cover slip. They were then examined under the low power of the microscope for tubal ova.

II. Results./

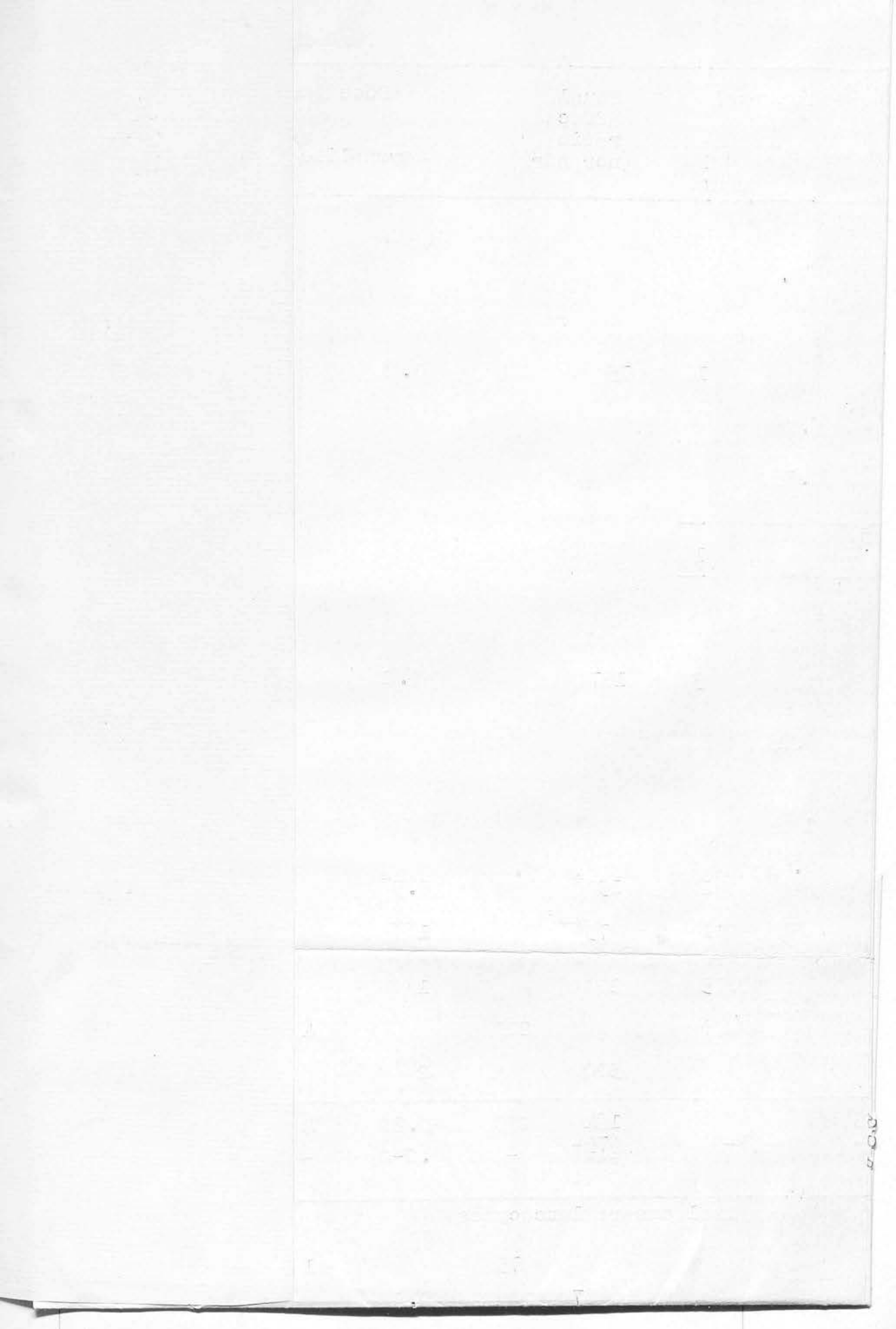


Table IV.

Inhibitory actions of adrenaline and noradrenaline. Tests on the isolated uterus. (Oestrus).

Uterus No.	Mode of stimulation	Equal effect ratio (nor./adr.)		Dose (µg.):-		Interval (hr) Between: - Rows Col- umns.	Equal Effect ratio (nor/adr.)	Dose (µg.):-		Interval (hr) Between: - Rows Col- umns.	Equal Effect ratio (nor/adr.)	Dose (µg.):-						
		Adrenaline	Nor- adrenaline	Adrenaline	Nor- adrenaline			Adrenaline	Nor- adrenaline									
Vaginal smear: nucleated epithelial cells.																		
29	Elect.	0.37		0.1	0.025	2	1	0.025	0.025									
36	Elect.	1		0.05	0.05	1.5	5	0.05	0.25									
39	Carb.		37		2		50 } 0.5											
	Elect.	2;3.7		0.2	0.4	1	10;15	0.2	2		1	75	0.2	10				
52	Elect.	1.5;3.75		0.2-0.5	0.2-2.5	0.5	7.4;9.8	1	10									
	Carb.							75	1			50 } 0.25						
	Elect.						10-20	1	5			50 } 0.75						
	Mean	2.0	37	0.18	2	0.46	50	9.0	75	0.46	1	3.4	50					
	Range	(0.37-3.75)	-	(0.05-0.5)	-	(0.025-2.5)	-	(0.5-2)	(1-20)	(0.025-1)	-	(0.025-10)	-	1				
Vaginal smear: cornified cells.																		
37	Elect.						4;6	0.05-0.1	0.2-0.6		3	187	0.2	20-50				
42a	Elect.						15	0.1	2									
	Carb.							75	1	50-100								
44a	Carb.							>100	2	200								
	Elect.						6	0.5	2									
	Elect.						>10 <sup>+</sup>	1 <sup>+</sup>	10 <sup>+</sup>		1	150 <sup>+</sup>	0.5 <sup>+</sup>	100 <sup>+</sup>				
49	Elect.						2	0.1	0.2		1	75 <sup>++</sup>	1 <sup>++</sup>	100 <sup>++</sup>				
56	Elect.						15	0.2	2			0.25 { 1.75	200	1	225			
	Carb.											1 {	200	4	200			
	Elect.												300	2	200			
	Mean	8.1	88	0.33	1.5	2.8	138					164	200	0.82	4	112	200	
	Range	(2-15)	(75-100)	(0.05-1)	(1-2)	(0.2-10)	(50-200)					(1-3)	(75-300)	-	(0.2-2)	-	(20-225)	-
Vaginal smear: leucocytes.																		
38	Carb.										2 {	75	1	100				
	Elect.											87	0.2	25				
42	Elect.											> 100	0.05-0.1	5				
44	Carb.										0.5 {	> 200	0.5	100				
	Elect.											200	0.5-1	100-200				
45	Elect.										0.75 {	100	0.1	10				
	Carb.											150	1	100				
	Elect.										1 {	300	0.5	200				
	Mean	157	175	0.32	0.83	78	100					157	175	0.32	0.83	78	100	
	Range	(87-300)	(75-200)	(0.05-1)	(0.5-1)	(5-200)	(100-100)					(87-300)	(75-200)	(0.05-1)	(0.5-1)	(5-200)	(100-100)	

+ Stilboestrol } see text  
 ++ Dihydroergotamine }

## II. Results.

Table III lists the results obtained from a series of uteri of rats in dioestrus when tested with electrical stimulation.

The ratio of equally active doses and the doses just producing a definite effect were similar to those found by Gaddum, Peart and Vogt (1949) when acetylcholine was used as stimulus.

Table IV shows the results obtained from uteri of rats in oestrus stimulated electrically and by carbachol.

The sensitivity of the electrically stimulated uterus to adrenaline and noradrenaline varied considerably with the stage of oestrus, whereas such a difference was much less pronounced when the strip was contracted by carbachol.

Electrical stimulation. In the earliest stage, i.e. when there were nucleated epithelial cells in the vaginal smear, the sensitivity to both adrenaline and noradrenaline was high and the mean ratio of equally active doses was 2.0 (0.37-3.75). When the uterus had been in the bath for some time (less than two hours), there was a slight decrease in the sensitivity to adrenaline but a considerable decrease in that to noradrenaline. The mean ratio now was 9 (1-20).

After/

After several hours the sensitivity to adrenaline was somewhat less still but the sensitivity to noradrenaline had decreased further and was of the order of that found in the uterus of rats in dioestrus. The mean ratio was 164 (75-300). When the rat from which the uterus was taken showed cornification of the vagina, the sensitivity to the two amines had a dose ratio of 8.1 (range 2-15). This figure was higher than the figure for the uteri excised in stage I and tested immediately but was the same as that of the same uteri kept in the bath for 0.5-2 hours. If the uterus tested was taken from a rat in metoestrus (first appearance of leucocytes), the ratio of equally effective doses of the two amines was higher still, with a mean of 157 (range 87-300). These figures are in close agreement with those for uteri excised in pro-oestrus and kept in the bath for about 6 hours.

The stages took longer to appear when oxygen was bubbled through the bath instead of air (uterus No.52, 56).

Carbachol. When the uterus was stimulated by carbachol the differences in the sensitivity to adrenaline and noradrenaline at different phases of the cycles/

cycles were not nearly so obvious. Between stage I and stage IV there was only a fourfold increase in the mean ratio compared with an eightyfold increase for electrical stimulation. Also in the same uterus ratios obtained during phase II and III with carbachol were consistently higher than those obtained with electrical stimulation. This was so even if the electrical stimuli were applied after the carbachol.

The examination of the ovaries and the tubes was carried out in the majority of cases in rats with cornified cells in the vaginal smear (i.e., in oestrus proper) and in which the ratio had average 8.1.

In the ovaries there were fresh corpora haemorrhagica and it was possible sometimes to express a drop of blood. This indicated recently ruptured follicles and stage III of oestrus. In two of the rats, tubal ova were seen as well.

In one experiment the ovary of a rat with cornified cells in the vaginal smear was examined and showed evidence of maturing follicles on the point of rupturing. No ova were seen in the tubes. The ratio given by the uterus was of the same order as those/

those of uteri of the other rats with cornified cells in the vaginal smear.

The results indicated that the extreme sensitivity of the uterus (mainly to noradrenaline) probably appeared early in oestrus, and certainly before ovulation because by that event (stage II-III) the sensitivity was already decreasing.

In one experiment (uterus No.44a) the Locke's solution was shaken up vigorously with stilboestrol after the first period of electrical stimulation and testing. Its presence in the solution thereafter did not prevent the changes (increase) in the ratio.

In another experiment (uterus No.49) dihydroergotamine in a concentration of  $2 \cdot 10^{-6}$  was continuously present in the Locke's solution after the first period of electrical stimulation and testing. It had no apparent effect on the ratio of equally inhibitory doses compared with the other experiments.

#### Discussion.

The assignation of the changes in the ratio of equally active doses in a uterus suspended in a bath to the stages of the oestrus cycle in the uterus in vivo though arbitrary, has some justification. When the/

the organ was taken from a rat in the stage of nucleated epithelial cells in the vaginal smear the ratio was smallest. The change to a bigger ratio in the isolated organ though more rapid than the change in sensitivity occurring in the organ in vivo, may have the same underlying cause.

The same consideration applies to uteri suspended during the stage of nucleated epithelial cells or of cornified cells in the vaginal smear and exhibiting ratios of over 150 when left in the bath for some hours; the transformation may correspond to that occurring in vivo when leucocytes appear in the vaginal smear.

While this correlation of the sensitivity to adrenaline and noradrenaline with the endocrinological stages may not be strictly true there is no doubt that the pharmacological stages do occur. It is possible that the changes the isolated uterus undergoes are similar in nature and only quicker than those of the uterus in vivo. In these experiments the pharmacological stages I-III took up to about 6 hours, which compares with about 36 hours in the living rat (Long and Evans, 1922).

The/



The appearance of the increased inhibitory action of noradrenaline at the onset of oestrus should be considered with the report of Mann (1949) that the point in the oestrus cycle at which the excitatory action of noradrenaline appears is when the vaginal smear consists entirely of nucleated epithelial cells. It seems that both actions keep together and are maximal early in oestrus.

It was disappointing that the uterus of the rat in oestrus could not be used as a sensitive test for noradrenaline because the sensitivity kept shifting. Those experiments have, however, extended the scope of usefulness of the organ for assaying adrenaline. With electrical stimulation, at least during oestrus, the sensitivity would be greater than in the method using carbachol. Also the presence of atropine in the test solutions would not be a bar. Gaddum, Peart and Vogt used potassium to stimulate the organ when atropine was present, as a choline ester would be ineffective. This had the disadvantage that after repeated administration the sensitivity to potassium decreased. This disadvantage is avoided with electrical stimulation.

Effect/

Effect of noradrenaline on the action of adrenaline on the rat's uterus.

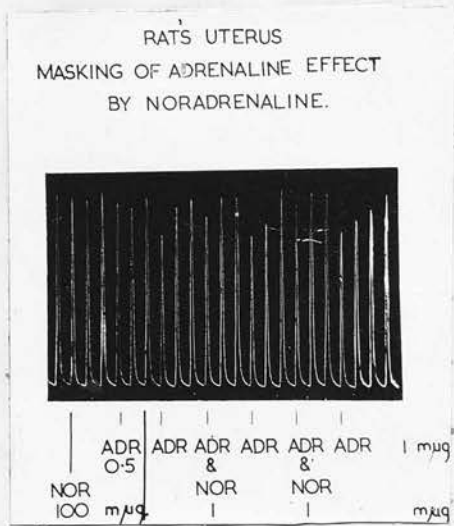
When parallel quantitative assays of a mixture are carried out the estimates of the substances present may be made by mathematical formulae with reasonable accuracy only if an essential condition be fulfilled. This is, that the substances estimated act purely additively on the test objects.

On account of its great sensitivity to adrenaline the rat's uterus is particularly useful in estimating that amine in mixtures containing noradrenaline. Some doubt arose however, as to the purely additive action of the two amines when it was found that in assays of known mixtures of adrenaline and noradrenaline the estimates of adrenaline fairly regularly were lower than the actual value instead of being evenly distributed about it.

Tests were made on simple solutions of adrenaline to which noradrenaline had been added to determine whether the non-methylated amine had a "masking" effect.

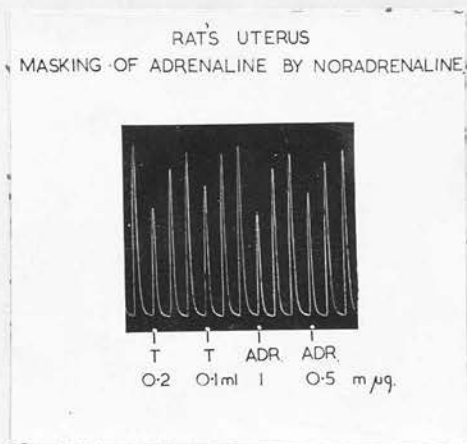
Experiment. A large volume of saline containing ascorbic acid  $10^{-5}$  was made up. This was used for making all solutions of the amines tested.

Fig. IX



The inhibition caused by 1 mμg of adrenaline when noradrenaline was present was less than that caused by the same amount of adrenaline alone.

Fig. X.



Set 4 of a statistical experiment in which 0.1 ml. T contained 1 mμg. and 0.2 ml. T contained 2 mμg. of adrenaline with 0.1 and 0.2 mμg. of noradrenaline respectively. The inhibitions were less than those of half the amounts of adrenaline alone.

A solution of adrenaline containing  $5\mu\text{g./ml.}$  of base was divided into two portions. One portion was used as "standard" adrenaline solution. To the other was added a quantity of noradrenaline in a small volume of the same saline such that the dilution of the adrenaline solution caused thereby did not exceed 0.01%. This "test" solution then contained  $5\mu\text{g./ml.}$  noradrenaline.

The solutions were compared on the rat uterus in the usual way.

Tests were also carried out with solutions containing larger amounts of adrenaline ( $10\mu\text{g./ml.}$ ) and various amounts of noradrenaline ( $1-100\mu\text{g./ml.}$ ) but always below the threshold of inhibitory action of the latter.

Results. In many cases, the comparison of the "standard" and "test" solutions on the uterus showed no appreciable difference. In some uteri, however, there was a difference and the accompanying tracing (Figure IX) is from one of them. The addition of  $1\mu\text{g.}$  each of adrenaline and noradrenaline together produced an inhibition corresponding to only 75% of the adrenaline present. When noradrenaline was added/

added alone 100µg. produced a hardly discernable inhibition. On the same uterus when a solution containing twenty times more noradrenaline than adrenaline was used and 1µg. of the methylated amine was added the amount of "masking" was the same.

The tracing in Figure 10 shows one set of a four-point assay in which a solution of adrenaline containing 10µg./ml. was compared with one containing 1µg./ml. noradrenaline as well. The threshold for the latter was about 50µg. The results were analysed statistically by the method described previously:-

<u>Solution</u>	<u>Low dose</u> (µg.)	<u>High dose</u> (µg.)
S - Adrenaline standard	0.5	1.0
U - Adrenaline with noradrenaline	1.0 0.1	2.0 0.2

The potency of U was 27.97% of S with a standard error of 10.67%.

In this case the solution of adrenaline which contained noradrenaline as well showed about 72% "masking".

Precaution/

Precaution was also taken that the effect was not due to the introduction of a small amount of acid from the stock noradrenaline solution ( $10^{-3}$  of dl'hydrochloride as 1' base). Stock adrenaline base was dissolved in just sufficient acid to leave the solution at pH 3-4. The noradrenaline salt being soluble in water had only sufficient acid added to produce the same pH. In both cases the pH was tested with indicator paper.

Discussion. While in many cases the presence of noradrenaline does not affect the assay of adrenaline with the rat uterus it would appear that sometimes there could be considerable vitiation of the estimate.

The amount of noradrenaline necessary to cause the "masking" was always below the threshold for inhibitory action. Increase of noradrenaline over an amount which produced "masking" did not increase the effect i.e., the minimal effective dose produced the full interference.

Another method of using the rat's uterus for assays.

Using this tissue after the manner described by King (1949) was tried.

A 2ml. bath was used and the uterus was set up in/

in unmodified Locke's solution at 35°-37°C. with air bubbled through. After 10-15 minutes to allow the tissue to relax, during which spontaneous contractions occurred occasionally, the solution was replaced by one containing more potassium but of the same tonicity. This was achieved by reducing the sodium chloride in the Locke's solution. The following table shows the relative concentrations of these two salts. The other constituents were unchanged.

Isotonic with Locke's solution:-

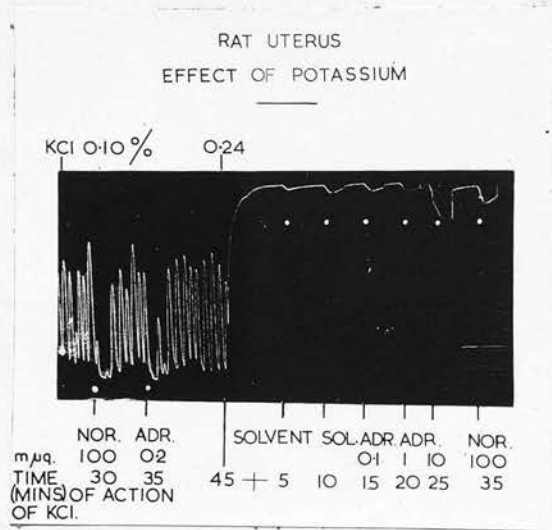
<u>Concentration of KCl (%)</u> <u>used in modified solution.</u>	<u>Corresponding concen-</u> <u>tration of NaCl (%)</u> <u>required.</u>
0.10	0.80
0.20	0.71
0.22	0.70
0.24	0.69
0.26	0.67

As soon as the solution containing about 0.1% KCl was introduced, the uterus generally started rapid, fairly regular spontaneous contractions. Adrenaline and noradrenaline in suitable doses were introduced every 5-10 minutes and they inhibited these spontaneous contractions.

The solution was then replaced by one containing still/

Figure XI

Effects of adrenaline and noradrenaline on the uterus in solution with high concentration of potassium.





still more potassium and the process continued till at a certain concentration (about 0.22-0.24% KCl) the uterus contracted and remained in that state throughout. When an inhibitory amine was now introduced the tissue relaxed. It contracted again when the amine was washed out. A 5 minutes schedule was found satisfactory with the added drug in the bath for 30-60 seconds.

In both types of test the adrenaline and noradrenaline solutions were made in the same modified Locke's solution as that in which the uterus was suspended but with the sodium bicarbonate omitted and with ascorbic acid  $10^{-5}$  added. Equal volumes of this solvent were added to the bath to ensure that the effects seen with adrenaline and noradrenaline did not occur with the solvent only.

The tracing (Figure XI) shows the record of an experiment in which the uterus was at first (spontaneous contractions) in a solution containing 0.1% KCl and later (steady contraction) in one containing 0.24% KCl. The inhibition of the spontaneous contractions shown was maximal (with 100 $\mu$ g. noradrenaline and 0.2 $\mu$ g. adrenaline). The preparation was sensitive to/

to about a quarter of these amounts. Plain solvent did not affect the contractions. In the state of steady contraction 100µg. noradrenaline produced an effect between that of 1µg. and 10µg of adrenaline. The threshold in this case was 2µg. adrenaline, i.e., about forty times as much as with the first method. This is shown in the table:-

KCl %	Method	Dose of adren. (µg)	Equal effect ratio Nor/adr.
0.1	Inhibition of spontaneous movements.	0.05-0.2	500-1000
0.22 - 0.24	Relaxation of contracted uterus.	1-5	10-100

The first method has the possible advantages over the method hitherto used of great sensitivity to adrenaline and a big ratio. It has the disadvantage of being liable to be readily affected by interfering substances, including dilution of the Locke's solution with saline (10% or less of the volume of the bath). This raises considerable difficulties in assays owing to the need for controls, especially/

especially for biological fluids, a constant volume technique being often necessary.

The second method has no advantage in sensitivity over the usual method of assay but is even more liable to interference.

Use of other stimulating agents. Acetylcholine was tried instead of potassium for the same purpose. This ester in a constant concentration of 10 $\mu$ g./ml. in the bath (the solution in the reservoir had to be changed frequently) produced an immediate contraction of the uterus but the tissue soon relaxed to the base line. It could not be made to contract again with this agent unless the bath was emptied, plain Locke's solution let in and some time allowed for the tissue to recover its sensitivity to acetylcholine.

Various concentrations of carbachol were also tried, 2 $\mu$ g./ml., 20 $\mu$ g./ml., and 200 $\mu$ g./ml. This substance only caused spontaneous contractions as with small amounts of potassium, but a steady contraction was not obtained. Inhibition of these contractions was produced with adrenaline and noradrenaline but the effect was unsatisfactory for assays as the preparation tended to become irregular quite/

quite quickly.

Application to the rat's colon.

A similar technique was tried with the rat's colon. This tissue did not show the regular spontaneous contractions of the rat's uterus. A steady contraction was produced with Locke's solution containing rather higher concentrations of potassium than for the rat's uterus (about 0.26% KCl). It was sensitive to the inhibitory action of 25-50 $\mu$ g. of noradrenaline and the ratio of equally active doses (noradrenaline/adrenaline) was about 0.25. It had little advantage over the usual method of assays.

Better results were obtained when the steady contraction was produced by carbachol (0.1 $\mu$ g./ml. was usually required). The sensitivity to adrenaline and noradrenaline was not increased but the responses were more regular. This was checked by the statistical method used by Gaddum and Lembeck (1949) and gave a value for  $\lambda$  of 0.068 with a standard error for the actual assay of just over 5%. This is about twice as good as the results of these workers, but the preparation had the disadvantage of being very sensitive to interfering substances. Also, after prolonged subjection to carbachol the tissue tended to show/

show a higher tone and to become less sensitive to the inhibitory amines tested.

The characteristic of the subject was that (1941) that stimulation of the respiratory system in animals by means of electrical means is liable to large errors produced by the development of a habit which for the separation of their substances even if present in quantities so small that biological effects were produced for their extraction. The habit described in the literature of the habit of the habit were not satisfactory owing to interference by means of chemicals from the electrographic process. The method of habituation studied and described here, although it was more sensitive than the electrographic method, was unfortunately not very sensitive to habituation.

The habit of habituation was studied in the habit of habituation and was described in a separate section.

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III. Quantitative separation of  
adrenaline and noradrenaline and assay.

The demonstration by Gaddum and Lembeck (1949) that estimation of adrenaline and noradrenaline in mixtures by parallel biological assays is liable to large errors prompted the development of a technique for the separation of these substances even if present in quantities so small that biological assays were required for their estimation. The tests hitherto described of using the rat's uterus and colon were not satisfactory owing to interference by traces of chemicals from the chromatographic processing. The modified methods studied and described here, although often more sensitive tests for the sympathomimetic amines, were unfortunately even more subject to interference.

The rat's blood pressure preparation was found to be the most satisfactory for these assays and is described in a separate section.

The method, which has been widely applied in the present work, has been described by Crawford and Outschoorn (1951). The paper chromatographic technique/

technique described by James (1948) for the qualitative separation of adrenaline and noradrenaline was adapted to the quantitative separation of these amines when present together in biological fluids and tissue extracts. It involves the separation of the amines by paper chromatography, elution of the separated amines from the appropriate portions of the developed chromatogram and finally, the determination of the adrenaline and noradrenaline contents of the eluates by assay on the rat's blood pressure preparation.

The method works well for quite small amounts of these amines and the sensitivity appears to depend on that of the assay method used. The limitations in the choice of this are described later. Chromatography also offers physicochemical evidence each time as to the identity of the separated substances because they are located at, or near, to the positions occupied by pure adrenaline and noradrenaline.

#### Method.

Separation of adrenaline and noradrenaline by paper chromatography.

Chromatographic technique. The chromatograms were carried out by the capillary ascent method of Williams and Kirby (1948) which gave more regular results/

results than the descending method. The solvent was water - saturated phenol.

Phenol (500g.) was distilled at atmospheric pressure from zinc dust (20g.) and the distillate, while still warm, shaken with a small excess of distilled water in a separating funnel. Sulphur dioxide was bubbled for ten minutes through the mixture which was then allowed to stand overnight to permit complete separation of the phases. Care was taken that this separation was allowed to take place at not too high a temperature (e.g., a warm night), because further separation was liable to occur when the water saturated phenol was placed in another container (for developing the chromatograms) if the weather suddenly got appreciably colder.

The lower yellow phenolic layer (500ml.) was then poured into one or more, clean glass tanks to give a layer of fluid at least 1cm. in depth. The sizes of the tanks used were 22x30x40cm. to accommodate two papers and 14x22x40cm. to accommodate one paper. The tank was closed by a glass plate carrying an inlet and an outlet tube at diagonally opposite corners so that the air inside the tank could be replaced by another gas. The union of the glass/

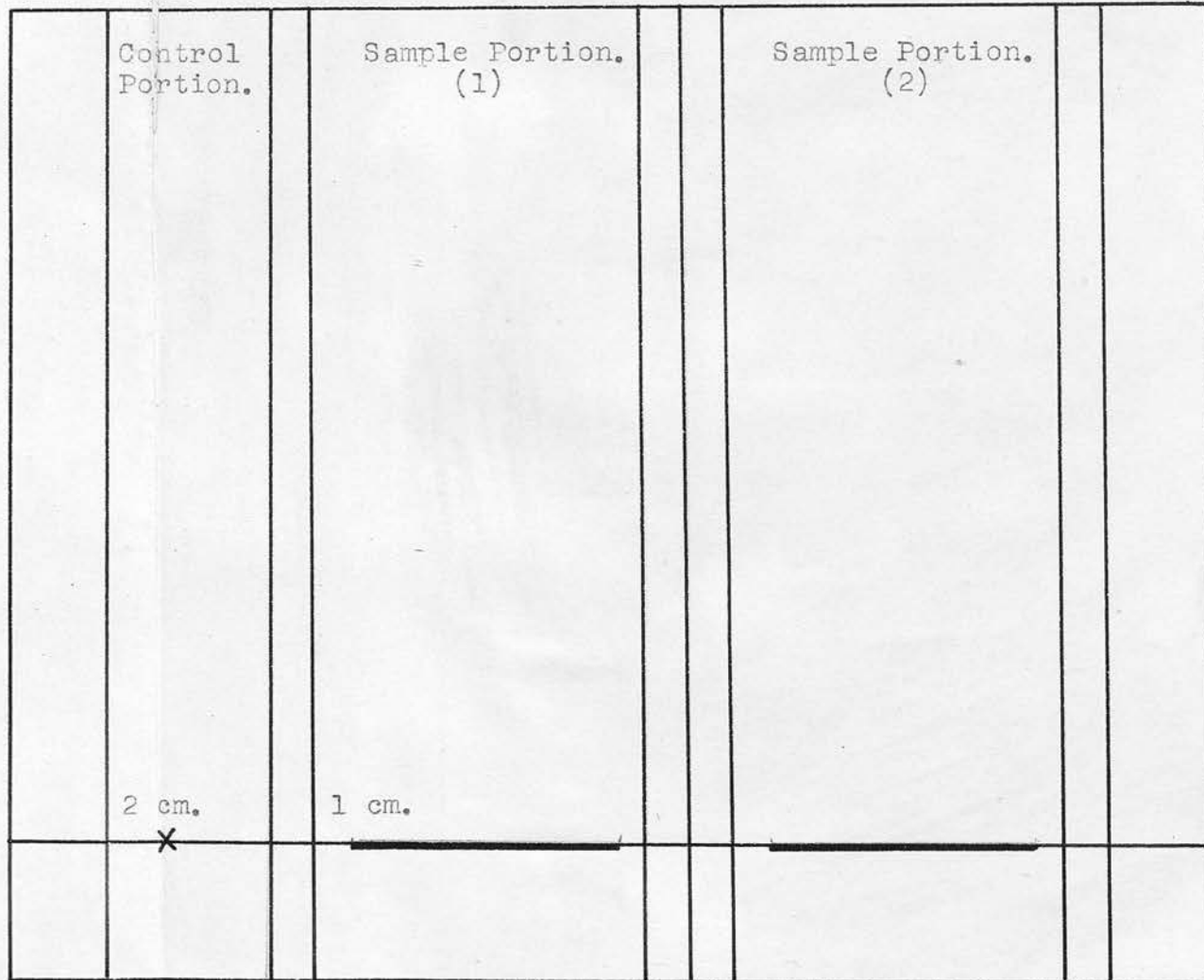


Fig. XII

Diagram of method of ruling sheet of filter paper  
for chromatographic separation of adrenaline and  
noradrenaline.

TOP. 3.5 6 1.5 12 1.5 1.5 12 1.5 3.5 cm.

LEFT EDGE.



2 cm.  
X

1 cm.

RIGHT EDGE.

30 cm.

5 cm. 35 cm.  
high.  
43 cm. wide.

BOTTOM.

glass plate to the top of the tank was made gastight by soft paraffin. The phenol was not used for longer than one week, but was used repeatedly within that time.

#### Preparation of the filter paper.

A sheet of Whatman No.1 filter paper was cut and ruled off in pencil in the manner illustrated in Figure XII. Usually, two samples, each containing adrenaline and noradrenaline were chromatographed on the one sheet of paper. The paper was fashioned into cylindrical form by joining the left and right sides edge to edge with cellulose tapes (1.6cm. wide) applied along the length of the join to within about 2 cm. from the bottom of the paper. The edges of the paper were not allowed to overlap, as if they did the solvent flow along the overlapping portions was different from the flow along the rest of the paper. The sample portion of the paper only i.e., not the control portion, were sprayed with a fresh solution of ascorbic acid (50mg./100ml.) with an all-glass spray. The paper was then allowed to dry at room temperature, about 1.5-2 hours being required.

#### Application of the test solution to the ascorbic acid treated paper cylinder.

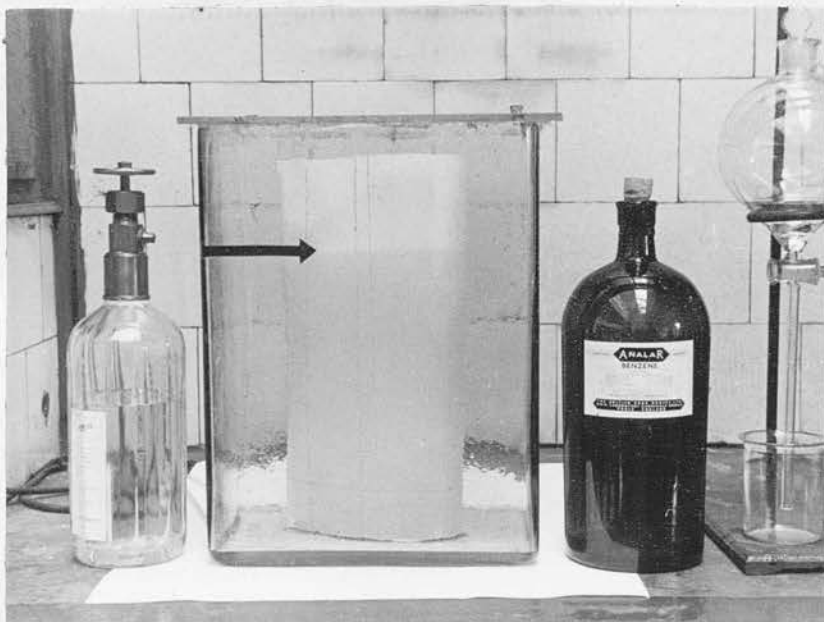
The material to be analysed was in the form of

a solution in either acid alcohol (0.1% conc. HCl/100ml. ethanol) or in acetone-alcohol (equal parts of acetone and acid alcohol), depending on the original, i.e., plasma, tissue extract etc. It was applied along the line at the foot of the paper cylinder (shown thickened in the diagram) so that it did not approach closer than 1cm. to the boundary line of the sample portion of the paper and was not wider than 1cm. along the length of the line of application. This was done by repeated application of the solution with a capillary pipette, a small quantity being applied and allowed to dry before the next application. This was continued till all the solution had been transferred to the paper (about 1 hour). Finally, on to the control portion of the paper, at a point on the line 2cm. from the left hand boundary (x in the diagram), was applied 0.01ml. of a solution containing 10mg./ml. each of adrenaline and noradrenaline.

Development of the chromatogram. After the test and control solutions had dried (without the application of heat to speed the process) the paper cylinder was stood with its lower end immersed in the solvent in the glass tank which was placed in a fume cupboard. The/

Fig. XIII.

Development of the chromatogram.



Photograph of a paper cylinder in the solvent -  
containing jar after about 20 hours development.  
The edge of the solvent front can be seen.

The tank was closed with the glass cover and sulphur dioxide passed through for 15 minutes, after which the inlet and outlet tubes were removed and the holes plugged with corks. The chromatogram was allowed to develop for at least 12 hours (overnight) but was often left for 20-24 hours. After about 12 hours the solvent had usually risen some 23-27cm. up the paper, and after the longer period about 29cm. (See Figure XIII).

Removal of solvent from the developed chromatogram.

The paper cylinder was removed from the tank and the cellulose tape, both backing and adhesive, stripped off. The filter sheet was then held at the dry end and pulled backwards and forwards three or four times through a layer of benzene (A.R.) in a large porcelain or enamel developing dish. It was ensured that the whole of the paper which had been wetted with phenol was washed with benzene. The paper was then hung up to dry in air. After about 0.5 hours it still felt slightly damp but was generally dry enough to handle.

Location of adrenaline and noradrenaline on the developed/

Location of adrenaline and noradrenaline on the developed and dried chromatogram. The control portion of the paper was cut off and sprayed with a solution of potassium ferricyanide ( $K_3Fe(CN)_6$  0.44g./100ml. 0.2 M-phosphate buffer pH 7.8 of James, 1948). This was done away from the sample portions of the paper to prevent any of the ferricyanide solution reaching them. Spraying with this solution causes the adrenaline and noradrenaline to form red oxidation products which show up on the paper strip as red spots, the noradrenaline being nearer to the starting line of the chromatogram. The distances from the starting line to the lower and upper extremities of the two spots were measured. These measurements were to locate the amines on the sample portions of the paper.

Allowance was made for slight differences in the rates of flow of the amines in the control and sample portions of the paper by including in the sample portion a width of paper 1cm. below each of the spots and 2cm. above the noradrenaline spot and 1cm. above the adrenaline spot. Transverse strips conforming to these extra allowance measurements of the/

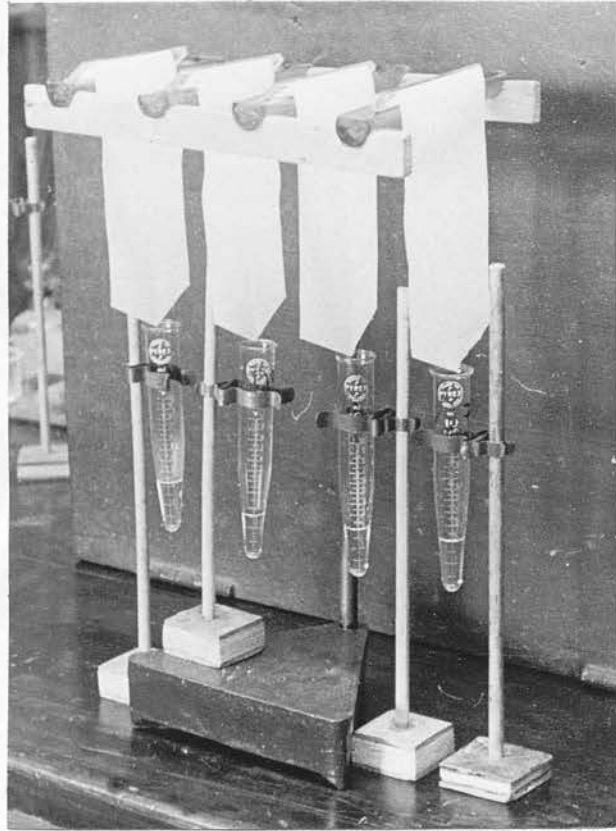
the location of the two amines were cut from the sample portions of the paper. They were usually 6-7cm. wide and were marked lightly in pencil for future identification. Usually, the 1.5cm. portion on one side of the boundary line was included to enable a suitable point to be cut (see below).

Elution of the amines from the paper strips.

The amine contained in each strip was eluted therefrom with a solution containing sodium acid phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  A.R.) and ascorbic acid (1mg./100ml.) The strength of phosphate solution used depended on the volume of eluate to be collected (see below). The manner of elution was similar to that described by Dent (1947). Each paper strip was cut to a point at one end, the 1.5cm. portion left on being adequate to do this without removing any paper on which adrenaline or noradrenaline was likely to be present. The other end was inserted to a depth of about 0.5cm. between two microscope slides held together by a narrow elastic band. The extruding portion of the strip was bent sharply over the edge of the slides so that it hung vertically when/

Figure XIV

Elution using a battery of troughs.





when the far sides of the slides were immersed in the eluting fluid. The pointed end of the paper just intruded into the mouth of a test tube or a 15ml. centrifuge tube. The eluting fluid passed between the microscope slides, on to and down, the paper and into the collecting tube. To prevent undue evaporation of the solvent from the papers the elutions were carried out in a closed chamber containing a beaker of water.

To carry out a number of elutions simultaneously, a battery of troughs for the eluting fluid was used (see Figure XIV). This was constructed by partitioning a half-section of celluloid tubing of 1.5cm. internal diameter into 8cm. lengths. Each of these troughs would hold about 8-9ml. fluid besides the sides of the slides holding the paper strip. When the elution was left for a long run (e.g., overnight) 7.5ml. of eluting fluid was placed in the trough and this yielded 3.5-4.5ml. of eluate in the collecting tube. If the elution could be supervised the trough was filled with as much fluid as it would hold and the elution stopped when 2ml. eluate was obtained. The following table gives the concentration/

concentration of phosphate used for different requirements of the eluates.

<u>Vol. of eluate to be collected.</u>	<u>Proposed final vol. of sample for assay.</u>	<u>Concentration of phosphate used.</u>
4ml.	1.0ml.	0.4%
4ml.	0.5ml.	0.2%
2ml.	0.5ml.	0.4%

By thus either restricting the supply of eluting fluid or by watching the elution as it proceeded constant volumes of eluates were obtained which, on further treatment, gave rise eventually to almost isotonic solutions for biological assay.

Preparation of eluates for biological assay. The aqueous eluate, containing traces of the chemicals used in the chromatographic process, was transferred with washing with 1ml. distilled water to a 50ml. round-bottomed flask and evaporated in vacuo at 55°-60°C. (external temperature). When the eluate was less than about 4ml. it was made up to that volume with distilled water before transferring to the evaporating flask. This was because traces of phenol in the eluate were removed by distillation in steam and the evaporation of less than 4-5ml. would have been inadequate for the purpose. Shaking up the eluate with quantities of benzene before evaporation/

evaporation was found not to be necessary (Vogt, 1951).

Heating the flask was stopped when the contents had evaporated almost, but not completely, to dryness. The flask and contents (layer of material on sides) were allowed to cool slowly to room temperature with the suction still on. The residue in the flask was then taken up in ascorbic acid solution (50 $\mu$ g./ml.) the volume being determined by the volume and strength of phosphate used for elution, i.e., either 0.5ml. or 1.0ml. as described above. This yielded a sample for assay which was of phosphate (about isotonic) with ascorbic acid and containing the separated amine to be tested with a minimum of other material which could possibly interfere with the test.

The solution was kept in ice until the assay could be carried out. If a considerable time had to elapse however, the residue was not taken up in the ascorbic acid solution till shortly before assay and kept in a refrigerator.

#### Notes.

Biological assay of the separated adrenaline and noradrenaline.

In the course of the development of the original method which has been described in the foregoing/

foregoing a suitable biological assay method had to be found. The rat's uterus and colon and the perfused rabbit's ear proved to be unsatisfactory owing to interference from traces of reagents used during the separation of the amines. The rat's blood pressure preparation was much less affected by these interfering substances and was used routinely, for the assays. The rat's uterus could still be considered a useful assay preparation if the amount of adrenaline available in the final sample would permit dilution (at least 10 times) and yet be above the assayable amount. The rat's blood pressure preparation proved to be one of the most sensitive tests for noradrenaline and was superior to any other for assaying that amine. It is described in detail in the next section.

Preparation of the test solution for application to the paper. Before obtaining a solution in acid alcohol or acetone-alcohol, biological material had to be deproteinised and purified. Some plasma samples had to be concentrated as well. A description of the various methods employed is given in each case of the series of experiments to which this separation/

separation technique has been applied.

Check on the rates of travel of adrenaline and noradrenaline. After the development of the chromatogram in phenol the edge of the solvent flow on the paper cylinder was generally the same or perhaps 0.5-1cm. less in the sample portion than in the control portion. The extra allowance above and below the measurements of each control spot in cutting transverse strips was then sufficient to include any slight difference in the rates of travel in the sample portions.

If the solvent flow in the sample portions was 2-3cm. less than in the control portion, as occasionally happened, the separated amines were "located" on the sample portion by recalculation from the  $R_F$  value (taken from the control portion) for the somewhat less solvent flow in the sample portion. ( $R_F$  value = mean height of travel of amine / mean height of flow of solvent).

A check was necessary to ensure that this procedure was adequate and that the amines did travel at the same rates in both the sample and control portions if the solvent flow was even.

Experiments/

Experiments. A plain paper cylinder cut to the same size as usual but ruled with only a transverse line 5cm. from the bottom of the paper had a series of drops of control adrenaline/noradrenaline solution applied 5cm. apart starting 1.5cm. from either edge. This was chromatographed in the usual way, washed, dried and developed with ferricyanide. The edge of the solvent flow showed no more than the usual, small irregularities. Measurement of the spots in the region of the paper corresponding to the control portion of the usual papers would have included all the spots across the paper with the exception of the two extreme ones 1.5cm. from the edges. As the usual papers have margins of 3.5cm. at the edges no portion of paper containing any adrenaline or noradrenaline would be likely to be left uneluted in practice.

Comparison experiments were also carried out in which samples from biological material were chromatographed in parallel with control adrenaline and noradrenaline. Those tested were 1ml. each of rabbit, rat and cat plasma to which adrenaline and noradrenaline had been added, and extracts of rat's adrenals/

adrenals and of human phaeochromocytoma tumour. There was sometimes a slight difference between the travel of the amines in the biological material and in pure solution which was apparently correlated to a slightly more uneven solvent flow. The "location" procedure based on calculation of the  $R_F$  value seemed to be adequate to determine the true positions of the separated amines.

Acid washing of filter paper. It was found recently that more regular flow of the solvent and non-occurrence of tiny adventitious spots (usually coloured blue or brown) were obtained, if the filter paper for chromatography had previously been washed in N/100 HCl. This was conveniently carried out with a glass trough of suitable large size for the paper by letting one edge dip in the acid contained therein. The edge was kept down by a glass rod which weighted it in position. The acid flowed up the sheet of paper and down along it similarly to the process taking place in elution. When the acid reached the bottom edge of the paper the washing was stopped, the paper hung up to dry in air, and then stored till needed.

Recovery/

Recovery of benzene. Considerable economy was effected by recovering the benzene used to wash the phenol off the developed chromatogram. This was first shaken up in a flask with dry calcium chloride and left for 2-3 days. It was then distilled twice after which it could be used again.



The rat's blood pressure preparation.

Historical. The technique of recording the blood pressure of the intact rat has been known for some considerable time.

Durant (1927), investigated the pressure changes in anaesthetised and heparinised Wistar rats of different ages (1 month to 2 years). He recorded the blood pressure in the carotid artery with a standard mercury manometer and used the jugular vein for injecting drugs. He found that adult rats had a mean normal blood pressure of about 119mm. mercury, that males had a slightly higher pressure than females, and that young rats (4-24 weeks) had lower pressures than adults. On injection of various drugs and on electrical stimulation of the vagi, rats exhibited the same changes and apparently had the same vaso-motor and cardio-inhibitory mechanisms typically possessed by mammals (cats and dogs).

For quantitative pharmacological work the preparation has been used by Simon (1937), in the investigation of certain pressor substances in blood serum.

Detailed/

Detailed investigation of its use for pressor assays of pituitary extracts and its response to histamine and adrenaline was carried out by Landgrebe, Macaulay and Waring (1946). They also studied different anaesthetics, with and without artificial ventilation, and the spinal animal with a view to finding the most suitable conditions for a particular purpose.

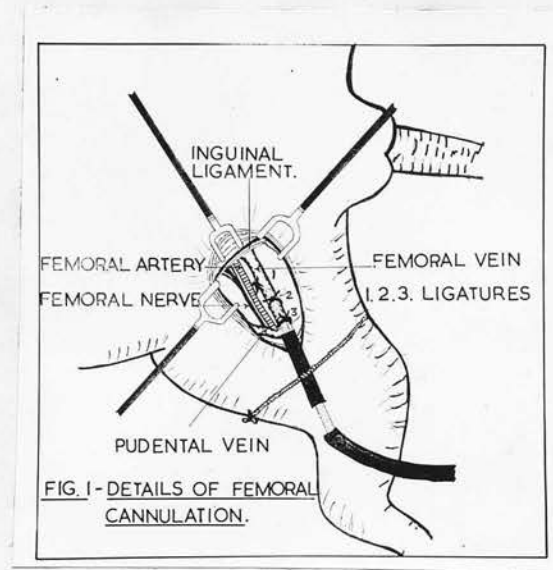
The preparation has been used as the method of choice for the assay of adrenaline and noradrenaline separated by paper chromatography (Crawford and Outschoorn, 1951).

#### Method of preparation.

A rat of either sex, weighing 200-250g. was anaesthetised with urethane B.P. (ethyl carbamate, a freshly prepared 25% solution in water, made by warming gently) given subcutaneously into the back of the neck in a dosage of 0.7ml.(175mg.)/100g. body weight of rat. When completely anaesthetic (in 0.5-1hour) the animal was tied in a supine position to a board.

A mid-line incision was made in the neck and the trachea cannulated. One carotid artery (usually the right) was dissected ready for cannulation and the other carotid tied off. Loose ligatures were placed/

Fig. XV.



Rat's blood pressure preparation.

Diagram of dissection for cannulation of femoral vein.

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placed round both vagi.

An incision was made on the inner side of one thigh (usually the right). The femoral vein was dissected carefully and all visible tributaries rigorously sought out and tied. The vein was then cannulated. The cannula was fixed firmly by means of a stout ligature including it and the thigh together (see Figure XV). The distal end of the venous cannula was connected by a short piece of rubber tubing to a 1ml. burette fitted just above the tap with a side arm connected through a piece of rubber tubing with a spring clip to a reservoir containing warm saline. This fitment enabled the burette to be refilled without detaching it from the venous cannula. Heparin ("Boots"), about 220 units or 2mg./100g. rat in 2ml. saline, was injected through the venous cannula and washed in with saline.

The arterial cannula was then inserted and tied in, held in position by a clip fixed to a stand, and connected to a special mercury manometer (described by Condon as an addendum to Crawford and Outschoorn, 1951). The connecting liquid medium was saline.

The/

The cutting of both vagi and the femoral nerve lateral to the venous cannula completed the preparation.

Cannulae. All the cannulae used were made of glass of conventional pattern but of suitable size for the rat. The orifices were of about 2.5mm. external diameter for the tracheal cannula and 1mm. external diameter for the arterial and venous cannulae, the internal diameters being slightly less.

Considerable improvement was effected by the use of "polythene" for the tracheal and venous cannulae. This material was extremely light and also enabled the dead space in the venous cannula to be reduced to 0.02ml.

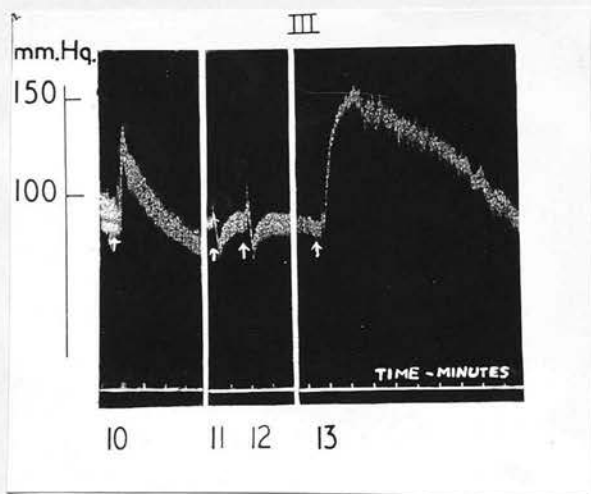
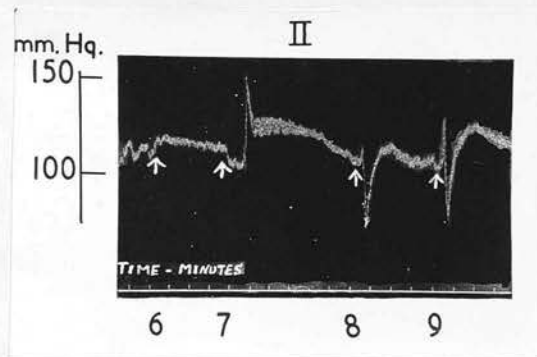
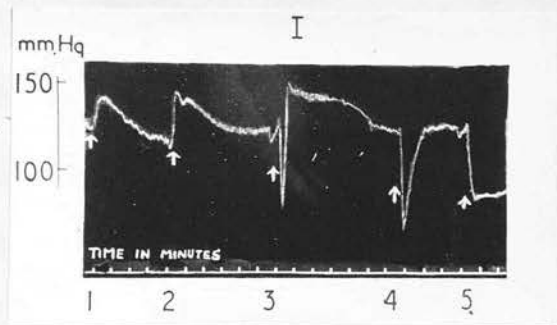
Anaesthesia. Urethane. Soon after the rat preparation was thus set up the blood pressure was generally high (100-140mm. mercury). With urethane anaesthesia, if the animal was left for about 20 minutes the pressure settled down to a steady low level (40-80mm. mercury) which was very suitable for assay of pressor substances. Other anaesthetics did not have this pressure lowering effect.

Nembutal. (Pentobarbitone Sodium "Abbott" 1 grain/cc. containing 10% alcohol). The dose was 0.1ml./100g. rat every hour intra-peritoneally. The blood pressure remained fairly high (60/

Figure XVI

Effect of drugs on the rat.

350g. Urethane 437mg.  
Heparin 7mg.



Tracings:-  
Carotid Blood Pressure

Time in minutes.

Injections:-

1. 0.5 $\mu$ g. adrenaline
  2. 1.0 $\mu$ g. adrenaline
  3. 100 $\mu$ g. nicotine. Note depressor phase.
  4. 0.5 $\mu$ g. acetylcholine
  5. 0.5 $\mu$ g. atropine. Blood pressure returned to "basal" level in 10 minutes.
  6. 0.5 $\mu$ g. acetylcholine. Note abolition of response after atropine.
  7. 100 $\mu$ g. nicotine. Note abolition of depressor phase after atropine.
  8. 50 $\mu$ g. acetylcholine.
  9. 100 $\mu$ g. acetylcholine. Note rise of blood pressure (adrenaline release).
- Between II and III 600 $\mu$ g. nicotine given in 8 minutes.
10. 1 $\mu$ g. adrenaline. Note increased response after ganglion "blockage" by nicotine.
- Between 10. and 11. ergotoxine 350 $\mu$ g. in 3x1 minute injections (50; 100; 200 $\mu$ g.)
11. 1 $\mu$ g. adrenaline ) Note "reversal"
  12. 2 $\mu$ g. adrenaline )
  13. 0.2 units posterior pituitary extract.

(60-100mm. mercury) but the depth of anaesthesia varied greatly at different times, rendering the preparation very unstable.

Barbitone soluble B.P. (Barbital Sodium). 80mg./100g. rat was given in 10% solution intra-peritoneally. Anaesthesia was not complete till after about 3 hours, but once developed was long lasting. The blood pressure was steady at a high level (120-140mm. mercury). This anaesthetic was unsuitable for assay of pressor substances but was satisfactory for estimating depressor agents. 0.5 $\mu$ g. of acetylcholine produced a fall of pressure of 60-80mm. mercury.

A small dose of urethane (one quarter the full anaesthetic dose) given subcutaneously to a rat already anaesthetised with barbitone soluble caused the blood pressure to fall within 10 minutes to the level usually found with urethane alone. This fact would enable a rat used for the assay of depressor substances to be made suitable for pressor assays in a short time if so desired.

Effect of drugs on the rat.

The illustration (Figure XVI) is the record of an/

an experiment in which various drugs were administered.

Note on histamine. The rat is relatively insensitive to this drug. Doses of histamine acid phosphate of 10-20 $\mu$ g. (as histamine base) had to be given to rats of the weight used in order to cause a fall of blood pressure. Slightly smaller doses sometimes showed a rise of pressure, presumably due to the release of adrenaline. In consequence, this preparation would be useful when biological material liable to contain small amounts of histamine is tested.

The carotid sinus reflex in the rat's blood pressure preparation.

Most of the studies on this reflex have been made in large mammals. It was believed at one time by Heymans (cf. Heymans, Bouckaert and Regniers, 1933) that the rise of blood pressure occurring when the carotid sinus is clamped off is largely due to the reflex liberation of adrenaline from the adrenals. The evidence mainly depended on the demonstration of other effects of this liberation at the same time, e.g. splenic contraction.

Recently/



Recently the mechanism of the reflex, particularly in relation to the spleen, in cats and dogs, was investigated by Driver and Vogt (1950). They found no evidence in the cat for the release of adrenaline during the pressor response to clamping the carotids. The main facts were that adrenalectomy hardly affected the pressor response or the splenic contraction and there was no detectable increase in the sympathomimetic amines in the adrenal venous blood.

Experiment. The rat's blood pressure preparation was used to see whether similar findings to those of Driver and Vogt would be obtained.

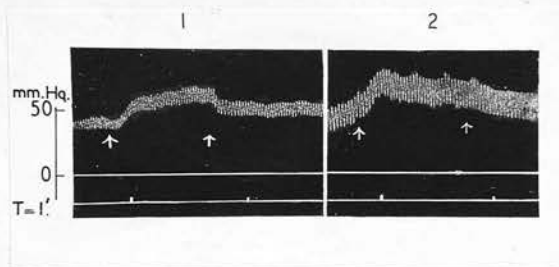
One carotid artery was used to record the blood pressure as usual. The other carotid was not tied off. A loop of thread was placed just below the carotid sinus and another loop well beyond the bifurcation round both the internal and external carotids. Tightening of the loop below the sinus produced the same effect as clamping the carotid, i.e., a rise of blood pressure. Tightening of the loop beyond the sinus had another effect, i.e., the blood pressure fell. The mechanism of this was not investigated.

Figure XVII

Carotid sinus reflex.

Effect of occlusion of the carotid artery below the sinus for 1 minute in the rat.

Between 1 and 2: both adrenals were removed.



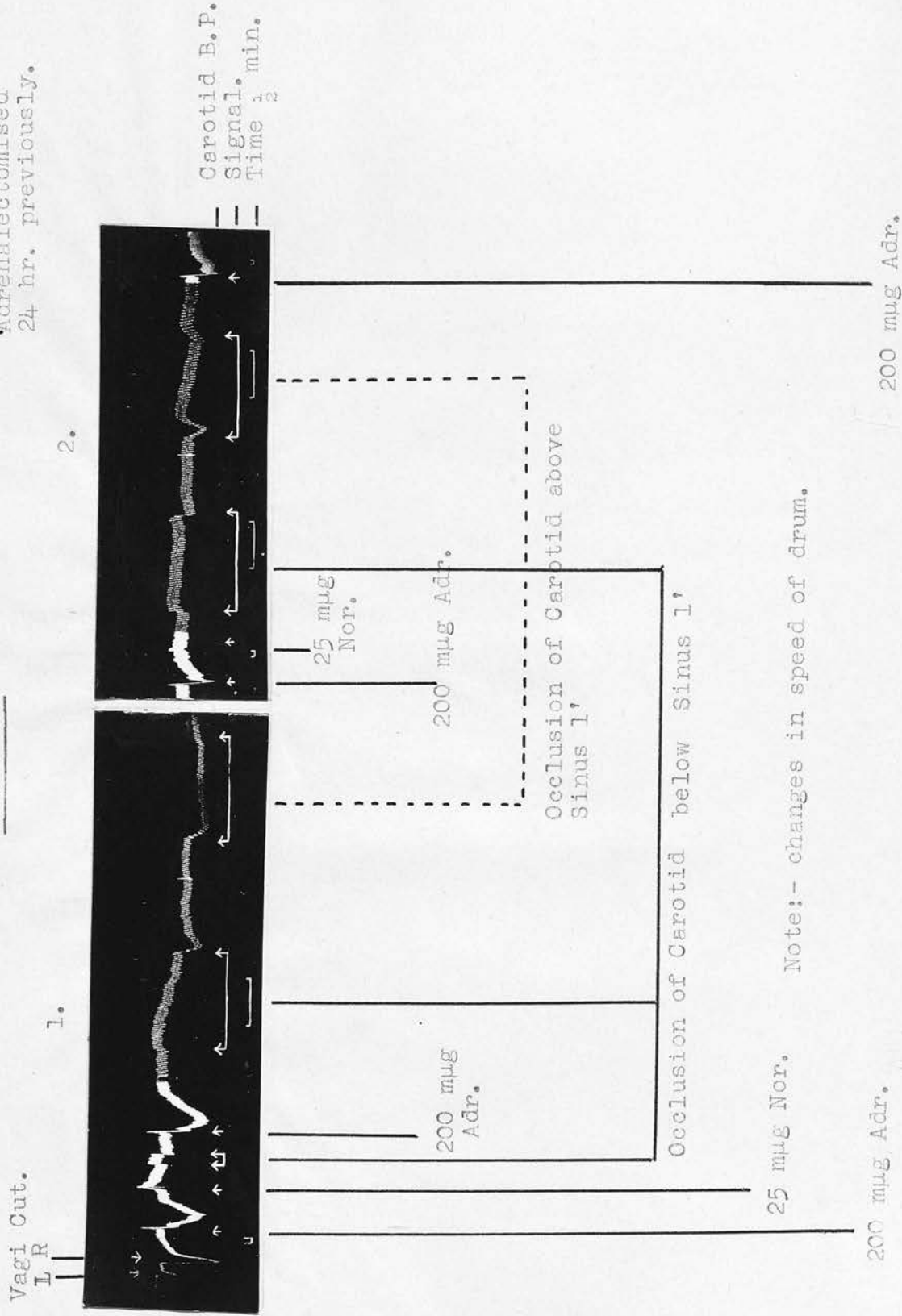
Recording of the carotid blood pressure.

The two tracings are directly comparable. Note greater respiratory excursion in 2.

Fig. XVIII.

CAROTID SINUS REFLEX  
IN THE RAT.

♀ 300 g.  
Adrenalectomised  
24 hr. previously.



Note:- changes in speed of drum.

Between 1. and 2. 40  $\mu$ g. DHE.

A preliminary record was obtained of the pressor response to tightening the lower loop. The rat was then disconnected from the manometer and venous inflow burette, turned over on its belly, and the adrenals rapidly removed from behind. On returning the animal to the supine position, reconnecting and resuming the record, the pressor reflex could be demonstrated unchanged. This is shown in Figure XVII.

For more detailed investigation, a rat which had been adrenalectomised about 24 hours previously and supplied overnight with food and saline to drink, was used.

Figure XVIII shows the record obtained. Both vagi were sectioned. The responses of the blood pressure to occlusion of the carotid artery below and above the sinus were recorded. Small doses of adrenaline (200 $\mu$ g.) and noradrenaline (25 $\mu$ g.) were given for comparison. In this particular rat the pressor effect of adrenaline was succeeded by a prolonged depressor phase.

Dihydroergotamine "Sandoz" (DHE) in a solution containing/

containing 100µg.ml. was then administered. After 40µg. DHE the pressor responses to adrenaline and noradrenaline were almost abolished but the sinus reflexes (both pressor and depressor) were unchanged.

Assay of pressor amines.

For the assay of adrenaline and noradrenaline the most convenient rats were those weighing 200-250g. The injection of 50µg. adrenaline or 25µg. noradrenaline usually produced a just detectable rise of blood pressure. Amounts of twice these quantities were regularly assayable. Smaller rats were even more sensitive but tended to be irregular in their responses. Larger rats were relatively insensitive. The ratio of equally active doses of noradrenaline/adrenaline (with the provisions hereinafter described) was 0.15-0.75 (average 0.25).

The sensitivity of the preparation to noradrenaline was often greater than that of the isolated colon of the rat. It is therefore one of the most sensitive tests for that amine. In view of this, it was decided to estimate the error of assaying two pure solutions of noradrenaline by the/

Fig. XIX.

Rat's blood pressure preparation.

Adrenaline and Noradrenaline dose - response curves.

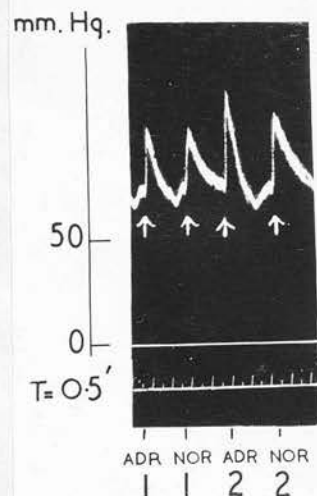
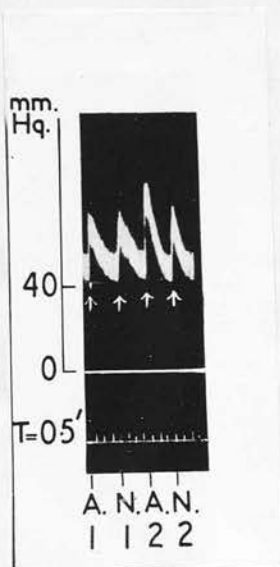
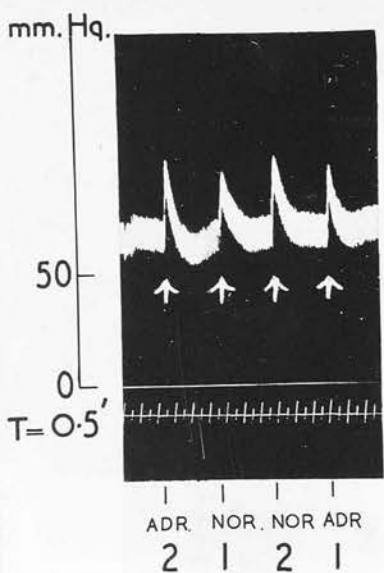


FIG XIX

RISE OF PRESSURE  
mm. Hg.

●—● ADRENALINE  
●- - -● NORADRENALINE

I

II

III

1 : 2

1 : 2

1 : 2

RATIO OF LOW : HIGH DOSES

the method of Noel (1945) as applied by Gaddum and Lembeck (1949). They pointed out that the best measure of the general accuracy of the test would be  $\lambda$ , the standard deviation of the log. dose, as this quantity is independent of the number of sets of results.

A mean (of 2)  $\lambda$  for noradrenaline of 0.096 was obtained, which compares with their value of 0.125 for the rat's colon.

#### Mixtures of adrenaline and noradrenaline.

The value of this preparation for estimation of these amines by parallel quantitative assays was examined. In spite of the sensitivity to noradrenaline and the small noradrenaline/adrenaline ratio of equally active doses there appeared to be one great disadvantage. The slopes of the dose-response lines for the two substances was often not the same. Two extreme cases are illustrated here, (Figure XIX), one in which the lines for adrenaline and noradrenaline are almost parallel (I), and another in which there was the greatest deviation from parallelism (II) out of a large number of rats investigated./

investigated. Usually, the difference in slopes is something between these two. One such is also shown (III).

This fact limits the use of the preparation in that it is not suitable for assays of mixtures.

#### Assay of separated amines.

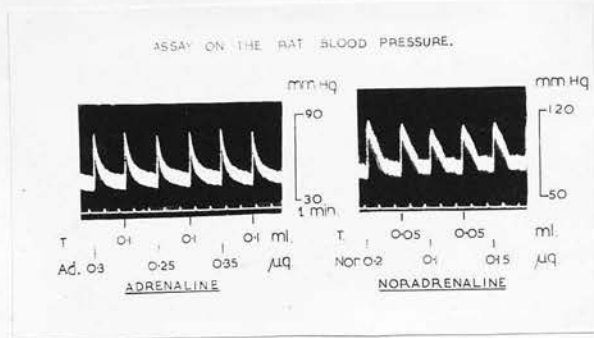
This disadvantage is of little significance when solutions of adrenaline and noradrenaline which have been separated by paper chromatography have to be assayed. The preparation has the additional advantage, referred to previously, that it is relatively insensitive to traces of chemicals from the chromatographic process.

#### Assay routine for adrenaline and noradrenaline.

Test samples containing adrenaline were assayed against standard adrenaline and those containing noradrenaline against standard noradrenaline solutions. If required dilution of a test solution was made in normal saline containing freshly dissolved ascorbic acid (50 $\mu$ g./ml.). Test and standard solutions, both at room temperature, were injected alternately into the venous cannula of the rat/



Figure XX



Typical records obtained in the assay of adrenaline and noradrenaline.

(Reproduced from Crawford and Outschoorn, 1951).

Adrenaline: 0.1ml. of the test solution produced an effect between those of 0.25 and 0.35µg. and about equal to that of 0.30µg. of standard adrenaline.

Noradrenaline: 0.05ml. of the test solution produced an effect between those of 0.1 and 0.2µg. and about equal to that of 0.15µg. of standard noradrenaline.

rat preparation with a 1ml. tuberculin syringe and washed in immediately with a quantity of saline. The volume of injected solution was 0.025-0.4ml. and the total volume of fluid given at any one time not more than 0.6ml. This total volume was varied with circumstances but was the same for both test and standard solutions in any one assay. The optimum total volume was 0.3-0.4ml.

Injections were made at regular intervals, usually every 2 minutes, but if after injection of adrenaline there was a prolonged depressor phase after the pressor effect, intervals of 3-5 minutes had to be adopted.

An assay consisted of the usual procedure of fixing the dose of one solution and varying the dose of the other to yield responses smaller than, larger than and equal to, that of the fixed dose solution. Typical records are reproduced in Figure XX.

#### Drugs to improve the preparation for assays.

Cocaine. Doses of cocaine hydrochloride (200-800µg./100g. rat) given intravenously were found to/

to produce some potentiation of injected adrenaline and less of noradrenaline. The drug did not appear to be very useful in improving the preparation for assays.

Ergot. The use of ergotamine tartrate (0.1mg./kg.) has been recommended by Euler and Schmitterl8w (1944), for stabilisation of the "base line" of the cat's blood pressure. Dihydroergotamine "Sandoz" (7.5µg./100g.) used in the rat did not improve the "base line" irregularities but did reduce the discrimination of the pressor effects between different doses.

Atropine. This drug was found useful when biological samples showed depressor effects, some of which could be attributed possibly to acetylcholine. A dose of atropine sulphate (100µg./100g.) was given subcutaneously, slowly over about 2 minutes and the animal left for about 10 minutes to recover.

Samples assayed before atropinisation sometimes showed as little as 50% of the adrenaline content shown by assay after atropinisation. The drug was not used as a routine however, in assays of samples from particular experiments when the amounts of/

of adrenaline and noradrenaline obtained were not "improved" by atropinisation. This was determined in one or two preliminary experiments.

Hexamethonium. (C6). This has proved most useful for stabilising the "base line" at a low level and increasing the sensitivity of the rat to adrenaline and noradrenaline. It was given soon after the rat was set up in a dosage of 400µg./100g. rat of hexamethonium bromide, intravenously, slowly over 2-3 minutes. The blood pressure usually fell to about 50mm. mercury and the rat could be used immediately for assays. After about 0.75-hour the blood pressure had often risen gradually to about 100mm. mercury. The C6 could then be repeated but often larger doses (about twice as much) than the first, were necessary for lowering the blood pressure to about the same extent.

This method of quickly producing a low stable blood pressure in the rat with C6 by paralysis of autonomic ganglia was found more satisfactory than leaving the animal till the blood pressure fell before using it for assays. In the latter, the effect was probably produced by slow bleeding and by the toxic effect of urethane over a fairly long period.

Standard/

Standard solutions of adrenaline and noradrenaline.

Stock solutions of those amines were prepared containing 1mg./ml. of the base from synthetic 1'adrenaline (Burroughs Wellcome and Co.) and 1'noradrenaline d'bitartrate monohydrate (Bayer) by solution in 0.01 N.HCl. In some of the earlier experiments, dl'noradrenaline hydrochloride was used and assumed to contain half its weight of the 1' isomer.

The table shows the relative molecular weights.

	<u>Mol. Wt.</u>	<u>Wt.for unit Wt.of 1'base.</u>
1'Adrenaline (base)	183	1.0
[1'Noradrenaline (base)	169	1]
dl'noradrenaline hydrochloride	2x205	2.4
1'noradrenaline d'bitartrate monohydrate.	337	2.0

Such stock solutions were stable for several months if stored in small stoppered test tubes at 5°C.

For use as standards, these stock solutions were diluted appropriately with saline containing freshly dissolved ascorbic acid (5mg./100ml.). This was used for all the biological tests except the perfused rabbit's ear for which the concentration of ascorbic/

ascorbic acid had to be reduced to  $1\text{mg./100ml.}$

For the rat's blood pressure preparation the standards were not made up in phosphate solution similar to the eluates from chromatographed samples. This was because it was found that if several assays had to be made, sometimes necessitating more than 100 injections for a rat, the administration of large amounts of phosphate depressed the responses of the animal which soon became insensitive and indiscriminating.

This did not occur if alternate injections of solutions in saline (standards) and in phosphate (test eluates) were given.

Several tests were carried out in which simple solutions of adrenaline and noradrenaline in saline and in phosphate were compared to verify that there was no difference in the responses to particular doses of the pressor amines attributable to the different solvents.

Summary.

1. Studies were made of some of the methods of estimation in general use for adrenaline and noradrenaline, including Shaw's chemical test and the biological tests using the rat's uterus and colon and the rabbit's ear.
2. Various methods of using the rat's uterus for assays, were tried and some of them tested on the rat's colon.
3. The relation of the oestrus cycle of the rat to the excitatory and inhibitory responses of the rat's uterus was studied from the point of view of usefulness for assay purposes.
4. The separation of adrenaline and noradrenaline by a paper chromatographic method and an assay method, using the rat's blood pressure, are described.
5. The rat's blood pressure preparation has been studied.

Part II.B. Studies on nerve sympathin and the  
adrenal medullary hormones.

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II. The amounts of the active principles in the rat's adrenal medulla stimulated by various agents.	181
III. The release of the adrenal medullary hormones in the cat.	229

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I. The nature of sympathin  
released in the rabbit's ear.

Introduction.

It is now generally accepted that most mammalian adrenergic nerves release a mediator consisting largely of noradrenaline which may be admixed with a varying but small proportion, of adrenaline. The adrenergic nerves in the rabbit's ear were investigated by Gaddum and Kwiatkowski (1938, 1939) and Gaddum, Jang and Kwiatkowski (1939), and found to release adrenaline. In view of the fact that no evidence was found for the release of noradrenaline these nerves have been quoted as exceptions to the general rule. Since there are now more sensitive and specific tests for the identification of the adrenergic mediators it was decided to reinvestigate the nature of those released in the rabbit's ear.

Method.

A large rabbit (over 2.5kg. where possible) was anaesthetised with Pentobarbitone Sodium ("Nembutal" Abbott containing 1 grain per cc) in a dosage/

dosage of 1.0ml./kg. intraperitoneally, repeated every 15 minutes till anaesthesia was complete (0.5-1.5hour ).

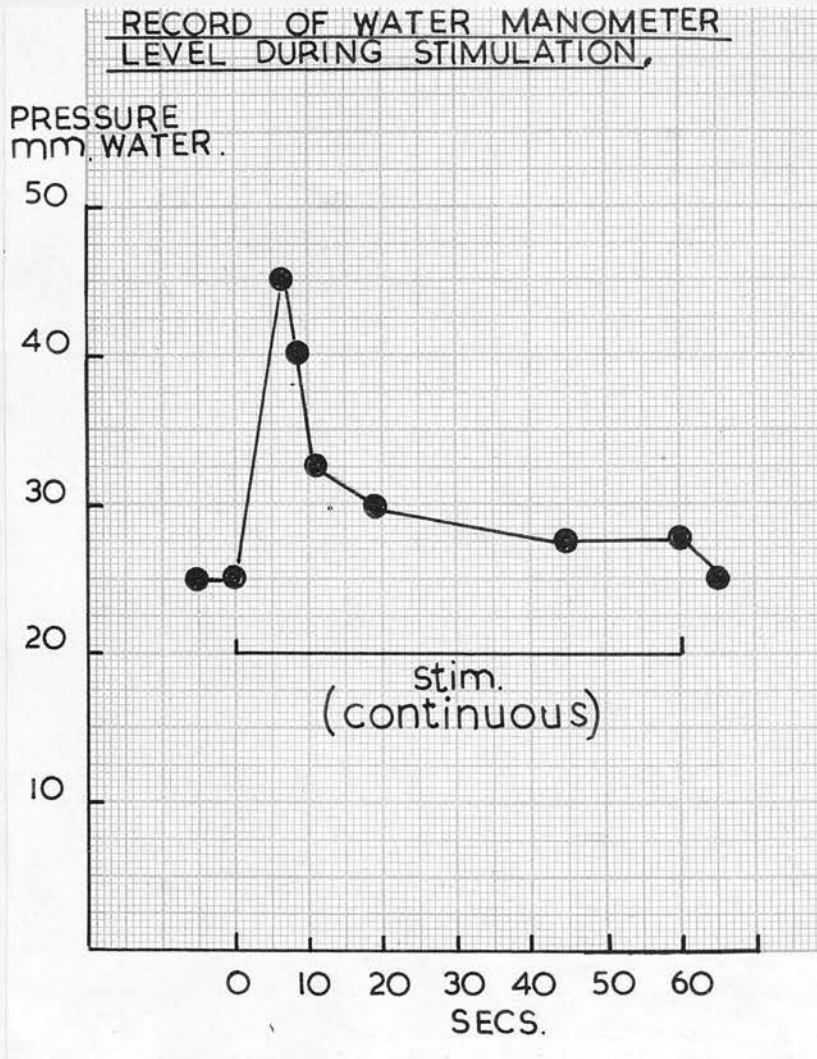
The animal was fixed in a supine position on a warmed table. The trachea was quickly cannulated and artificial respiration instituted. The common carotid artery was identified and followed headwards to its terminal branches. The aim of the dissection throughout was to identify the arterial branches rather than to clean the structures surrounding the vessels. All the branches of the common carotid artery were tied off, including the terminal ones, but excluding the artery to the ear which arises laterally about the level of the superior cervical ganglion. The tied vessels were not divided.

The cervical sympathetic trunk was traced up to the superior cervical ganglion and the fine post-ganglionic fibres running laterally, carefully dissected for 0.2-0.5cm. Where these post-ganglionic fibres were in close proximity to the auricular artery no attempt was made to separate them from the vessel, but where the anatomy permitted, the nerves were cleared separately. The great auricular vein was/

was freed. For identification, a loose ligature was placed around its union with the external jugular vein. The common carotid artery was then cannulated and the perfusion with salt solution commenced. The arterial cannula was usually of a simple straight type but where the preparation was to be used later for assays (Gaddum, Peart and Vogt, 1949) the special cannula described by Gaddum and Kwiatkowski (1938) was used. As soon as the salt solution was flowing into the ear the external jugular vein was cut with scissors to allow the perfusate to flow out freely. The tissues of the neck were completely severed with a scalpel as low down as possible, and the vertebral column was cut with bone forceps, thus decapitating the animal. To obviate leakage, the tissues of the neck were tied with a stout cord at a level below the arterial cannula and the cut in the jugular vein. Any possible leaking vessels were thus compressed against the cervical vertebrae. The vertebral canal was blocked tightly with plasticine and a cork.

A straight glass cannula was then inserted into the great auricular vein through the orifice in the/

Fig. XXI.



the jugular vein and tied therein. The reservoir containing the perfusing fluid was fixed about 2-3m. above the preparation. The fluid passed through a 3-4cm. length of capillary tubing fitted with a bypass of rubber tubing with a clip. When the clip was closed the only passage available was through the capillary. The bypass enabled the preparation to be washed out. The clip was generally opened slightly when the perfusion commenced but tightened when the effluent from the ear no longer contained evident traces of blood. A water manometer of small-bore glass tubing was interposed between the capillary tube and the arterial cannula. The apparatus is similar to that devised by Gaddum and Kwiatkowski (1938) and is intended to make the rate of outflow independent of the degree of contraction of the vessels of the ear. The "resting" perfusion pressure was usually 10-15cm. water, but on stimulation of the nerves the pressure rose owing to vasoconstriction. Figure XXI is a record of the changes in the manometric level during one such stimulation.

The perfusing fluid was equilibrated with 5% carbon dioxide in oxygen before perfusion started, and/

and as finally evolved had the composition NaCl 9, KCl 0.21,  $\text{CaCl}_2$  0.24,  $\text{NaHCO}_3$  0.12, Glucose 1g./litre with ascorbic acid ( $10^{-5}$ ) and ephedrine hydrochloride ( $2.5 \cdot 10^{-6}$  of base). In a few experiments the ephedrine was omitted at first and introduced later. The purpose of using half the usual concentration of potassium was to reduce interference in the biological assays, and of one quarter the usual bicarbonate to render the solution somewhat less alkaline (Bülbring, 1944) to preserve any amines liberated into the vessels. The perfusion was carried out at room temperature.

For stimulation of the nerves a unipolar electrode was used, the "earth" terminal being a piece of brass gauze wrapped in saline-wetted cotton wool and placed behind the head. Stimulation was by an alternating current (50 cycles, 5-10volts) for 10-20 seconds in every minute for 10-15 minutes. The voltage in the first stimulation period was that which elicited a good contraction when tested on a voluntary muscle, but this was increased in succeeding periods to ensure that adequate stimulation was obtained. Continuous stimulation for the whole period of collection/

collection of the sample and intermittent stimulation by "square" pulse waves of different durations and frequencies were also tried but possessed no advantages.

#### Procedure.

When the perfusing fluid was clear of blood the collection of samples was commenced. The outflow from the venous cannula was collected in ice-cooled centrifuge tubes, containing solid ascorbic acid (1mg./10ml.). Each sample was collected over 10-15 minutes and in any particular experiment the samples obtained were of approximately the same volume, since the rate of flow was kept constant by the perfusion device used. As soon as possible after collection the samples were centrifuged (2,000 r.p.m. for 5 minutes) to bring down any cells, and the supernatant fluid was transferred to test tubes and immediately acidulated with 2-3 drops of 0.15 N. hydrochloric acid.

After one or more control samples had been taken the electrode was placed under the cleared nerves, or under the nerves and artery if the two had not been separated by dissection. Stimulation was applied/

applied intermittently during the sampling. Usually several "stimulation" samples were collected, interrupted by periods of rest during which control samples were again taken.

#### Estimation of adrenaline and noradrenaline.

In the first series of experiments these substances were estimated by using several different tests on each sample and calculating the concentrations of the two amines by means of appropriate formulae. The biological methods were those described by Gaddum, Peart and Vogt (1949), and Gaddum and Lembeck (1949). Shaw's colorimetric test was also used (Shaw, 1938).

In some assays on the rat's uterus and colon the control samples had a stimulant action due to interfering substances. When these were present the effects of active samples were compared with those of the control samples with added adrenaline (cf. Peart, 1949).

In order to test for the presence of interfering substances which had a "masking" effect on small amounts of adrenaline, that amine was added to control



control samples and this mixture was compared with standard solutions of adrenaline. Up to an adrenaline concentration of 25µg./ml. not more than a 20% discrepancy on the rat uterus was found, and in the majority of cases there was no appreciable difference.

In the second series of experiments after preliminary direct tests with the rat's uterus, the two amines were estimated independently after separation by paper chromatography.

Each sample (4-6ml.) was placed in a 2.5x9cm. centrifuge tube with a further 2-3 drops of 0.15 N. HCl and 1mg. ascorbic acid. To each sample 10ml. alcohol (ethanol) was added. The tubes were left in the refrigerator for 0.5 hour and then centrifuged (2,000 r.p.m. for 15 minutes). The supernatant fluid was transferred to a 50ml. round-bottomed Quickfit and Quartz flask, the tube being washed with about 2 ml. alcohol which was also transferred. A little whitish residue was sometimes left in the tube. The mixture in the flask was evaporated to dryness in vacuo at a temperature not exceeding 60°C. (external temperature) and the flask allowed/

allowed to cool with the suction still on. The flask was then opened and the inside leached three times with 1.0; 0.5; 0.5ml. alcohol saturated with sodium chloride. Potassium salts are very insoluble in this mixture and thus possible interference in the assays by potassium is minimised (Barsoum and Gaddum, 1935). The leachings from two or more control or stimulation samples were usually pooled by transferring them to another 50ml. flask. The contents were evaporated in vacuo and taken up in 0.75ml. acid alcohol (0.1% conc. HCl in ethanol). This was applied to a cylinder of filter paper for chromatography. The flask was washed with a further 0.2ml. acid alcohol which was also applied. Endeavours were made to keep the total original volumes of the pooled control samples and the pooled stimulation samples approximately equal.

The application of the acid alcohol extracts to the paper, development of the chromatogram with phenol as solvent, elution of the approximate strips of paper containing adrenaline and noradrenaline and the assay of these amines using the rat's blood pressure preparation (Crawford and Outschoorn, 1951) have been described in the section on methods.

Factors/

Factors possibly affecting the tests.

The effects of ascorbic acid on the biological tests were investigated. Ascorbic acid ( $10^{-5}$ ) was used in the tests described by Gaddum, Peart and Vogt (1949), and did not cause interference. The tests were repeated with five and ten times this amount, the solutions having been acidified with HCl similarly to the samples of the rabbit's ear effluent and made almost neutral with solid sodium bicarbonate before assay on the rat's uterus and colon. The sodium ascorbate did not affect the responses of these tissues. In the chromatographic method, however, much higher concentrations might have occurred in the solutions used in the assay. Standard mixtures of adrenaline and noradrenaline (2 $\mu$ g./ml. of each) containing ascorbic acid in concentrations of 1, 5, 10 and 50mg./100ml. were therefore chromatographed and assayed on the rat's blood pressure preparation. The percentage recoveries all agreed closely, irrespective of the amounts of ascorbic acid present originally and were of the order found by Crawford and Outschoorn (1951). These results indicate that for good recoveries of adrenaline and noradrenaline concentrations/

concentrations of ascorbic acid above  $10^{-5}$  are unnecessary but not disturbing.

The same amounts of adrenaline and noradrenaline were chromatographed with ephedrine hydrochloride ( $10^{-5}$  as base) alongside "blank" ephedrine solutions of the same concentration. Assayed on the rat's blood pressure preparation the "blanks" showed no difference from equal volumes of saline, while the adrenaline and noradrenaline were recovered to the same extent as without ephedrine. In addition, noradrenaline added to "blank" ephedrine eluates after chromatography showed no "masking" when compared with standard solutions of noradrenaline.

Attempts were made to find out the fate of ephedrine subjected to this method of chromatography and to compare it with that of adrenaline and noradrenaline. About 2mg. of ephedrine in  $2 \cdot 10^{-3}$  solution was applied on a 12cm. line alongside a control spot of adrenaline and noradrenaline mixture. After 22 hours development the paper was washed in benzene and dried. The ephedrine portion was sprayed with 10% ninhydrin in water, allowed to dry again and sprayed with 10% sodium carbonate solution. The adrenaline/noradrenaline strip was developed with/

with ferricyanide in the usual way. The ephedrine showed up at the edge of the solvent, having travelled much faster than the adrenaline. For an average solvent flow of 27.5cm. the mean R<sub>F</sub> values (taken from the centres of the coloured areas) were ephedrine: 0.85, adrenaline 0.57 and noradrenaline 0.29. The ephedrine was thus well away from the portion of paper which would have contained the adrenaline had this been present in the solution analysed.

The effect of ephedrine on the rat's blood pressure preparation was tested by injecting volumes of up to 0.4ml. of solutions from  $10^{-3}$  to  $10^{-6}$ . None of the effects were distinguishable from those of saline injections. Dilutions made in the same way, and using the same pipettes from the same stock solutions of adrenaline and noradrenaline in saline, and in saline containing ephedrine ( $10^{-5}$ ) were also compared. No differences were observed in the pressor responses. West (1947), has quoted workers using the spinal cat and has himself examined the actions of ephedrine on the responses to adrenaline and noradrenaline of the Straub heart. It is generally/

generally agreed that low concentrations ( $10^{-6}$ - $10^{-7}$ ) synergise while high concentrations ( $10^{-4}$ ) antagonise. Neither effect was noted on the rat's blood pressure over a period of about two hours. It is possible that intermittent injections of ephedrine over a longer period might have made these effects evident but this was not tested. In any event, as estimates of the amines were made by comparison with standard solutions it is unlikely that any sensitisation by ephedrine would have introduced an error into these estimates.

On the other biological tests concentrations of ephedrine above  $10^{-5}$  were found to produce definite effects and to potentiate those of standard adrenaline solutions. They were most marked on the rat's colon and were less on the rabbit's ear and rat's uterus. Smaller concentrations of ephedrine did not have these effects and one fourth the average threshold concentration was selected as suitable for the perfusing fluid. The existence of unusual sensitivity to ephedrine in any of the biological tests would have been brought to light when control samples containing ephedrine only, were tested. No effects of any significance were found./

found.

Ephedrine is known to be without effect on the results of Shaw's chemical test (Gaddum and Kwiatkowski, 1938). An experiment was performed in which different amounts of adrenaline were added to volumes of the fluid used for perfusing the ear, i.e., of the same composition and containing ephedrine to which was also added a few drops of rabbit's blood. This was to reproduce as nearly as possible the type of samples obtained in the sympathin experiments. They were then assayed by Shaw's method and using the rat's uterus. The results by either method showed good agreement with the original amounts of adrenaline added. They are shown in the following table.

Solu- tion No.	Conc. of adr. (µg./ ml.)	Solvent used	Results by Shaws test.	Results by assay on rat's uterus
1	10	Perfusing fluid	7	9
2	15	" "	14	15
3	25	" "	21	21
4	10	Saline	Stand- ard.	Stand- ard.
5	25	"	"	—

In/

In assays of the final eluates, using the rat's blood pressure preparation, injections of samples from some experiments had depressor effects, while those from others, did not. The results of assays were checked by diluting standard noradrenaline (1 in 10) with eluates of the noradrenaline or adrenaline strips of the control samples collected when the nerves were not stimulated. The concentrations of these dilutions were made to contain 100-250µg. noradrenaline per ml.; if the eluates had depressor properties, these concentrations of noradrenaline were often completely "masked"; if the eluates were pressor, their "masking" effect was either small (20%) or absent.

The results described below were those obtained by actual comparison with standard noradrenaline and not multiplied by a factor calculated from the "unmasked" proportion of the noradrenaline added to the control sample. They are therefore often underestimates. Since the occurrence of depressor effects did not appear to be related to the volume of original sample concentrated and chromatographed, and since all the samples from a particular experiment either did, or did not, prove depressor, the ability to obtain/



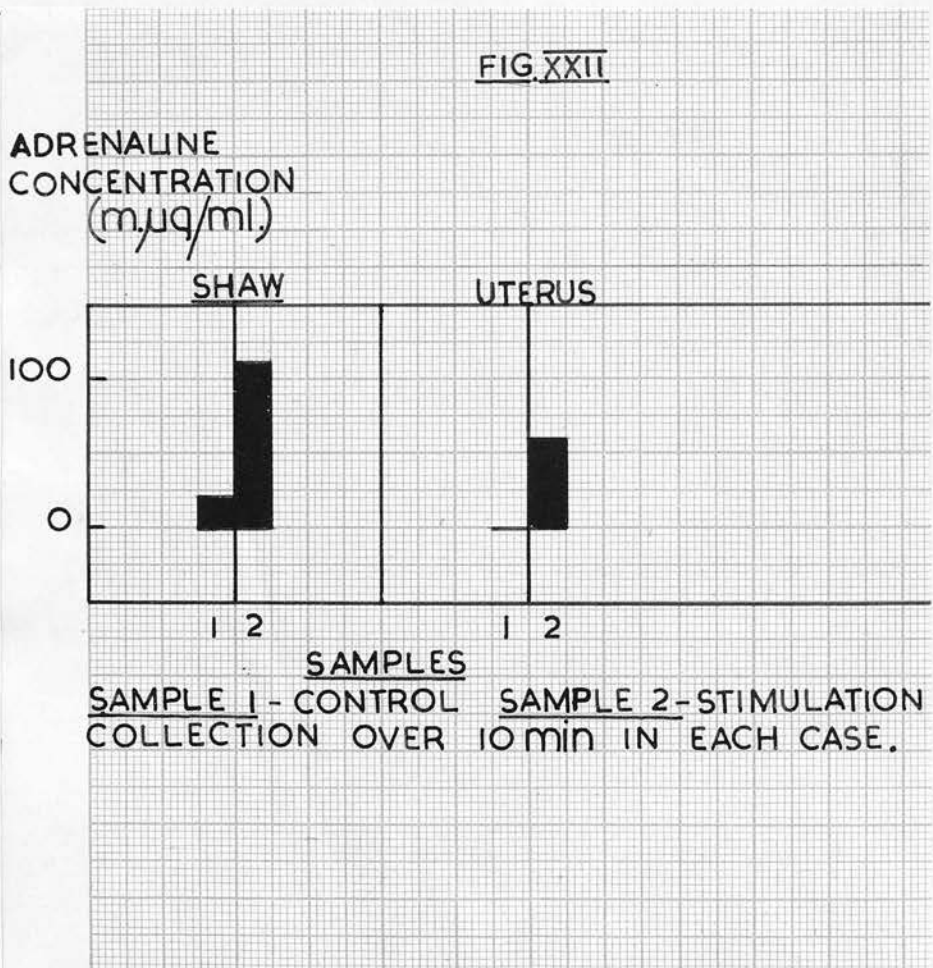
obtain a result of an assay apparently depended on the relative insensitivity of the rat used for the assay to the interfering substances present. It was impracticable to do a preliminary test on one (control) sample and in the event of there being depressor effects prepare another rat in the hope that it would prove more satisfactory.

### Results.

In some experiments the activity of the effluent was too low to be detected by direct tests, and in others, stimulation of the nerve had no apparent effect, but in eight experiments there was clear evidence by at least two methods that following stimulation the samples obtained were more active than control samples. This was obtained not only by individual assays of the samples against standard solutions but also by direct comparison of the control and stimulation samples in the same biological tests. The results obtained by each of the tests used are given in Table V. By this direct comparison and assay increased activity due to stimulation was shown by the rat's uterus 9/10 times, by the rat's colon 3/5 times, by the rabbit's ear 2/2 times, and by Shaw's test 5/5 times. Table VI shows/

Comparison of assays by Shaw's test and  
with rat's uterus.

Experiment No.13.



shows the results of tests on the active samples collected during and after stimulation when the increase due to stimulation was shown by at least two tests.

The estimates of the adrenaline equivalent with the uterus were nearly always much lower than those given by Shaw's test or the colon or the ear. Such a pair of results is shown in the graph alongside. This fact itself shows that adrenaline was not the only substance present, and could be explained by the presence of noradrenaline. The equivalent concentrations of these two substances were calculated from the results obtained with the uterus and colon by the formulae used by Gaddum and Lembeck (1949) and others.

In Table VI the concentrations of adrenaline and noradrenaline calculated in this way show in some instances "negative" values for adrenaline (experiments 4 and 5). These minus values are a measure of the error of the assay methods when a small amount of adrenaline is present in a mixture with a relatively larger amount of noradrenaline, i.e., when the percentage methylation is below ten. Gaddum and/

Table V.

Results of assays of samples by different tests.

Test	Expt. No.	Dose ratio Nor./Adr.	Adrenaline equivalents (µg./ml.)		
			Control	During stimulation	After stimulation.
Rat's uterus	4	100	< 2	2.5	1.8
	5	15	1	1	7.9
	6	150	1.87	5.8	3.3
	9	150	1.8	3.7	5
	11	100	< 2.5	< 1.25	< 1.25
	12	100	1.8	3.7	< 1.25
	13	75	< 1.25	60	-
	16	100	3.7	15	-
	17	150	< 5	5	5
19	75	< 2.5	3.7	-	
Rat's colon	4	1	< 125	750	375
	5	1	187	187	375
	6	0.75	< 125	< 125	< 125
	13	1.1	-	750	-
	16	1.5	-	33	-
	17	2	< 250	< 50	82
	19	0.5	< 300	< 300	-
Rabbit's ear	5	3	-	-	10
	6	1.5	4	8	7.5
	13	0.75	-	3000	-
	16	3.7	7.5	15	-
Shaw's test	9	13.5	9	106	17
	11		26	25	70
	12		15	30	-
	13		20	112	-
	16		38	110	-

Table VI.

Results of tests on samples collected  
during and after stimulation.

Expt No.	Adrenaline equivalents (µg./ml.)				Concentrations calcu- lated from uterus and colon results. (µg./ml.)		Percentage Methyl- ation.
	Shaw's test	Uterus	Colon	Ear	Adrena- line.	Noradrena- line.	
4	-	2.5	750	-	( -5)	747	} 0
	-	1.8	375	-	(-1.9)	373	
5	-	7.9	375	10	(-17.1)	367	0
6	-	5.8	<125	8	-	-	
	-	3.3	<125	7.5	-	-	
9	106	3.7	-	-	-	-	
	17	5	-	-	-	-	
12	30	3.7	-	-	-	-	
13	112	60	750	3000	50	759	6.2
16	110	15	33	15	14.5	28	34
17	-	5	82	-	4.5	72	6

Table VII.

Concentrations of amines calculated from results of  
different combinations of tests.

Expt. No.	Tests	Adrenaline (m $\mu$ g./ml.)	Noradrenaline (m $\mu$ g./ml.).
13	Uterus and Colon	50	759
	Uterus and Ear	30	2205
	Shaw's test and Colon	51	702
	Shaw's test and Ear	(-55)	2166
16	Uterus and Colon	14.5	28
	Uterus and Ear	14.4	0
	Shaw's test and Colon	106	(-116)
	Shaw's test and Ear	106	(-352)

Lembeck (1949) have shown that in such cases only the calculated noradrenaline figure can be accepted as reasonably accurate. The replacement of the minus values by zero (i.e., no detectable adrenaline) is considered unjustifiable.

When several tests have been applied to the same samples the amounts of the amines could be calculated similarly from other combinations of tests. This has been done for two experiments and the results are shown in Table VII. In experiment 13 whichever combination of tests is used there is definite evidence of both amines. In experiment 16 the evidence for the presence of an active substance in addition to adrenaline, is not so convincing, only the colon which is the most sensitive test for noradrenaline, when considered with the rat's uterus indicating the presence of that substance. It is doubtful whether all the results can be explained by the presence of these two amines, but the data do not justify speculation about the possible presence of other substances.

The results in Table VIII provide better evidence for the conclusion that adrenaline and noradrenaline/

Minute output of adrenalinine and noradrenalinine, calculated from results of estimates in pooled samples of effluent. Adrenalinine estimated directly with the rat's uterus; noradrenalinine estimated with the rat's blood pressure after chromatographic separation.

Expt. No.	Control samples.		Samples collected during stimulation.		Percentage Methylation
	Adrenalinine (µg.)	Noradrenalinine (µg.)	Adrenalinine (µg.)	Noradrenalinine (µg.)	
25	< 0.33	Depressor	0.60	Depressor	-
27	0.12	< 4.17	0.34	< 6.25	> 2.5
29	0.68	< 8.33	1.00	12.50	7.4
30	< 1.41	Depressor	1.45	Depressor	-
31	< 0.81	< 3.33	0.82	1.43	36.5
32	< 0.45	< 3.38	0.71	3.75	15.9
34	0.84	< 2.07	0.60	< 2.07	> 22.5
35	0.42	< 1.67	0.48	3.12	13.3
36	< 0.52	< 8.33	0.30	< 3.12	> 8.8
39	< 0.43	< 0.83	0.59	1.10	34.9



noradrenaline are both released when the nerve is stimulated. In these experiments, noradrenaline was estimated on the rat's blood pressure after chromatographic separation from adrenaline and impurities. It was not possible to estimate adrenaline in the same way since the rat's blood pressure is not sensitive enough to detect these very small amounts of adrenaline, and the rat's uterus is too sensitive to interfering substances coming from the paper itself. The estimates of adrenaline given in the table were therefore obtained by testing samples taken from the unconcentrated extracts before chromatography. The amounts of noradrenaline found would not affect the results of those tests since the rat's uterus was never less than 100 times as sensitive to adrenaline as it was to noradrenaline. The evidence in Tables VI and VIII that the substance measured by these tests on the rat's uterus was adrenaline was thus not very good; it was certainly not noradrenaline or any other substance which is known to occur in the body. If it is true that these results give an estimate of adrenaline then this substance/

substance was present in all of the samples collected during stimulation. The amounts present in control samples were generally too small to be detected and in 9 of the 10 experiments (Table VIII) there was evidence that adrenaline was present after stimulation in higher concentration than before.

Noradrenaline was detected in 5 of the 10 samples (Table VIII) collected during stimulation and in each of these cases the concentration was greater than in the corresponding control samples. The identification of this substance as noradrenaline depends upon the fact that it moved up the paper at the same rate as noradrenaline.

These results show that both amines were present in increased amounts after stimulation. The percentage methylation in the samples collected during stimulation varied between 7.4 and 36.5%.

#### Discussion

The results recorded here show that both adrenaline and noradrenaline were released during stimulation of the nerves. The percentage of adrenaline was often much higher than that found for other nerves, so that it is perhaps not surprising/

surprising that Gaddum and his co-workers failed to detect the simultaneous liberation of noradrenaline. The concentrations are very low and it is difficult to get satisfactory results even with the improved methods now available. The results of the direct tests shown in Table II prove that adrenaline was not the only substance present, since the test on the uterus gave lower estimates than the other tests. This test has a higher dose-ratio (noradrenaline/adrenaline) than any other known test and is therefore particularly useful in experiments of this type. Gaddum and Kwiatkowski (1939) used the hen's rectal caecum, which also has a fairly high dose-ratio, and did find that the adrenaline equivalent in this test was sometimes lower than that on the frog's heart. This might have suggested the presence of noradrenaline, but the evidence was not convincing and was neglected. The specific test described by Shaw (1938) proved that adrenaline was present and the facts did not justify the theory that the two amines were both present.

In the present experiments, only Shaw's ordinary test was applied and although the results are/

are similar to Gaddum and Kwiatkowski's the discrepancy found between the adrenaline equivalent obtained by it and with the rat's uterus indicates that in the chemical method some other reactor with the colour reagent was also being estimated, since the effluent did not contain any substances which "masked" the effect of adrenaline added to control samples and assayed on the rat's uterus.

As previously shown by Gaddum and Kwiatkowski (1938), the addition of ephedrine to the perfusing fluid was essential to obtain a high yield of sympathin. The reason they assumed for this phenomenon was the inhibition of amine oxidase by the ephedrine. Recent evidence in favour of their hypothesis was obtained when Thompson was able to demonstrate the presence of the enzyme in rabbit ear vessels and the potentiation of the effects of injected amines was lost when the enzyme had apparently disappeared after prolonged perfusion (Burn and Robinson, 1951). The following results show/

show the effect of adding ephedrine to the perfusing fluid.

Experiment 31

Results of assays on the rat's uterus.

<u>Plain Locke 1.15p.m.</u>			<u>Ephedrine Locke 3.30p.m.</u>		
Sample	Time p.m.	Adrenaline equivalent (µg./ml.)	Sample	Time p.m.	Adrenaline equivalent (µg./ml.)
Control	2.15	≪ 1.25	Control	4.00	≪ 1.25
Stim.	2.30	≪ 1.25	Stim.	4.15	1.25
Stim.	3.00	≪ 1.25	Stim.	4.50	1.87
			Stim.	5.05	1.25

The low concentrations obtained show that it would not have been possible, even now, to prove that the two amines were both present without the use of concentrated extracts and chromatography. The evidence still does not exclude the possibility that other active substances besides adrenaline and nor-adrenaline are released.

Summary/

Summary.

1. The sympathetic nerves were stimulated in rabbit's ears perfused with salt solutions and the effluent tested for sympathomimetic amines by colorimetric and biological methods aided by chromatography.
2. The release of adrenaline is confirmed, but evidence is presented that it is accompanied by larger amounts of noradrenaline.
3. The percentage methylation was generally higher than that usually found after stimulation of other adrenergic nerves but there is no evidence of any qualitative differences between sympathetic nerves to the rabbit's ear and those to other tissues.

II. The amounts of the active principles in the adrenal medulla of rats stimulated by various agents.

Over seventy years ago it was suggested by Johannes Müller that there were two ways available to determine the secretory function of an endocrine gland. One could look for secretory products in extracts of the organ or in the venous blood draining it. Both methods have been used extensively.

Among the earliest studies on the amounts of adrenal medullary hormone were those of Elliott (1912) who determined biologically the adrenaline content of cat's adrenals after the glands had been stimulated; Stewart and Rogoff (1916) who attempted to relate the amounts of adrenaline in the glands to the quantities released into the circulation; and Cramer (1918) who demonstrated histo-chemically granules of adrenaline in the adrenals of various animals enabling the glands to be studied after different stimulating agents had been used.

Many/

Many chemical substances are known to cause a release of adrenal medullary hormone. They may be broadly classified (LeLoir, 1934) into two groups. The first consists of substances which act directly on the adrenal medulla and independently of the nervous system because section of the splanchnic nerves or adrenal denervation fails to abolish their effects. Examples are histamine, nicotine, acetylcholine (and eserine), potassium and quaternary ammonium compounds. The second category produce their effects on the adrenal medulla through the splanchnic nerves, i.e., reflexly, like insulin, caffeine, strychnine and ether. Emotion, cold, asphyxia and haemorrhage also act through the nerve.

Three drugs belonging to LeLoir's second category were selected for study in the present series of experiments, particularly because they differed widely in their other pharmacological effects, viz. insulin, morphine and  $\beta$ -tetrahydro-naphthylamine.

#### Modes of action.

Insulin. It was shown by Cannon, McIver and Bliss (1924) that the injection of insulin evoked a discharge of adrenal medullary hormone as soon as the/  
the/



the blood sugar fell below 70mg. per 100cc. The experiments were carried out on unanaesthetised cats and the adrenaline release was demonstrated by an increase in the animals' heart rate. The effect was absent if one adrenal was removed and the other denervated and prevented if the blood sugar was kept above the critical level by an injection of glucose.

Using Tournade-Chabrol (1923) vaso-anastomosed dogs (adrenal vein of donor to jugular vein of recipient) Houssay, Lewis and Molinelli (1924a) by administration of insulin to the donor (causing a hypoglycaemia) produced glycogenolysis and hyperglycaemia in the recipient. This only occurred if the donor's splanchnic nerves were intact and also if the hypoglycaemia was not counteracted by glucose.

Using a similar technique, La Barre (1937) and La Barre and Kettenmeyer (1937), carried out the isolated perfusion of a dog's brain with hypoglycaemic blood from a donor animal. This caused a release of adrenal medullary hormone into the blood which they collected through a venous fistula and assayed on isolated rabbit's intestine. It was unaffected by vagotomy, but preventible if the spinal cord was cut/

cut in the cervical region. La Barre and Kettenmeyer (1941) also demonstrated the release of hormone by decreased gastric movements in the same animal. They showed that thalamic paralysis by barbitone (sodium barbital) or magnesium sulphate had a similar effect to cutting the cervical spinal cord.

Morphine. Besides the direct demonstration of the effect of morphine on the adrenal medulla by Elliott (1912) using the method already mentioned, this drug has been chiefly studied through the hyperglycaemia it produces (Ross, 1918). Stewart and Rogoff (1922) and Lewis (1923) showed that the adrenals played a part in the production of this hyperglycaemia but Houssay, Lewis and Molinelli (1924b) using the same technique as they used in the study of insulin effects on the adrenals showed that the injection of morphine into the donor produced hyperglycaemia in the recipient. This was chiefly due to the release of adrenal medullary hormone for it did not occur if the splanchnic nerves of the donor were cut. The effect was almost abolished in cats and dogs by denervation or demedullation (Bodo, CoTui and Benaglia, 1937) and was absent in rabbits/

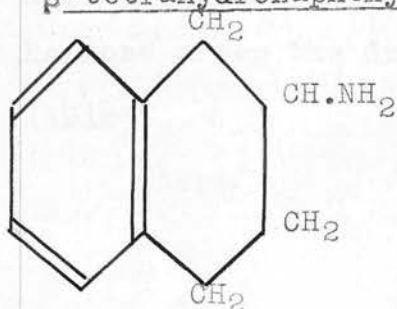
rabbits after adrenalectomy (Chon and Chin, 1943).

The direct estimation of medullary hormone released after morphine was made by Sato and Ohmi (1933) in non-anaesthetised dogs. The maximum output occurred within 10 minutes of intra-venous and 0.5hour of subcutaneous injection.

The output of adrenal medullary hormone (as shown by hyperglycaemia) was prevented by section through the midcollicular region of the brain in cats (Brooks, Goodwin and Willard, 1941) and by infusion of 20mg./kg./30 minutes of tetraethylammonium in dogs (Morrison, 1947). The last drug had a similar effect in rabbits (Heuvel, 1950) and acts presumably by virtue of its ganglion "blocking" properties.

It has been shown that when prolonged administration of morphine established tolerance to its narcotic effect a marked reduction in the release of adrenal medullary hormone also occurred, as evidenced by absence of its effects (Wada, Tanaka, Hirano and Taneiti, 1938).

$\beta$ -tetrahydronaphthylamine.



ac-2-amino-1,2,3,4-tetrahydronaphthylamine.

The effects of administration of this drug had received attention from as early as 1889 but they were reinvestigated in detail by Mutch and Pembrey (1911), who described the results of injection of a 3% solution into rabbits. Although they did not make the following distinction, its effects can be divided into (a) those now known to be the result of sympathetic stimulation, e.g., rise of blood pressure, mydriasis and (b) increased muscular activity, hyperthermia and convulsions. The combination of hyperthermia, hypertension and mydriasis occurring with this drug came to be known later (1913) as "Cloetta and Waser's triad".

The drug was one of the agents used by Elliott (1912) in his investigation into the adrenaline content of the glands of the cat and also by Cramer (1918, 1919, 1920) using the histo-chemical method of staining the granules of adrenaline. The latter found that no release of hormone occurred after administration of tetrahydronaphthylamine if the splanchnic nerves of the animal were cut. Hyperglycaemia caused by release of adrenal medullary hormone after the drug was described by de Corral (1918).

There/

There is evidence that the rise of body temperature described by Mutch and Pembrey (1911) is independent of the blood sugar level, because if it be kept low by simultaneous injection of insulin, section of the splanchnic nerves or adrenalectomy, the temperature reaction is unaffected (Bouckaert and Heymans, 1928).

#### Methods.

Rats of both sexes, weighing 150-200g. were used. For the insulin experiments only they were fasted overnight (17 hours) and during the course of the experiments. Groups of 4-8 rats were divided into two equal lots. One lot was injected subcutaneously in the back of the neck with a drug and the other lot served as a control. The drugs used were:

Soluble insulin, 20 units/ml., (Burroughs Wellcome and Co., ) - 1 unit  
or 0.05ml./100g. rat.

Morphine hydrochloride, (Macfarlane, Edinburgh)  
2mg. or 0.1ml. of a fresh 2%  
aq. solution per 100g. rat.

ac-Tetrahydro- $\beta$ -naphthylamine carbonate (B.D.H)  
7.5mg. or 0.375ml. of a fresh  
2%/

188.

2% aq. solution per 100g. rat, prepared as follows: 100mg. powder was dissolved in about 3.5ml. of 0.15N.HCl. A small amount of brownish material remained undissolved (impurities). The solution was filtered and the filter paper washed through with distilled water. The filtrate was made up to 5ml. with distilled water, and partially neutralised with solid  $\text{NaHCO}_3$ . It was still distinctly acid (red with B.D.H. Universal Indicator) before use.

Both injected and control animals were killed together by a blow on the head at various times after administration of the drugs. The adrenals were quickly dissected out, weighed on a torsion balance, and the pair of glands from each animal placed in 0.5ml. of 0.15 N.HCl. in a "Pyrex" centrifuge tube. When all the glands from the particular experiment had been thus collected they were ground up in each tube with the assistance of a little acid-washed sand./

sand. To each tube was added 10ml. of alcohol (ethanol), the whole thoroughly mixed and left at room temperature for 30 minutes. The tubes were then centrifuged at 2,000 r.p.m. for 15 minutes and the supernatant fluid from each poured into a 50ml. round-bottomed Quickfit and Quartz flask, the residue being washed with a further 2ml. of alcohol. The solutions were evaporated to dryness in vacuo at 55°-60° (external temperature) and then taken up, each in 0.75ml. acid alcohol (0.1ml. conc. HCl. per 100ml. ethanol). This solution was applied to a cylinder of filter paper for chromatography in the usual way, the flask being washed with a further 0.25ml. acid alcohol which was also applied.

The development of the chromatogram in water-saturated phenol, elution of the separated amines and assay with the rat's blood pressure preparation (Crawford and Outschoorn, 1951) have been described in the section on methods. Each rat yielded finally two samples, each in a volume of 1ml. of 1.6%  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (almost isotonic) containing 50 $\mu\text{g.}/\text{ml.}$  ascorbic acid, one containing the adrenaline and the other the noradrenaline from both its adrenals.

### Results/

FIG. XXIII.

INJECTED AS  
PERCENTAGE  
OF CONTROLS

INSULIN

PERCENTAGE CONCENTRATIONS

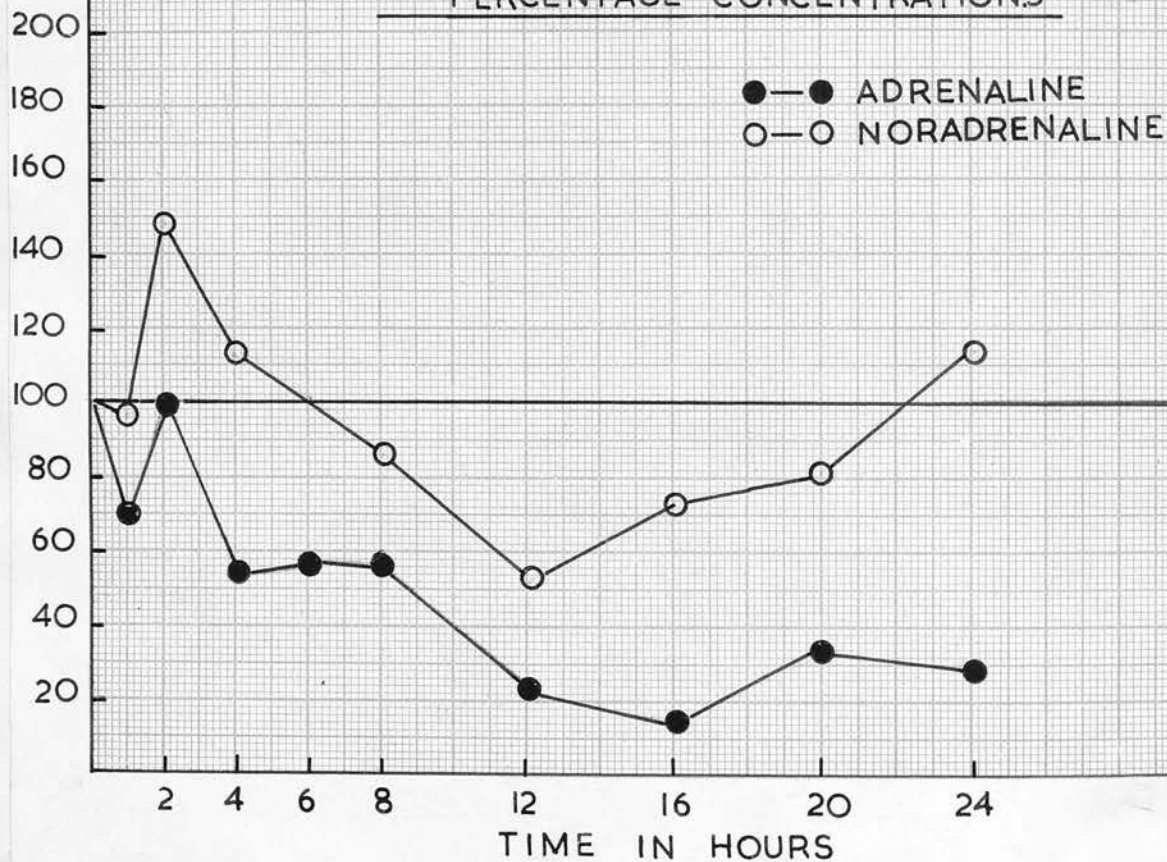




FIG. XXIV.

INJECTED AS  
PERCENTAGE  
OF CONTROLS

200

$\beta$ -TETRA

PERCENTAGE CONCENTRATIONS

180

●—● ADRENALINE.  
○—○ NORADRENALINE.

160

140

120

100

80

60

40

20

2

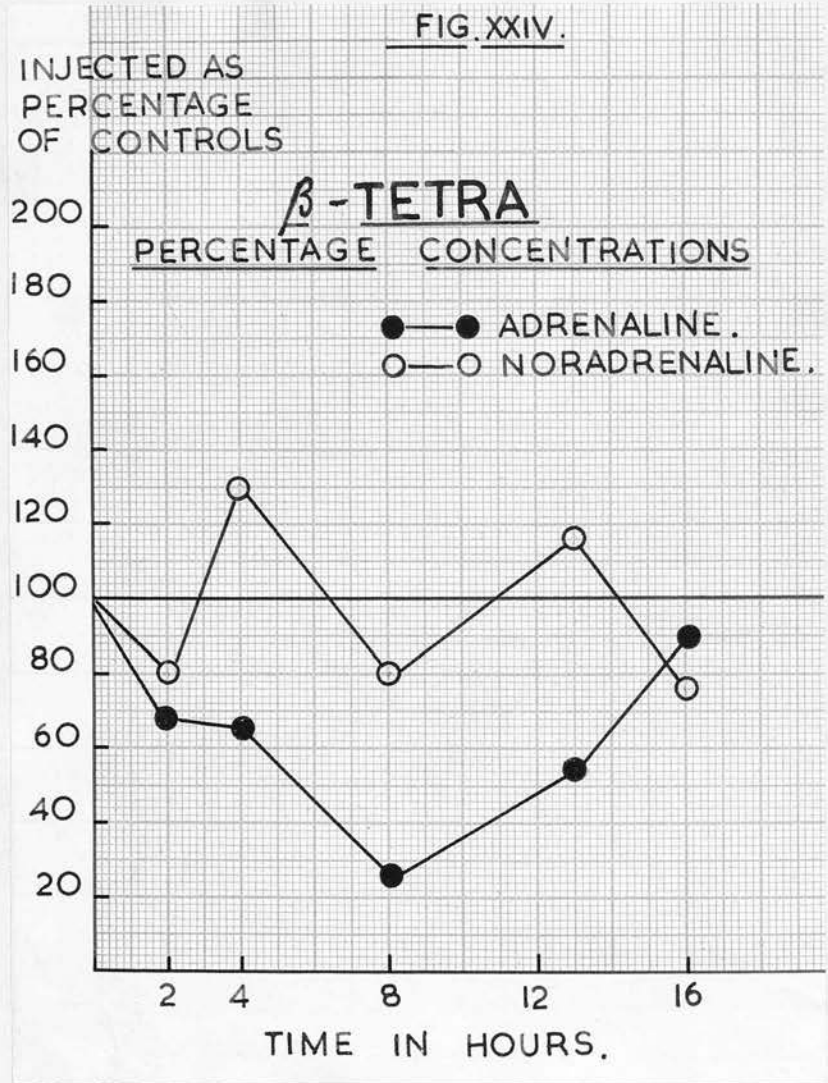
4

8

12

16

TIME IN HOURS.



Results.

The concentrations of adrenaline and nor-adrenaline (in terms of body weight of rat) found in each individual's pooled glands are recorded in Tables IX, X, and XI. These results show the changes in the absolute amounts of the amines in the glands of the injected and control animals. The changes produced by the drugs may be more distinctly seen however, if the amounts of the amines in the injected animals are calculated as percentages of the mean control values obtained in the same experiment. This has been done and the results are shown in Tables XII, XIII and XIV. The mean percentages for various times of action of insulin and tetrahydronaphthylamine, but not morphine, are shown in Figures XXIII, and XXIV.

Adrenaline. Insulin produced a depletion of adrenaline. In 12 hours the injected animals had about 20% of the amount in the controls and in 16 hours only 10-20%. Thereafter, and up to 24 hours a tendency to recovery was noticeable. The depletions of adrenaline from 12 hours onwards were significant.

Tetrahydronaphthylamine/

Table IXaConcentrations of active substances in rats' adrenals.( $\mu\text{g.}/100\text{g.rat}$ )Injected with insulin.

Hours after insulin	Expt. No.	ADRENALINE	NORADRENALINE
1	24	18.60; 16.67	1.16; 1.88.
	25	11.63; 12.50; 16.16	2.03; 1.88; 3.03.
2	19	11.97; 13.33.	2.99; 2.50.
	20	12.97; 9.41; 3.64.	2.16; 2.35; 1.82.
	33	18.18; 16.95; 9.70; 16.36.	1.82; 1.13; 1.82; 2.15
	38	14.28; 11.36; 16.22.	1.00; 1.56; 1.62.
	39	22.22; 18.33; 15.01.	4.44; 1.88; 2.42.
	40	14.41; 14.41; 15.50.	0.72; 1.08; 1.16.
	41	11.11; 12.50; 20.00.	1.11; 0.78; 3.33.
4	21	15.00; 3.75.	2.34; 1.82.
	22	8.82; 25.00; 13.64.	2.94; 2.08; 1.82.
6	23	25.97; 21.23; 8.08.	2.60; 1.70; 1.21.
	30	10.78; 10.67.	1.80; 1.33.

191a.

Table IXa (Contd.)

Hours after insulin	Expt. No.	ADRENALINE	NORADRENALINE
8	27	3.85; 13.07.	1.16; 1.74.
	29	9.14; 10.00; 6.72.	1.71; 1.72; 2.35.
12	26	2.14; 3.44.	1.07; 1.61.
	28	6.45; 1.52; 4.04.	1.29; 0.97; 1.21.
16	31	2.73; 2.94.	0.55; 1.76.
	32	4.62; 4.85; 5.00.	2.31; 1.82; 1.25.
20	34	3.54; 3.58; 3.08.	2.01; 2.18; 1.35.
	35	15.97; 4.26.	1.20; 1.14.
24	36	6.17; 6.13; 3.88.	1.23; 1.84; 0.89.
	37	8.88.	1.69.

Table IXbConcentrations of active substances in rats' adrenals.( $\mu\text{g.}/100\text{g. rat}$ )Controls (no insulin)

Hours after "zero".	Expt. No.	ADRENALINE	NORADRENALINE
1	24	24.24; 25.16; 21.39.	1.82; 1.68; 1.60.
	25	26.67; 20.23; 13.33.	3.00; 1.73; 2.00.
2	19	28.57; 18.62.	1.71; 2.13.
	20	18.93; 17.88; 22.82.	1.78; 1.12; 1.07.
	33	25.00; 15.69; 20.25; 15.78.	1.25; 1.18; 1.26; 1.18.
	38	11.98; 1.16; 2.13.	1.80; < 0.29; < 0.13.
	39	24.24; 19.35; 27.59.	1.52; 1.94; 1.72.
	40	18.87; 15.24; 19.38.	1.57; 2.28; 2.31.
	41	27.59; 20.51; 12.12.	1.38; 1.02; 0.81.
4	21	13.33; 26.67.	1.67; 2.50.
	22	20.20; 34.29; 25.00.	2.12; 2.29; 1.25.
6	23	25.00; 40.16; 30.30.	1.56; 1.36; 1.70.
	30	24.10; 16.34.	1.81; 2.61.

192a.

Table IXb (Contd.)

Hours after "zero".	Expt. No.	ADRENALINE	NORADRENALINE
8	27	17.78; 16.67.	2.67; 4.28.
	29	16.67; 12.90.	1.52; 1.77.
12	26	18.82; 26.67.	1.03; 3.56.
	28	14.65; 13.79; 12.81.	2.75; 3.45; 1.03.
16	31	13.54; 27.97; 52.29.	1.78; 2.80; 3.92.
	32	23.26; 27.03.	1.16; 2.70.
20	34	15.78; 19.32; 15.41.	1.58; 1.11; 3.47.
	35	17.34; 21.88.	1.16; 2.50.
24	36	21.88; 23.95; 20.23.	1.88; 1.80; 1.54.
	37	22.86.	0.95.

Table Xa.Concentrations of active substances in rats' adrenals.( $\mu\text{g.}/100\text{g. rat}$ )Injected with morphine.

Hours after morphine	Expt. No.	ADRENALINE	NORADRENALINE
		( <u>Single dose</u> ).	
1	48	9.41; 6.67; 4.32.	1.18; 4.00; 2.16.
	49	9.41; 10.32; 8.67.	2.35; 2.58; 4.00.
2	47	8.33; 15.97; 8.82.	1.11; 5.99; 2.35.
4	42	11.22; 11.66; 13.97.	1.32; 0.86; 1.68.
8	45	8.02; 8.02; 3.94.	1.07; 1.43; 0.45.
12	43	14.26; 16.67; 13.51.	1.60; 2.00; 2.16.
16	44	7.13; 7.62; 7.02.	0.53; 1.71; 1.58.
20	46	8.59; 8.33; 9.38.	1.47; 1.25; 3.12.

(Repeated doses)/

193a.

Table Xa (Contd.)

Hours after morphine	Expt. No.	ADRENALINE	NORADRENALINE
		(Repeated doses)	
4	50	6.06; 1.11; 0.36.	0.45; 0.83; 1.62.
	54	16.16; 8.08; 8.60.	3.03; 1.82; 1.94.
5	51	6.25; 6.94; 12.12.	2.00; 3.17; 2.69.



Table Xb

Concentrations of active substances in rats' adrenals.

( $\mu\text{g.}/100\text{g.rat}$ )

Controls (no morphine)

Hours after "zero"	Expt. No.	ADRENALINE	NORADRENALINE
		(Single dose)	
1	48	6.61; 10.81; 8.24.	4.62; 3.09; 0.88.
	49	8.89; 9.26; 8.08.	3.81; 1.67; 2.42.
2	47	6.49; 7.69; 10.00.	2.60; 0.51; 2.86.
4	42	11.15; 18.72; 12.00.	2.15; 2.00; 1.88.
8	45	7.41; 8.00; 9.70.	1.00; 1.00; 0.81.
12	43	15.29; 8.56; 16.13.	0.64; 0.80; 1.61.
16	44	7.33; 7.33; 11.36.	1.37; 1.43; 1.70.
20	46	7.96; 9.68; 8.93.	1.50; 1.29; 0.95.

(Repeated doses)/

Table Xb (Contd.)

Hours after "zero"	Expt. No.	ADRENALINE	NORADRENALINE
		<u>(Repeated doses)</u>	
4	50	11.76; 10.00; 13.64.	4.41; 3.75; 1.82
	54	12.50; 23.53; 14.28.	1.88; 2.35; 2.28.
5	51	13.89; 12.86; 15.85.	3.17; 1.52; 1.30.

Table XI

Concentrations of active substances in rats' adrenals.

( $\mu\text{g.}/100\text{g.rat}$ ).

Injected with tetrahydronaphthylamine

Hours after THN	Expt. No.	ADRENALINE	NORADRENALINE
2	52	14.70; 6.67; 9.70	1.47; 1.50; 2.27.
4	53	5.88; 10.26.	3.36; 2.93.
8	55	5.00; 3.87; 4.30.	2.00; 0.83; 1.61.
13	56	6.67.	2.67.
16	57	9.14; 12.12; 10.00.	- ; 1.62; 3.33.

Controls (no injections).

Hours after "zero"	Expt. No.	ADRENALINE	NORADRENALINE
2	52	20.00; 15.62; 10.00.	3.33; 1.25; 1.88.
4	53	12.12; 12.90; 11.76.	3.03; 2.58; 1.57.
8	55	27.57; 12.12; 12.50.	2.07; 1.82; 1.56.
13	56	10.53; 14.70; 12.50.	2.10; 2.35; 2.50.
16	57	12.50; 13.33; 8.89.	5.00; 2.67; 2.00.

Table XIIIa

Insulin Experiments.

Percentage concentration of active substances in rats' adrenals.

(Injected animals as percent. of mean of control animals)

Hours after injection.	Expt. No.	Mean conc. of controls ( $\mu\text{g.}/100\text{g}$ )	Conc. of injected as percent. of control mean.	Mean percent. of injected for time.
<u>Adrenaline.</u>				
1	24	23.60	79; 71.	70
	25	20.08	58; 62; 80.	
2	19	23.60	51; 56.	99
	20	19.88	65; 47; 18.	
	33	19.18	95; 88; 50; 85.	
	38	5.09	280; 223; 319.	
	39	23.73	94; 77; 63.	
	40	17.83	81; 81; 87.	
	41	20.07	55; 62; 100.	
4	21	20.00	75; 19.	54
	22	26.50	33; 94; 51.	
6	23	31.82	82; 67; 25.	56
	30	20.22	53; 53.	

Table XIIIa (Contd.)

Hours after injection.	Expt. No.	Mean conc. of controls ( $\mu\text{g.}/100\text{g}$ )	Conc. of injected as percent. of control mean.	Mean percent. of injected for time.
<u>Adrenaline.</u>				
8	27	17.22	22; 76.	55
	29	14.78	62; 68; 45.	
12	26	22.74	9; 15.	22
	28	13.75	47; 11; 29.	
16	31	31.27	9; 9.	15
	32	25.14	18; 19; 20.	
20	34	16.84	21; 21; 18.	33
	35	19.61	81; 22.	
24	36	22.02	28; 28.	28
	37	22.86	17; 39.	

Table XIIbInsulin Experiments.Percentage concentration of active substances in rats' adrenals.(Injected animals as percent. of mean of control animals)

Hours after injection.	Expt. No.	Mean conc. of controls ( $\mu\text{g.}/100\text{g.}$ )	Conc. of injected as percent. of control mean.	Mean percent. of injected for time.
<u>Noradrenaline.</u>				
1	24	1.70	68; 110.	98
	25	2.24	91; 84; 135.	
2	19	1.92	89; 111.	147
	20	1.32	163; 178; 138.	
	33	1.22	150; 93; 150; 177.	
	38	0.60	167; 260; 270.	
	39	1.73	257; 109; 140.	
	40	2.06	35; 52; 56.	
	41	1.07	104; 73; 311.	
4	21	2.08	112; 88.	112
	22	1.89	156; 110; 96.	
6	23	1.54	169; 110; 78.	100
	30	2.21	81; 60.	

197a.

Table XIIb (Contd.)

Hours after injection.	Expt. No.	Mean conc. of controls ( $\mu\text{g./100g.}$ )	Conc. of injected as percent. of control mean.	Mean percent. of injected for time.
<u>Noradrenaline.</u>				
8	27	3.48	33; 50.	87
	29	1.64	104; 105; 143.	
12	26	2.30	46; 70.	52
	28	2.41	54; 40; 50.	
16	31	2.83	19; 62.	72
	32	1.93	120; 94; 65.	
20	34	2.05	98; 106; 66.	80
	35	1.83	66; 62.	
24	36	1.74	71; 106.	112
	37	0.95	94; 178.	

Table XIIIaMorphine Experiments.Percentage concentration of active substances in rats' adrenals.(Injected animals as percent. of mean of control animals)

Hours after injection.	Expt. No.	Mean conc. of controls ( $\mu\text{g.}/100\text{g.}$ )	Conc. of injected as percent. of control mean.	Mean percent. of injected for time.
<u>Adrenaline.</u>			<u>(Single dose)</u>	
1	48	8.55	110; 78; 50.	
	49	8.74	108; 118; 99.	94
2	47	8.06	103; 198; 109.	137
4	42	13.96	80; 84; 100.	88
8	45	8.37	95; 95; 47.	79
12	43	13.33	107; 125; 101.	111
16	44	8.67	82; 88; 81.	84
20	46	8.86	97; 94; 106.	99

(Repeated doses)/



Table XIIIa (Contd.)

Hours after injection.	Expt. No.	Mean conc. of controls ( $\mu\text{g.}/100\text{g.}$ )	Conc. of injected as percent. of control mean.	Mean percent. of injected for time.
<u>Adrenaline.</u>			<u>(Repeated doses)</u>	
4	50	11.80	51; 9; 3.	43
	54	16.77	96; 48; 51.	
5	51	14.20	44; 49; 85.	59

Table XIIIbMorphine Experiments.Percentage concentration of active substances in rats' adrenals.(Injected animals as percent. of mean of control animals)

Hours after injection.	Expt. No.	Mean conc. of controls (µg./100g.)	Conc. of injected as percent. of control mean.	Mean percent. of injected for time.
<u>Noradrenaline.</u>			<u>(Single dose)</u>	
1	48	2.86	41; 140; 76.	
	49	2.63	89; 98; 152.	99
2	47	1.99	56; 301; 118.	158
4	42	2.01	66; 43; 84.	64
8	45	0.94	114; 152; 48.	105
12	43	1.02	157; 196; 212.	188
16	44	1.50	35; 114; 105.	85
20	46	1.25	118; 100; 250.	156

(Repeated doses)/

Table XIIIb (Contd.)

Hours after injection.	Expt. No.	Mean conc. of controls ( $\mu\text{g.}/100\text{g.}$ )	Conc. of injected as percent. of control mean.	Mean percent. of injected for time.
<u>Noradrenaline.</u>			<u>(Repeated doses)</u>	
4	50	3.33	14; 24; 49.	
	54	2.17	140; 84; 89.	67
5	51	2.00	100; 158; 134.	131

Table XIV

Tetrahydronaphthylamine experiments.Percentage concentration of active substances in rats' adrenals.(Injected animals as percent. of mean of control animals)

Hours after injection.	Expt. No.	Mean conc. of controls ( $\mu\text{g.}/100\text{g.}$ )	Conc. of injected as percent. of control mean.	Mean percent. of injected for time.
<u>Adrenaline.</u>				
2	52	15.21	97; 44; 64.	68
4	53	12.26	48; 84.	66
8	55	17.40	29; 22; 25.	25
13	56	12.58	53.	53
16	57	11.57	79; 105; 86.	90
<u>Noradrenaline.</u>				
2	52	2.15	68; 70; 106.	81
4	53	2.39	140; 122.	131
8	55	1.82	110; 46; 88.	81
13	56	2.32	115.	115
16	57	3.22	50; 103.	76

Tetrahydronaphthylamine produced a similar effect but more quickly. There was about 25% of the adrenaline left in 8 hours. This was followed by recovery which was almost complete by 16 hours. The depletion at 8 hours was just below the significant level, i.e., not significant.

With morphine, a transient depletion of adrenaline, to 43% of the original level, was achieved with 4 doses of the drug at hourly intervals four hours after the commencement. After only 1 injection there was no appreciable depletion of adrenaline over twenty hours observation. This depletion was significant.

Noradrenaline. The amounts of noradrenaline did not follow the trend of the methylated amine. Neither morphine nor tetrahydronaphthylamine produced any lowering of the content of noradrenaline below the range of the control animals. The most powerful and longest acting (in the dosages employed) of the three agents, insulin, produced no more than a transient depletion of less than 50% of the noradrenaline at 12 hours. Recovery was complete in 16-20 hours. This depletion was not significant.

On the other hand, 2-4 hours after injection  
of/

of the drugs there was an indication that the noradrenaline content was increased 30-50% above that of the controls. Although this increase was small, just significant for insulin and not significant for the other two drugs it occurred following all three of the agents used.

The amounts of medullary amines in the adrenals of an animal at any given moment are determined by a balance between utilisation (release into the circulation and/or destruction) and synthesis (formation and retention in the glands). It would appear that with a sufficiently powerful and prolonged stimulus the utilisation of adrenaline increasingly outpaces synthesis. This is shown by the depletion. It was most marked in the insulin experiments, less pronounced in the tetrahydronaphthylamine experiments and could only just be demonstrated in the experiments in which repeated doses of morphine were given. On the other hand, under the conditions of those experiments the noradrenaline level kept within the normal range. This may be because there was either no output of noradrenaline or an output with which synthesis could keep pace. If the latter, it may be owing to the fact that the rat/

rat is better able to synthesize the non-methylated amine than the methylated even when its medullary resources are heavily taxed.

Owing to the much greater utilisation of adrenaline than of noradrenaline which occurred after prolonged stimulation of the adrenal medulla there tended to be a relative increase in the proportion of noradrenaline, i.e., the percentage methylation was reduced, 12 hours after insulin and 8 hours after tetrahydronaphthylamine. There was no evidence of an absolute increase of noradrenaline at these times. In fact, in the case of the insulin injected rats the noradrenaline may even have been somewhat decreased after 12-16 hours.

There was evidence of an absolute increase of noradrenaline 2-4 hours after injection of the drugs. After this transient effect the amount of noradrenaline returned to the normal range again. During this increase of noradrenaline the amounts of adrenaline in the injected rats were hardly less than those found in the controls. There thus seemed to be a tendency for the earliest response of the glands to be an increased production of noradrenaline, even/

even while the demand for adrenaline could still be met. Non-significance of the results may be due to the fact that the response did not occur in every rat or did not occur at the same time in all the rats.

#### Discussion.

The effect of insulin on rats' adrenals was investigated by Vogt (1947) using a staining method similar to that of Cramer (1918). The adrenals were fixed in Orth's fluid, embedded in gelatine, sectioned and stained with Haematoxylin or Sudan III or IV. This method showed up stained granules of adrenaline in the medulla. Given a single dose of insulin, the rats were killed after 2.5 to 6.5 hours and the glands examined. With 0.12 units of insulin per 100g. rat there was no visible depletion of adrenaline, which only became obvious with 0.24 units per 100g. rat. Larger doses still produced more conspicuous effects. In the present series, four times the dose which just showed an effect in Vogt's experiments caused about 50% depletion of adrenaline in 6 hours, which agrees well with the histo-chemical results. Vogt points out, however, that in sections of/



of obviously depleted glands the reduction in the density of the granules was not uniform but patchy. There were clumps of granules here and there which had not apparently participated in the general release of hormone.

Cramer's (1919) method consisted of suspending a suitably sized piece of adrenal gland in osmic acid vapour at about 40°C. for 1.5 hours after which it was dehydrated in alcohol, embedded in paraffin and sectioned. The medullary cells showed numerous black granules of "adrenaline" of which there appeared to be two kinds - very fine granules evenly distributed throughout the cell, staining dull black, and another type, fewer in number, of larger round black spheroids irregularly scattered throughout the cell. After stimulation of the glands by cold or by injection of tetrahydronaphthylamine there was a reduction in the number of fine black granules but even after almost complete depletion of the medulla, as shown by the absence of fine granules, many cells still contained black spheroids. In view of the fact that in the present experiments even when there was marked depletion/

depletion of adrenaline there was hardly any reduction in the amount of noradrenaline, whatever the stimulating agent used, Vogt considers that the fine granules easily discharged probably are adrenaline, while the black spheroids apparently not reduced in numbers may be noradrenaline.

Burn, Hutcheon and Parker (1950) estimated both adrenaline and noradrenaline in rats' adrenals after insulin by a method involving the simultaneous recording of the blood pressure and the normal nictitating membrane of the cat. Extracts of pooled rat adrenals were injected and the rises of blood pressure obtained were matched with those of various equipressor mixtures of adrenaline and noradrenaline. The proportions of the two amines present could then be estimated by the effects on the nictitating membrane which is more sensitive to adrenaline than to noradrenaline. They showed that after 0.2 units per 100g. rat there was 50% depletion of the total amount of medullary hormone in the adrenals after 8 hours. At this time however, there was a decrease in the percentage methylation. They concluded/

concluded that an increase in the amount of noradrenaline which they obtained by their method was due to the process of methylation being slower than the processes by which fresh noradrenaline was accumulated after the adrenals had been active for 4-6 hours. In their experiments the pooled adrenals of several rats were extracted and assayed. In the present series the amines present in the adrenals of each animal were estimated individually and separately. As already pointed out, under the conditions of the present experiments the percentage methylation was reduced but there was no evidence of an absolute increase in the amount of noradrenaline when the glands had been active for some hours.

The absolute increase in the amount of noradrenaline which occurred early (2-4 hours) after injection of the drugs has also been found by West (1951) in rabbits. In this animal there is so little noradrenaline in the adrenals that it cannot be detected by the usual pharmacological tests. Two hours after the injection of insulin however, West found there was just sufficient noradrenaline to assay. At that stage, the depletion of adrenaline which/

which occurred following the administration of insulin had not yet become conspicuous.

There was some difficulty in producing a definite depletion of adrenaline with morphine in the present series of experiments on rats. This contrasts with the results of Elliott's (1912) experiments on cats. After a preliminary series of experiments to show that the amount of adrenaline in each gland of the same animal was the same, his procedure was to cut one splanchnic nerve, administer the drug and then determine the amount of adrenaline in each gland separately. The amount of depletion in the intact (stimulated) gland was then considered in terms of the splanchnotomised (control) one. His figures give the amounts of "adrenaline" per gland and yield the following percentages of amine left:-

	Hours after injection.		
	6	8	24
Tetrahydronaphthylamine (2-3cc. of 2% solution)	-	50	37
Morphine (20mg.)	32	15	-

Morphine seems more efficient in depleting the adrenals/

adrenals of cats than of rats, while the opposite is true of tetrahydronaphthylamine.

Although a fall in the blood sugar of an animal below a certain level is necessary to initiate a release of adrenal medullary hormone after insulin it would appear that the subsequent changes in the amounts of the medullary amines bear little relation to the blood sugar level. This was shown in an experiment in which rats were given the same dose of insulin used in the present investigations under the same conditions in which they were kept. Blood sugar estimations (Hagedorn-Jansen method) resulted as follows:-

Mean of 3 rats	Hours after insulin			
	0	2	4	5
Blood sugar (mg./100cc blood)	88	54	51	89

Evidently the depletion of adrenaline continued long after the blood sugar had returned to normal.

In the longest acting agent used, insulin, even after 24 hours the amount of adrenaline in the glands had not returned to normal limits. This persistence/

persistence of the depletion may have been accentuated by the prolonged starvation of the animals (17 hours before the experiments commenced followed by the period of action of the drug). However, it agrees with the time course found by Edmunds and Smith (1932) in dogs after subcutaneous injection of eserine. The amount of adrenaline was 60-75% of the normal after 4-5 hours and only 10-20% after 24 hours. Not till after 48 hours were the glands almost replenished and this replenishment occurred even if the splanchnic nerves were cut at the time of estimated maximum depletion.

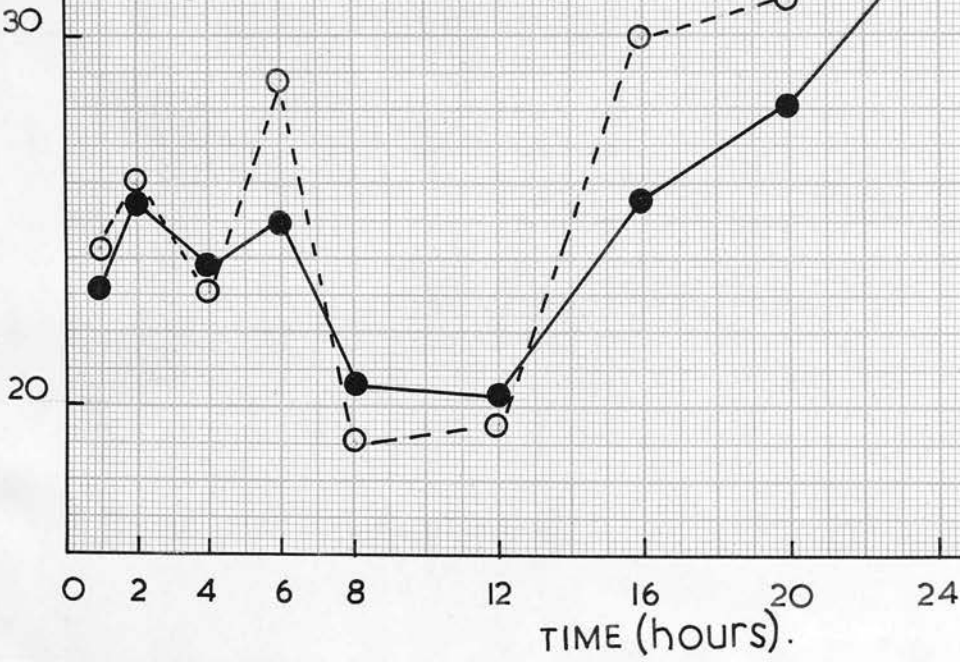
FIG. XXV.

CHANGES IN WEIGHT OF RATS' ADRENALS.

MEAN WEIGHT  
mqm. / 100 Gm.

CONTROLS.

AFTER INSULIN.



Calculation and statistical treatment of results.

The results obtained could be presented in several ways. The concentrations of the amines could be calculated in terms of gland weight of adrenal or of body weight of rat.

In the insulin experiments, the factors had to be considered of starvation and the drug itself influencing the weights of the adrenals. Vogt (1947) cited examples from the literature and studied the changes which occur in the adrenal cortex as a result of these influences. Briefly, they amount to cell proliferation in the cortex as a result of starvation and changes in the lipid content owing to insulin. After a single dose (similar to that used in the present series) there was lipid depletion for about 6 hours followed by lipid storage. The weights of the glands in relation to body weight of rat thus tended to decrease at first and increase later.

Table XV shows the adrenal weights and Figure XXV the changes in them in the insulin experiments. Both control and injected groups agree fairly closely in showing a fall in weight up to about 6-8 hours and a great increase in weight/



weight after 16 hours. Owing to these changes, the concentrations of adrenaline and noradrenaline in these results have been expressed not in terms of gland weight, but in terms of body weight of rat, which presumably varied much less during the periods of the experiments.

In considering the concentrations of adrenaline and noradrenaline, it was first necessary to decide whether **those** obtained on different days could be taken together in the calculation of means, either for particular times after the drug or (in the case of the controls) for all the results of the entire series. Analyses of variance were therefore made of the sets of results.

Table XV/

Table XV

Relationship of adrenal weights to periods of  
fasting and of action of insulin.

Hours after insulin	No. of rats	Insulin injected.			Corresponding controls.			
		Total weight of rats (g.)	Weight of adrenals (mg.)	Mean wt. of glands (mg./100g.rat).	No. of rats	Total weight of rats (g.)	Weight of adrenals (mg.)	Mean wt. of glands (mg./100g.rat).
1	5	829	200	24.1	6	984	228	23.2
2	21	3644	950	26.1	21	3558	910	25.6
4	5	815	189	23.2	5	800	191	23.9
6	5	793	227	28.6	5	821	207	25.2
8	5	831	158	17.8	5	780	161	20.6
12	5	817	158	19.3	5	792	159	20.1
16	5	848	255	30.1	5	813	206	25.3
20	5	862	268	31.1	5	855	239	28.0
24	4	666	208	31.2	4	675	222	32.9

Analyses of variance

of concentrations of amines in control animals (ug/100g)					Tabular ratio
Source of variance.	D.f.	Sum of squares	Mean squares	Variance ratio	(P=0.05)
<u>Insulin experiments.</u>					
<u>Adrenaline:</u>					
Times	8	913.8013	114.2252	4.59+	(8 <del>+</del> 29d.f.) 2.28
Days	22	1990.8482	90.4931	3.63	(22 <del>+</del> 29d.f.) 1.90
Error	29	721.9866	24.8960		-1.94
Total	59	3626.6361			
<u>Noradrenaline:</u>					
Times	8	10.4503	1.3062	3.72+	(8 <del>+</del> 29d.f.) 2.28
Days	22	20.8967	0.9498	2.70	(22 <del>+</del> 29d.f.) 1.90
Error	29	10.1825	0.3511		-1.94
Total	59	41.5295			
<u>Morphine experiments.</u>					
<u>Adrenaline:</u>					
Times (days)	7	140.0415	20.0045	3.42	(7 <del>+</del> 17d.f.) 2.62
Error	17	99.4662	5.8510		
Total	23	239.5077			
<u>Noradrenaline:</u>					
Times (days)	7	8.6303	1.2329	2.08	(7 <del>+</del> 17d.f.) 2.62
Error	17	10.0637	0.5920		
Total	23	18.6940			
<u>β-tetra experiments.</u>					
<u>Adrenaline:</u>					
Times (days)	4	71.2225	17.8056	1	(4 <del>+</del> 10d.f.) 3.48
Error	10	226.1010	22.6101		
Total	14	297.3235			
<u>Noradrenaline:</u>					
Times (days)	4	3.2522	0.8130	1	(4 <del>+</del> 10d.f.) 3.48
Error	10	8.5645	0.8564		
Total	14	11.8167			

All the variance ratios in the insulin experiments were significant, indicating that the variations in/

in the control values from day to day and for different periods of starvation (corresponding to periods of action of the drug as well as in the values from injected rats) were too great to be attributed to chance. In the other experiments since smaller groups of animals were used and one group for each "time" after administration of the drug the items "times" and "days" coincide. In the morphine experiments, the adrenaline ratio was significant but the noradrenaline ratio was just below the significant value, so that the same considerations apply as in the case of the insulin experiments. In the tetrahydronaphthylamine experiments however, the variance ratios were not significant. This was probably due to the smaller number of animals used and not to less variation among them, for which there would be no apparent reason.

Owing to the significance of some of the variance ratios it is evident that the adrenaline and noradrenaline content of the glands should be considered in groups of control animals treated in the same way and killed at the same time. The amount of/

of adrenaline (or noradrenaline) in the adrenals of each injected rat was therefore calculated as a percentage of the mean of the amounts found in the control rats of the same experiment. It was then possible to take the mean of the percentages in order to obtain a mean value for the amount of the amine found after a particular time of action of a drug. It is these mean values which are plotted in Figures XXIII and XXIV.

#### Tests of significance.

The simplest way of testing the significance of the differences between the injected and control groups would be to consider all the injected animals for a particular time of action of a drug with all the control animals killed with them. This has been done in the tests of significance which are referred to in the text. As an example, the calculations for the depletion of adrenaline 12 hours after insulin are shown.

#### Insulin/

Insulin experiments.

Concentration of adrenaline (in  $\mu\text{g./100g}$ )

	n	D.f.	Sum of observations.	Mean.	Sum of squares.	Correction term.	Corrected sum of squares.	Variance (Corr. sum of sq.) / (n-1)
			( $\Sigma Y$ )	( $\Sigma Y$ )/n	( $\Sigma Y^2$ )	( $\Sigma Y$ ) <sup>2</sup> /n	( $\Sigma Y^2$ ) - ( $\Sigma Y$ ) <sup>2</sup> /n	
Insulin injected	5	4	17.59	3.518	76.6477	61.8816	14.7661	3.6915
Controls	5	4	86.74	17.348	1634.3640	1504.7655	129.5985	32.3996

Difference of means.  $\frac{13.830}{144.3646}$  Total

$$t = \frac{13.830}{\sqrt{\frac{5 \times 5 \times (5 + 5 - 2)}{(5+5)} \times 144.3646}}$$

$$= \frac{13.830}{\sqrt{0.13854}} \quad \text{Tabular } t \text{ (8 d.f.)}$$

$$= 13.830 \times 0.3722 \quad P = 0.05 \quad P = 0.01$$

$$= \frac{5.148}{++} \quad P = 0.05 \quad P = 0.01$$

$$= \frac{2.306}{++} \quad P = 0.05 \quad P = 0.01$$

The/

The important values in the tests of significance calculated in this way for the results of these rat's adrenal experiments are listed hereunder.

Times of action of drug. (hr.)	D.f.	Tabular t		<u>Adrenaline:</u>	<u>Noradrenaline:</u>
		P=0.05	P=0.01	Calculated t.	Calculated t.
<u>Insulin</u>					
2	40	2.021	2.704	-	2.129 <sup>+</sup>
12	8	2.306	3.355	5.148 <sup>++</sup>	-
16	8	2.306	3.355	3.862 <sup>++</sup>	-
20	8	2.306	3.355	4.306 <sup>++</sup>	-
24	6	2.447	3.707	12.357 <sup>++</sup>	-
<u>Morphine - Single dose.</u>					
2	4	2.776	4.604	-	0.706
<u>Repeated doses.</u>					
4	16	2.120	2.921	3.344 <sup>++</sup>	-
<u>β-tetra.</u>					
4	3	3.182	5.841	-	1.297
8	4	2.776	4.604	2.551	-

The legitimacy of this procedure is however, questionable/

questionable in one respect. It was found by the analyses of variance that within a given "time" there was a significant variance between experiments carried out on different "days". The procedure adopted in effect amounts to the application of a less stringent test.

Table XVI/



Table XVI

Insulin experiments.Control animals.Concentrations of amines found in each experiment.( $\mu\text{g.}/100\text{g.}$ )

Expt. No.	n	<u>Adrenaline:</u>		<u>Noradrenaline:</u>	
		Mean concentration	Variance	Mean concentration.	Variance
19	2	23.595	49.5013	1.920	0.0441
20	3	19.877	6.7730	1.323	0.1047
21	2	20.000	88.9778	2.085	0.1722
22	3	26.497	51.3120	1.887	0.2075
23	3	31.820	59.1892	1.540	0.0195
24	3	23.597	3.8636	1.700	0.0083
25	3	20.077	44.5066	2.243	0.2984
26	2	22.745	30.8113	2.295	1.6002
27	2	17.225	0.6161	3.475	0.6480
28	3	13.750	0.8476	2.410	1.0339
29	2	14.785	7.1065	1.645	0.0156
30	2	20.220	30.1088	2.210	0.1600
31	3	31.267	383.5416	2.833	0.7638
32	2	25.145	7.1065	1.930	0.5929
33	4	19.180	19.5858	1.218	0.0014

Table XVI (Contd.)

Expt. No.	n	<u>Adrenaline:</u>		<u>Noradrenaline:</u>	
		Mean concentration	Variance	Mean concentration	Variance
34	3	16.837	4.6594	2.053	1.0403
35	2	19.610	10.3058	1.830	0.4489
36	3	22.020	3.4743	1.740	0.0211
37	1	22.860	-	0.950	-
38	3	5.090	35.8393	0.600	0.7200
39	3	23.727	17.1720	1.727	0.0294
40	3	17.830	5.0961	2.057	0.1187
41	3	20.073	59.9732	1.070	0.0554

Logarithmic transformation. Insulin experiments.

The desirability of more elaborate statistical treatment was investigated. For the insulin experiments the mean concentrations of amines and variances for the control animals of each experiment were determined. These are shown in Table XVI. The values of the individual variances do not show any reasonable relation to the corresponding values of the means. In such a case, there is a possibility that by the use of a suitable transformation a degree of homogeneity of the variances might be achieved. A log. transformation was likely to be the most suitable.

Logs. were taken of all the observations (concentrations of adrenaline and noradrenaline) of both injected and control animals and these log. values treated as follows for each "time" of action of the drug:-

Mean of injected animals =  $m_2$  for  $n_2$  animals.

Mean of control animals =  $m_1$  for  $n_1$  animals.

Then log. ratio of means =  $m_2 - m_1$

Concentration of amine in injected animals (as percent. of control animals) = antilog.  
 $(m_2 - m_1) \times 100.$

The/

The table below compares the results obtained by this method with the simple arithmetical method used in the text.

Insulin experiments:-

Mean concentration of amines (as percent. of controls) in injected animals.

Time after injection. (hr.)	<u>Adrenaline:-</u>		<u>Noradrenaline:-</u>	
	Arithmetical method.	Using logs.	Arithmetical method.	Using logs.
1	70	72	98	92
2	99	109	147	160
4	54	48	112	112
6	56	54	100	93
8	55	50	87	79
12	22	19	52	61
16	15	15	72	68
20	33	31	80	84
24	28	32	112	125

In order to test whether there was more justification for pooling the experiments for each "time" after the log. transformation Bartlett's test for homogeneity of variances was applied to all the groups of/

of animals, i.e., each experiment provided two groups, one of injected and the other of control, animals. The computation was carried out as described by Snedecor (1948) but the detailed calculations are not reproduced here. The results obtained were:-

	Corrected $\bar{x}$	D.f.	P
Adrenaline	80.37	43	<0.01
Noradrenaline	64.80	43	<0.01

This showed that the variances were not homogeneous and confirmed that the tests of significance should be applied to the two groups in each experiment and not pooled for each "time". A combined probability for each time of action of insulin could then be calculated from the individual probabilities. The t test was applied as before and an example is shown.

Insulin experiments.

Log. concentration of adrenaline.

12 hours after injection

Expt. No.	D.f.	Differ- ence of log. means	Total corr- ected sums of squares.	Calcu- lated t	Probabi- lity.
26	2	-0.915	0.0349	6.926	0.022
28	4	-0.604	0.2086	3.242	0.032

The/

The exact value for P could not be obtained from tables of t, so a graph was constructed for the purpose. On double log. paper (4 cycles x 4 cycles) the values of t (abscissa) were plotted against the probabilities (ordinate) obtained from t tables. A separate graph was needed for each degree of freedom being applied. When a few points had been plotted a smooth curve could be drawn. From the graphs, the values of P corresponding to the calculated t obtained could be read (last column of previous table).

Combination of probabilities.

The individual probabilities for each "time" were combined by the method of Fisher (1948).

Insulin experiments.

Adrenaline, 12 hours after injection.

Expt. No.	P	$-\frac{1}{2} \chi^2$ ( $-\log_e P$ )	D.f.	Sum of $-\frac{1}{2} \chi^2$	$\chi^2$	Combined P.
26	0.022	3.8157	2			
28	0.032	3.4420	$\frac{2}{4}$	7.2577	14.5154	0.005

The/

The combined value of P was read from a graph of  $\chi^2$  for 4 degrees of freedom as again the exact value could not be obtained from tables.

The table below compares the probabilities obtained in the insulin experiments by the simple arithmetical method with the log. transformed combined probabilities.

Time after insulin. (hr.)	<u>Adrenaline:</u>		<u>Noradrenaline:</u>	
	Arithmetical method.	Using logs.	Arithmetical method.	Using logs.
2	-	-	0.02 - 0.05	0.002
12	<0.001	0.005	-	-
16	0.001 - 0.01	<0.001	-	-
20	0.001 - 0.01	0.001-0.003	-	-
24	<0.001	<0.001	-	-

These figures indicate that there is little advantage in applying this elaborate statistical treatment to the results of these experiments as no effect of the drug is uncovered which was not evident before. Also, the reduction in the range of the observations was still inadequate. Only the simple/

simple arithmetical form was used in presenting the results of these experiments with the three drugs although only the insulin figures were examined. One of the difficulties was the small number of degrees of freedom for each experiment. This was due to limitations in the experimental technique which necessitated the use of small groups of animals.

Summary/



Summary

1. Insulin, morphine and tetrahydronaphthylamine which are known to cause a release of adrenal medullary hormones, were injected into groups of rats.
2. By assay with the rat's blood pressure after paper chromatographic separation estimations were made of the amounts of adrenaline and noradrenaline in the adrenal glands of control rats and of the injected rats after different periods of action of the drugs.
3. All three agents produced significant depletion of adrenaline after some hours.
4. No significant depletion of noradrenaline occurred with any of the three drugs although there was a tendency to depletion with insulin.
5. At the time of maximum depletion of adrenaline, the percentage methylation was reduced owing to the relative increase of noradrenaline.
6. An absolute increase of noradrenaline only occurred/

occurred within the first few hours of stimulation with the drugs, at which time the amounts of adrenaline were still normal.

7. Some points in the calculation and treatment of the results statistically are discussed.

III. The release of the adrenal medullary hormones in the cat.

Evidence has been offered by several workers that noradrenaline is one of the hormones released by the adrenal medulla (Meier and Bein, 1948,; Holtz and Schümann, 1949; Bülbring and Burn, 1949a). The study of these hormones has been limited by the fact that the methods of estimation are required to function when adrenaline and noradrenaline are present together in adrenal venous plasma. Two main types of biological methods have been used. One, to record two different effects in the same animal, e.g., both nictitating membranes of which one has been denervated (Bülbring and Burn, 1949b), one normal nictitating membrane and the blood pressure, Burn, Hutcheon and Parker, 1950a), the nictitating membrane and the non-pregnant uterus both being denervated (West, 1949); the other by independent assays on different preparations (Bülbring and Burn, 1949c; Gaddum and Lembeck, 1949). The last named workers showed precisely by statistical analysis that such assays did point to the presence of both amines/

amines but that the combined errors of the tests often made the estimates of each substance unreliable.

The separation of the two amines by paper chromatography followed by biological assay would provide better evidence of the changes the amounts undergo in different conditions.

#### Method.

A cat of either sex, over 2kg. if possible, was anaesthetised with ether and chloralose (60mg./kg. in approximately 2% solution intravenously). The animal was eviscerated and both splanchnic nerves dissected for a good length and cut between ligatures. A straight glass cannula was inserted into the coeliac artery. This was fitted with a short length of rubber tubing to which a syringe could be attached for injecting drugs. A clip kept this tubing compressed except when an injection was being made. Both renal arteries and veins were tied off as were the aorta and inferior vena cava at the level of the bifurcation of the former. A glass cannula was tied into the inferior vena cava just below the junctions with the (tied) renal veins. A clip was placed just above the cannula on the vein. A thread was passed under the inferior vena cava where it entered the liver and formed into a loop. Blood from the adrenals/

adrenals now flowed into the inferior vena cava and up to the heart. If the clip on the vein was transferred to the tightened loop of thread the blood flowed out through the cannula and was collected. In some experiments, when only one gland (the left) was stimulated and the adrenal vein joined the left renal vein the ligature confining the renal blood was placed distal to the union with the adrenal vein. The cannula was tied into the left renal vein. The blood which now drained into it was almost entirely from the adrenal. The blood was shunted through rubber tubing into one external jugular vein. The rubber tubing carried a T piece and another short length of tubing which was clipped. If the clip was transferred to the part of tubing through which blood flowed into the jugular vein collection of blood could be made from the free limb of the T piece.

One carotid artery was cannulated for recording the blood pressure, one external jugular vein for infusion of saline or blood and the trachea for artificial respiration, if necessary. When all the cannulae were in position heparin was injected (10mg./kg. i.e., about 1,000 units/kg. in 2-3ml. saline).

Samples of adrenal venous blood were collected when the glands were at "rest" and when they were stimulated/

stimulated by various means. The following were used.

Electrical: by an alternating current (50 cycles)

5-7.5 volts to the splanchnic nerves. The electrodes were usually of a simple type (bipolar) but when prolonged stimulation of one nerve was carried out, a Collison's electrode, in which the nerve is constantly in Locke's solution, was used. The voltage in the latter case was 40-50 owing to the greater resistance through the salt solution.

Acetylcholine. A solution of 100 $\mu$ g./ml. in saline

was used. For long stimulation 0.5-1ml. was given over 5-10 minutes timed with a stop watch. For a quick stimulation, 0.75ml. was given quickly at a given moment.

Potassium chloride. A solution of 10mg./ml. in

distilled water was used. For long stimulation 0.5-1ml. was given similarly to acetylcholine over 5-10 minutes. For quick stimulation, 0.5ml. was given in a moment.

Injections/

Injections were made through the coeliac artery while the outflow blood from the vein was being collected.

In the first series of experiments, the periods of stimulation and collection were long (5-10 minutes) and the cat's blood pressure fell progressively with each collection. An arterial sample was also taken. The substances in the venous plasma were estimated by parallel assays.

In the second series, the plasma was treated by chromatography and the separated amines assayed. In those experiments, the samples were collected over a much shorter period (30-60 seconds). The fall in blood pressure was much less but even that was counteracted in a few experiments by infusion of blood from another cat between samples. In most experiments, however, it was sufficient to leave the animal for a few (about 5) minutes when the pressure rose to the pre-collection level. Each sample was thus collected with the animal's blood pressure at the same height at the commencement. This was to avoid the influence of haemorrhage on the adrenals as much as possible. In addition, the types of stimulation were repeated thus Elec., A.Ch., KCl, A.Ch., Elec. in each experiment. This was to prevent/

prevent any change in the amounts of the amines and/or the methylation due to a general shift caused by the condition of the animal passing unnoticed.

The samples of blood were collected in ice-cooled graduated centrifuge tubes, centrifuged as soon after collection as possible (3,000 r.p.m. for 10 minutes) and the supernatant plasma pipetted off. The plasma was stored in the refrigerator until used for assay or chromatography.

Estimation of the amounts of adrenaline and noradrenaline.

In the first series, the amines were assayed with the rat's uterus and colon and occasionally, also with the rabbit's ear. In the second series, after preliminary tests with the rat's uterus the plasma was prepared for application to a paper cylinder for chromatography and assay with the rat's blood pressure as described previously. The method of preparation was as follows.

A measured volume of plasma, up to 2ml. was added dropwise to 6 volumes of acid alcohol (0.1% conc. Hcl in ethanol) chilled in ice and left chilling for 30 minutes. The tubes were then/



then centrifuged (3,000 r.p.m. for 5 minutes) and the supernatant fluid transferred to a 50ml. round-bottomed Quickfit and Quartz flask. The precipitate was washed with 2ml. acid alcohol, left to chill for 10 minutes, centrifuged (3,000 r.p.m. for 3 minutes) and the supernatant fluid also transferred to the same flask as the first fraction. The contents were evaporated in vacuo at a temperature of 55°-60°C. (external temperature). The residue was transferred with 5 washings of 1ml. each of acid alcohol to a 10x1cm. hard glass centrifuge tube. This was chilled **in ice** for 15 minutes, centrifuged (3,000 r.p.m. for three minutes) and the supernatant fluid transferred to another similar round-bottomed flask. The residue was washed with another 1ml. of acid alcohol, chilled for 5 minutes, centrifuged (3,000 r.p.m. for 3 minutes) and the supernatant fluid transferred to the round-bottomed flask. The contents in the flask were evaporated in vacuo at a temperature of 55°-60°C. (external temperature) and the residue taken up in/

in 0.7ml. acid alcohol which was applied to a paper cylinder. The flask was washed with a further 0.2ml. acid alcohol which was also applied.

In some experiments, extracts of the adrenal glands were made and chromatographed. One gland was removed before cannulation of the inferior vena cava and the other served for stimulation by the splanchnic nerve electrically. Continuous stimulation was given for 1 hour during which samples of blood were collected. At the end of the period, the remaining gland was removed. Each was weighed on a torsion balance soon after removal and then placed in 10ml. of 0.15N.HCl. When about to be chromatographed, the gland was ground up in a mortar with the acid and 1ml. of the liquid pipetted off for treatment.

The gland extract (1ml.) so obtained was added to 10ml. acid alcohol, chilled in ice for 30 minutes and centrifuged (3,000 r.p.m. for 5 minutes) the supernatant fluid was transferred to a 50ml. round-bottomed flask as above. The residue was washed with 2ml. acid alcohol, chilled for 10 minutes, centrifuged (3,000 r.p.m. for/

for 3 minutes) and the supernatant fluid was also transferred to the flask. The contents were evaporated in vacuo at 55°-60°C. (external temperature) and the residue taken up in 0.7ml. acid alcohol, which was applied to a paper cylinder. The flask was washed with a further 0.2ml. acid alcohol which was also applied.

Table XVII

Cat: Estimates of adrenalinine and noradrenalinine in  
adrenal venous plasma by parallel assays.

Expt. No.	Adrenalinine equivalents (mcg./ml.)				Calculated conc. (mcg./ml.)				+Statisti- cal. Prob- ability of differ- ence not signifi- cant.
	Serial Samples	Assay with uterus	Ru	Assay with colon	Adren- aline	Nor- adren- aline	% methyl- ation.		
55	Control	< 2.5	150	< 50	< 2.5	< 24	-	-	
	A.ch.	300	150	200	299	(-50)	100	100	
	A.ch.	375	150	375	374	0	100	100	
	Control	75	75	87	75	6	93	93	
	Control	12	75	< 50	12	< 19	> 39	100	
57	KCl	3000	75	3000	2979	0	100	100	
	Control	500	75	1500	490	500	49	49	
	Control	75	160	125	73	125	37	37	
58	A.ch.	562	160	750	550	470	54	54	
	A.ch.	1200	160	1500	1177	750	61	61	
	Control	1250	160	1750	1223	1250	49	49	
59	Control	30	175	< 5000	> 10	< 3727	-	-	
	A.ch.	4876 <sup>+</sup>	175	790 <sup>+</sup>	4873	(-3064)	100	100	
59	Control	< 5	150	Inter.	< 5	-	-	-	
	Electr. Control	3200	150	9333	3153	4600	41	41	
	Control	1500	150	2000	1490	375	80	80	
	Arterial	Inter.	150	Inter.	-	-	-	-	

Table XVII (Contd.)

Expt. No.	Serial Samples	Adrenaline equivalents ( $\mu\text{g.}/\text{ml.}$ )			Calculated conc. ( $\mu\text{g.}/\text{ml.}$ )			
		Assay with uterus	Ru	Assay with colon	Rc	Adrenaline	Adrenaline	methylation, %
60	Control	2.5	250	< 250	3	(-0.5)	< 742	-
	Electr. Control	2500 562	250 150	2000 1500	3 0.5	2476 558	(-1500) 469	100 54
61	Arterial	< 2.5	250	< 250	3	-	< 742	-
	Control Electr.	15 6000	37 37	500 6000	0.75 0.75	$\geq$ 5 5880	< 364 0	- 100
62	Arterial	Inter.	37	Inter.	0.75	-	-	-
	Control Electr.	7.5 1500	75 75	< 250 3000	0.37 0.37	$\geq$ 6.2 1485	< 92 555	- 73
63	Control Electr.	7.5 1000	100 100	< 500 1000	1 1	$\geq$ 2.5 900	< 492 0	- 100
	A.Ch.	12,360 <sup>+</sup> (s.e. 16.8%)	100	17,866 <sup>+</sup> (s.e. 25.7%)	1.5	12,092	8259	59
64	Control Electr.	10 6480 <sup>+</sup> (s.e. 28.3%)	75 110 <sup>+</sup> (s.e. 3%)	< 500 1693 <sup>+</sup> (s.e. 51.2%)	0.75 0.7 <sup>+</sup> (s.e. 51.2%)	$\geq$ 5 6469	368 (-3351)	- 100
	A.Ch.	19,540 <sup>+</sup> (s.e. 27.9%)	110 <sup>+</sup>	10,720 <sup>+</sup> (s.e. 36.7%)	0.7 <sup>+</sup>	19,472	(-6174)	100

Assay with rabbit's ear.  
Re 10,000 1.8

+ statistical cal. Probability of difference:  $P > 0.1$

+ statistical cal. Probability of differences not significant.

Cat: Estimates of adrenaline and noradrenaline in adrenal venous plasma separated by chromatography with the rat's blood pressure preparation.

Expt. No.	Sample: Control or method of stim.	µg./min.		Adrenaline	µg./min.		methylation.
		Adrenaline equivalent with the rat's uterus.	Estimates of separated amines with the rat's blood pressure.		Noradrenaline		
65	Control	-	< 551	412	< 57		
	Electr.	-	1332	1248	52		
	A.Ch.	-	3268	612	84		
	KCl	-	4960	1550	76		
66	A.Ch	-	4799	2800	63		
	Electr.	-	2400	600	80		
67	Control	180	< 300	< 375	-		
	Electr.	720	775	< 186	> 81		
	A.Ch.	8000	5000	< 625	> 89		
	Control	< 10	< 1000	< 500	-		
68	Electr.	2800	4375	3500	56		
	A.Ch.	4560	7600	5700	57		
	KCl	5000	10000	5000	67		
	A.Ch	1440	3000	1500	67		
69	Electr.	-	3256	4888	40		
	Control	19	< 950	357	< 73		
	Electr.	1001	600	581	51		
	A.Ch.	6400	3240	8000	29		
69	KCl	2855	2800	2500	53		
	A.Ch.	4800	3528	1500	70		
	Electr.	925	1388	462	75		
	Control	2.6	< 525	< 262	-		
69	Electr.	1040	975	455	68		
	A.Ch.	1600	1250	625	67		
	KCl	4400	3300	2475	57		
	A.Ch.	2666	2500	1500	62		
69	Electr.	3466	3250	1950	62		

Comparison of different methods of stimulation:

- Control: unstimulated.
- Electr.: electrical stimulation (see text)
- A.Ch.: Acetylcholine injection.
- KCl: Potassium chloride injection.

Results.

The results of parallel assays on the samples of adrenal venous blood are shown in Table XVII. Some assays were carried out with the complete statistical analysis used by Gaddum and Lembeck (1949). The release of large amounts of active substances on stimulation is evident. In view of the fact that, where the comparison could be made the increase in the stimulation samples over the control ones was accompanied by an increase in the percentage methylation, the active principles released must have consisted mostly of adrenaline. Likewise, control samples collected after stimulation ones showed a decrease both in total activity and percentage methylation. There was an indication of the presence of noradrenaline in the plasma in one experiment (No. 63) where statistical analysis was carried out. These results are similar to those of Gaddum and Lembeck. No conclusions can be drawn as to differences owing to different methods of stimulation because the data are insufficient to reveal any.

In Table XVIII, the results of assay of the two/

two amines after chromatographic separation give a better idea of the proportions of the two amines. The percentage methylation in the control samples could not be calculated from the rat's blood pressure assays only, but if the rat's uterus assays be considered as well, it appears that the percentage must be small. In two experiments (Nos. 65 and 68) there was assayable noradrenaline in the "resting" plasma. On stimulation, the considerable increase in the amounts of both amines over the control values was accompanied by an increase in the percentage methylation, again indicating that the substance released was mainly adrenaline. No differences in the relative amounts of the two amines were seen which could not be accounted for by the error of the tests. The three methods of stimulation used, while differing in their individual powers to release the medullary hormone, yet produced no differences in their proportions.

The relation of the amounts in the adrenal glands with those in the venous plasma, was investigated. The results are in Table XIX. In two experiments/

Table XIX/



Table XIX

Cat: Estimations of adrenaline and noradrenaline in adrenal glands and adrenal venous plasma; separated by chromatography and assayed with the rat's blood pressure.

Expt. No. of (wt. of cat)	Stim. for (hr.)	Adrenaline equiv: Rat's uterus.	Venous plasma:-			Weight	Adrenal glands:-		
			Adren- aline	Nor- adren- aline	Per- cent. methy- l.		Adren- aline	Nor- adren- aline	Per- cent. methy- l.
70 (2.85 kg.)	Control	< 2	< 48	< 21	-	{ Control (14.2mg) (14.4mg) Stim.	150	100	67
	0 ½	27 30	< 62 < 62	15 63	< 80 < 50				
71 (3.4 kg.)	Control	< 2.8	< 79	< 25	-	{ Control (26.4mg) Stim.	75	25	75
	0 ½	36 75	113 188	74 75	60 71				
72 (4.2 kg.)	Control	< 8.5	< 36	70	< 34	{ Control (238mg) (226mg) Stim.	100	15	87
	0 ½	360 315 286	24.0 275 262	450 275 131	35 50 67				
73 (2kg)	Control	>> 180	84.1	599	58	{ Control (120mg) (108mg) Stim.	100	37.5	73
	0 ½	800 75 87	333 125 87	500 62 54	40 67 62				
74 (3.6 kg.)	Control	< 9	< 30	< 15	-	{ Control (190mg) Stim. (218mg)	100	75	57
	0 ½	256 408 90	426 303 100	426 454 150	50 40 40				

Mean depletion (percent.)

34

38

Effect of continuous stimulation.

Control gland removed before stimulation.

Stimulated gland removed at end of period of stimulation.

experiments (Nos.70 and 71) the left adrenal was removed as a control at the commencement of the experiment and the right adrenal was stimulated. This proved unsatisfactory because the yield of active material was small due to a small blood flow. This was due to partial occlusion of the adrenal vein by the thread round the inferior vena cava because there was very little room between the liver and the junction with the adrenal vein. In the other experiments, the right adrenal was removed as a control and the left was stimulated. This was more satisfactory.

Electrical stimulation was applied to the splanchnic nerve continuously for the period of the experiment. Samples of blood were taken at the commencement, after half and one hour's stimulation. This was sufficient usually, to produce some depletion of the glands, but the proportions of adrenaline and noradrenaline in them did not change. The proportions in the plasma samples too showed no change, but the percentage methylation in the plasma was generally less than in the glands.

The high output of active substances in the control plasma in Experiment 73 can be explained.  
The/

The nerve was generally fixed in the electrode before the collection of the control sample. For stimulation, only the current then needed to be switched on. In this experiment, the electrode was fixed in an unsuitable position in that it was moved up and down by the respiratory movements of the liver, the metal holder having enough elasticity to make the electrode follow these movements. This caused mechanical stimulation of the nerve by pulling. It also apparently damaged it because the amounts of hormones released decreased as the stimulation proceeded.

#### Discussion/

Discussion

The suggestion of Meier and Bein (1948), that there was probably a constant discharge of noradrenaline from the resting adrenals is supported by the finding that this amine could be detected in the venous outflow plasma of the control samples in some experiments. It is not likely that its presence was due to stimulation of the adrenals by shock, haemorrhage or mechanical stimulation in operation because it was not accompanied by even an equivalent amount of adrenaline. The experiments show throughout that stimulation by any of the methods used caused a release of a mixture in which adrenaline predominated. Whether there is a constant supply of noradrenaline as part of the normal physiological function of the adrenals is, on this evidence, only speculative.

No differences were observed between the three methods of stimulation in respect of the percentage methylation. It was not likely that there would have been a difference between electrical stimulation and/

and stimulation by acetylcholine because their mechanisms are essentially the same. Any differences would most likely have been seen in the stimulation with potassium because that method did not at the same time, supply the gland with methyl groups. None, however, were detected. Either the method of investigation was not sensitive enough to detect them or the methylation is rather dependent on the supplies of noradrenaline available to the glands than on that of methyl groups. Bülbring and Burn (1949c) found that noradrenaline added to the perfusing fluid of a dog's isolated adrenal resulted in an increase of adrenaline in the outflow.

When the splanchnic nerves were stimulated continuously, there was no very marked change in the total amounts of the amines or their proportions in the plasma over the period of stimulation. There was some indication of a reduction in the total activity, but this may be due to the nerve working less efficiently as time went on. Bülbring and Burn (1949b) and West (1949), however, noticed a fall in the percentage methylation on prolonged stimulation of the adrenals of the cat. The former workers had some/

some evidence that this change was dependent on methionine deficiency in the animals' diets, but it may also be related to the intensity of the stimulus. Partial depletion of the glands was obtained in these experiments and here both adrenaline and noradrenaline were depleted to the same extent. This is in sharp contrast to the findings in the rats' adrenals experiments in the preceding section where little or no depletion of noradrenaline occurred in the glands even after prolonged stimulation of several hours duration.

Summary/

Summary.

1. The amounts of adrenaline and noradrenaline in the adrenal venous plasma and in the adrenal glands of eviscerated cats was estimated in two ways: by parallel assays on different preparations and with the rat's blood pressure after paper chromatographic separation.
2. Small amounts of adrenaline and sometimes larger amounts of noradrenaline, were detected in control plasma from the resting gland.
3. Stimulation of the splanchnic nerves or injection of either acetylcholine or potassium chloride through the stump of the coeliac artery, caused the release of large amounts of active substances into the plasma.
4. More adrenaline than noradrenaline was released on stimulation as judged from the percentage methylation in the effluent.
- 5./

5. There was no difference in the percentage methylation in the plasma from the stimulated glands whichever method of stimulation was used.
6. On prolonged stimulation for 1 hour there was no change in the proportions and little change in the amounts of adrenaline and noradrenaline in the plasma during the period of stimulation.
7. When one adrenal gland was removed as a control and the other was stimulated for 1 hour the stimulated gland was depleted of about 35% of the amount of each amine present in the control gland.
8. The fact that in the cat's gland, depletion of adrenaline and of noradrenaline occurred to an equal extent, shows a difference from conditions in rat's adrenals where only adrenaline was depleted appreciably.



Acknowledgments

No work of this nature can be completed without the assistance and co-operation of many persons. I have been particularly fortunate in drawing richly from many sources of aid, generously offered and gratefully accepted. To the following I have especially to express my thanks.

To Professor J.H. Gaddum for suggesting this problem and for constant advice and encouragement throughout the course of the work.

To Dr. M. Vogt, no less, for her encouragement and constant aid in many details of the work.

To Dr. T.B.B. Crawford with whom it is a pleasure and privilege to have been associated.

To Miss M.L. Clark for her careful technical assistance, as well as help in the preparation of this thesis.

To Mr. N.E. Condon, whose help was always evident and could not be too highly assessed.

To many others in the Department of Pharmacology for assistance, given without stint, on occasions too numerous to detail.

This work was carried out during the tenure of a Ceylon Government Scholarship.

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Many of the references for the period 1856 to 1905, and some for the period 1905 to 1910, have not been consulted in the original, but have been culled from reviews. Most of the references in English, for the latter period, have been read. A few references of later dates, which were not read, have been asterisked, or have their sources stated.

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