J. Physiol. (1949) 108, 467–481 577.174.5.612.129

THE ESTIMATION OF ADRENALINE AND ALLIED SUBSTANCES IN BLOOD

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(*Received* 10 *September* 1948)

Much work has been devoted to the search for a really sensitive and specific method of detecting and estimating adrenaline and allied substances in blood. This search has gained importance in recent years because of the desire to identify the substances liberated by adrenergic nerves. These substances can only be expected to appear in very low concentrations, and sensitive and specific tests are needed for their study. This paper describes part of the search for such tests and is a continuation of the work of West (1947*a*, *b*) in this department.

The most sensitive tests for adrenaline are pharmacological tests, but no single test is really specific by itself. A number of other sympathomimetic amines are known to have effects like those of adrenaline, but they can sometimes be distinguished by the method of parallel quantitative assays. If the adrenaline-equivalent of a solution is estimated quantitatively by several different methods, and the results differ significantly among themselves, adrenaline cannot be the only active substance in the solution. If the results agree among themselves, then the evidence supports the theory that the solution contains adrenaline, but its value depends on the use of pharmacological methods which vary independently in their sensitivity to drugs closely allied to adrenaline. If small changes in the molecule affect all the tests equally, then parallel quantitative assays are of little value. One of the objects of the present investigation was to discover a set of tests which would vary independently in their response to sympathomimetic amines, so that they could be used to distinguish these amines from one another.

One of the main difficulties in the estimation of adrenaline in blood is due to the fact that when blood is removed from the body various other pharmacologically active substances are formed in it or released from the cells (Gaddum, 1936; Reid & Bick, 1942; Zucker, 1944). Two of these interfering substances are histamine and an adenosine compound (Barsoum & Gaddum, 1935), but there is at least one other active substance which has not yet been identified.

Acetylcholine can be estimated in blood by using pharmacological tests which are specific for choline esters, but the known tests for adrenaline and allied substances are not specific, and can only yield useful information if measures are taken either to prevent the liberation of the interfering substances, or to remove them from the plasma afterwards. These two possibilities have been explored and the results are discussed below. The presence of these active substances complicates most pharmacological experiments with blood and the techniques used in the study of blood-adrenaline may have applications in the study of other substances.

METHODS

Dilute solutions of adrenaline and allied drugs are apt to be unstable. Drugs have therefore been dissolved in 0·9% NaCl containing ascorbic acid (10^{-5}). Such solutions are stable for many hours.

Doses are generally given in terms of mμg. of the base (1 mμg. $= 10^{-6}$ mg.). In comparing different drugs it would have been better to use equimolar solutions, but this would not have made much difference, since the molecular weights of most of the substances studied are nearly equal.

TABLE 1. Drugs used

		3, 4$(HO)_2C_6H_3$C———CH———N		
		‖	|	‖
Hydroxytyramine HCl	Light and Co.	H_2	H	H_2
Noradrenaline HCl (L and DL)	Sterling Winthrop	H.OH	H	H_2
L-Adrenaline	B.D.H.	H.OH	H	$H.CH_3$
Epinine HCl	Burroughs Wellcome	H_2	H	$H.CH_3$
Adrenalone	Light and Co.	O	H	$H.CH_3$
DL-corbasil HCl	Sterling Winthrop	H.OH	CH_3	H_2
DL, α-ethylnor-adrenaline HCl		H.OH	C_2H_5	H_2
DL, *N*-ethylnor-adrenaline HCl		H.OH	H	$H.C_2H_5$
DL, *N*-isopropylnor-adrenaline HCl		H.OH	H	$H.CH(CH_3)_2$
DL, *N*-methyladrenaline	I.G. Farben	H.OH	H	$(CH_3)_2$

Tyramine HCl, ephedrine HCl, histamine acid phosphate, ergotoxine methanesulphonate (B.D.H.), neoantergan (pyranisamine maleate, May and Barker), dihydroergotamine methanesulphonate (Sandoz).

Interfering substances. In experiments on interfering substances heparinized blood from cats and man was centrifuged at 2000–3000 r.p.m. for 10 min. and various fractions were removed with teat pipettes. The plasma still contained platelets, which were separated in some experiments by centrifuging at 4500 r.p.m. for 20 min. The other fractions contained mostly erythrocytes, or mostly leucocytes. Each was mixed with an equal volume of distilled water to lyse the cells and then used in pharmacological tests. Some of these fluids were dialysed for 4 hr. in apparatus like that described by Verney (1926), but made of perspex, using cellophane 400S of the British Cellophane Company. The receptor fluid was either distilled water, 0·005% ascorbic acid, 1% glycine in N/100-HCl or N/100-HCl. The dialysates were adjusted to various pH values with N/100-HCl and shaken with alumina of the kind used by Shaw (1938). This adsorbs adrenaline at pH 8·5, but not at pH 4.

The collection of plasma. The simplest method of avoiding complications due to interfering substances is to prevent them from being liberated. Adrenaline and noradrenaline were found to be stable for several days in plasma at 4° C. The presence of platelets in the plasma did not appear to affect the stability of adrenaline or to lead to the appearance of undesirable quantities of inter-

fering substances. Blood was therefore centrifuged at 3000 r.p.m. for 5 min. as soon as possible, and the following precautions were taken to prevent the liberation of interfering substances before the plasma could be separated:

(1) Heparin was injected intravenously into the cat in sufficient doses to prevent clotting (1000 units/kg.). Later, after various other precautions had been introduced, it was found beneficial also to add heparin (10 units/c.c. of blood) to the tubes in which the blood was collected. This seemed to be particularly important when the plasma had to be tested on the rat's uterus and colon.

(2) The blood was collected in centrifuge tubes standing in ice, and ice was also used in the centrifuge. After separation the plasma was kept at 0–4° C. and warmed up just before testing.

(3) Glass connexions and the centrifuge tubes were treated with silicone (General Electrical Co., Drifilm 9987) to render their surfaces water-repellent.

These precautions were largely successful, but some experiments were nevertheless complicated by the release of interfering substances.

It is known that when adrenaline is added to blood it is taken up by the cells, and when the concentration is low a large proportion of it may disappear from the plasma in this way (Bain, Gaunt & Suffolk, 1937), but the process is a slow one and would not be expected to affect the results of experiments in which the adrenaline is only in contact with the cells for a few minutes. A control experiment showed that this was so. Adrenaline (10^{-7}) was quantitatively recovered from the plasma of cats' blood when centrifuged after 5 min. contact with the cells.

ASSAYS

(1) *Isolated plain muscle*

de Jalon's method. Some of our best results have been obtained by a procedure recommended by de Jalon, Bayo & de Jalon (1945). Observing that the rat's uterus is very sensitive to adrenaline, but liable to show excessive spontaneous activity, these workers reduced this organ to quiescence by lowering the temperature and the calcium-content of the Locke solution in which it was immersed. They then produced contractions with acetylcholine and inhibited those contractions with small amounts of adrenaline added to the bath about 1 min. before the acetylcholine. We have used their method, except that the muscle was in a smaller bath (2 c.c.), and we have also applied the same technique to other tissues.

In our experiments on the rat's uterus the composition of the Ringer solution was as follows (g./l.): NaCl, 9, KCl 0·42, $CaCl_2$ 0·06, $NaHCO_3$ 0·5, glucose 0·5. A higher Ca/K ratio produced greater sensitivity to acetylcholine, but the maximum inhibitory effect of adrenaline was obtained with a low ratio. The temperature was 29–30° C. Large uteri appeared to be more sensitive than small ones, but pregnant or recently pregnant uteri were discarded since they did not become quiescent. Acetylcholine (0·5–1 μg.) was added to the bath every 2 min. and washed out when the effect had reached its maximum (after 30–40 sec.). The doses of adrenaline, dissolved in not more than 0·2 c.c., were added to the bath exactly 1 min. before one of the doses of acetylcholine. Another dose of adrenaline can be given as soon as the response to acetylcholine is normal. Instead of stimulating the uterus by acetylcholine, contractions may be produced by KCl in doses of 1–2 mg. This is useful if tests have to be carried out on plasma which contains atropine. Some preparations, however, lose their sensitivity to KCl after repeated administration.

A similar technique has been applied to the first 3 cm. of the rat's ascending colon which is easily recognized by the diagonal striations on its surface. This tissue was less easily reduced to quiescence than the uterus and was often used with less calcium and at a lower temperature. The solution was either the same as for the uterus or contained (g./l.): NaCl 9, KCl 0·4, $CaCl_2$ 0·03, $NaHCO_3$ 0·15, glucose 1. The temperature was $25 \pm 2°$ C. and was adjusted in each experiment so as to reduce the spontaneous activity to reasonable dimensions. The dose of acetylcholine was lower (0·001–0·01 μg.) than with the uterus.

In experiments with other tissues the conditions were altered according to the spontaneous activity. The rabbit's ileum usually remained spontaneously active at 20° with low calcium

concentrations and seems to be unsuitable for this technique. The guinea-pig's uterus or colon was used in low $CaCl_2$ concentrations (0·05 g./l.) at 37°. The other tissues showed little spontaneous activity and were used in normal Locke solution at 37° C. In experiments with the bladder the whole organ except the trigone was used.

Direct excitor effects. Various pieces of plain muscle were suspended in Locke solution at 37° C. In the case of the splenic capsule suitable strips were cut from the organ. Frogs' hearts were studied by Straub's method.

(2) *Perfusion with salt solutions*

Rabbit's ear. The ear was perfused with Locke solution at room temperature as described by Gaddum & Kwiatkowski (1938) with small modifications. The rabbit was first killed by a blow on the head. This was followed by low decapitation. A cannula was then tied in the right common carotid artery and perfusion started as quickly as possible at a pressure of 10–20 cm. of water; the external jugular vein was freed to allow the fluid to escape. When the blood had been washed away the branches of the artery, except that to the ear, were tied, and a cannula was inserted in the external jugular vein and connected to the outflow recorder. Injections were made in small volumes (0·05–0·2 c.c.) using the special cannula described by Gaddum & Kwiatkowski (1938).

Other tissues. Similar methods were used to perfuse various other tissues. Whole frogs were perfused from the left arch of the aorta to the posterior vena cava and frogs' hind limbs were perfused from the abdominal aorta to the abdominal vein. These preparations were left for 4 hr. to gain their maximum sensitivity before use. Various other tissues were perfused (Table 2) at room temperature, using Locke solution for mammalian tissues and Clark solution for frogs' tissues.

In perfusing the hind limbs of rats it was found important to wash out the blood quickly because clotting is rapid. The rat was killed by a blow on the head and the aorta cannulated just above the diaphragm. Locke solution was perfused at high pressure (60–70 cm. of water) for 5 min. and then at a lower pressure (10–20 cm. of water). A cannula was then inserted in the inferior vena cava. The preparation behaved consistently for at least 24 hr., but it was often necessary to increase the perfusion pressure temporarily after doses of adrenaline to restore the flow to its initial value. The perfusion of the hind legs of mice gave similar results.

(3) *Perfusion* in situ

Various organs were perfused *in situ* in heparinized cats by a method comparable with that of Richards & Plant (1915). The blood flowed continuously from one of the larger arteries to the apparatus which pumped it into a smaller artery and so returned it to the cat. The inflow pressure was recorded and various drugs and samples of plasma were injected through the rubber tubing near the inflow cannula. The cats were anaesthetized with ether, pentobarbitone or chloralose. The details of the technique underwent various changes, but the apparatus in the later experiments is shown in Fig. 1. In some cases a small piston pump was used and in others a Dale-Schuster pump. The main disadvantage of the latter pump was that it was not easy to adjust the stroke to a small enough value. This stroke was transmitted by water to the base of a glass tube *T*. The blood entered and left this tube through two holes in the rubber bung *B*, and was separated from the water by a thin rubber membrane which covered the inner surface of the bung.

In order to facilitate the removal of air the blood left this part of the apparatus by the upper hole. The valves used were Guy Ross valves and were obtained from manufacturers of dental equipment. On emerging from the second valve the blood passed through an air space so that the flow was visible and then flowed past a filter *F* and a thermometer to the inflow cannula. The pressure was transmitted to a mercury manometer by means of saline.

Some trouble was experienced owing to the formation of thrombi in the circuit, and the following precautions were therefore taken. The cat received a fairly large dose of heparin (1000 units/kg.), the glass parts of the apparatus were made unwettable with silicone and a filter was included. This consisted of a small ring of perspex across which a piece of nylon gauze was tied. Thrombi did eventually form on this filter but did not interrupt experiments lasting 4 hr.

In some of the experiments an open circuit was used in which the blood flowed from the cat into an open reservoir at a rate which was controlled by a screw clip, which needed constant attention. With this open circuit changes of pressure were not transmitted through the apparatus, but no trouble was experienced from this cause even with the closed circuit, provided the perfusion pressure was kept higher than the carotid blood pressure of the cat. In some cases a second cat was used to provide extra blood, but this was not always necessary. An electric lamp was placed near the apparatus to keep it warm and the temperature recorded by the thermometer was usually 32–36°

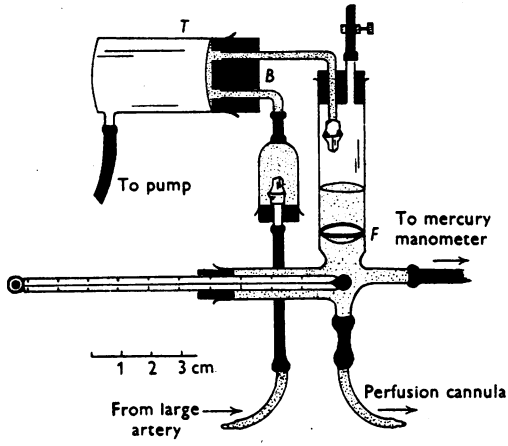

Fig. 1. Pump for perfusion *in situ*.

(4) *Nictitating membrane and lower eyelid of cat*

The technique was like that used by others (Cannon & Rosenblueth, 1937; Isola & Bacq, 1946) except that injections were made into the carotid artery instead of intravenously. The anaesthetic was allobarbitone (Dial compound Ciba) or pentobarbitone (Nembutal, Abbott) or chloralose (B.D.H.). The membrane, or lid, was sensitized by aseptic removal of the superior cervical ganglion under ether 1–2 weeks before the main experiment, and by the intravenous injection of cocaine HCl (8 mg./kg.). The contractions were recorded isotonically. To facilitate the injections a special cannula was introduced in the carotid artery. The two short horizontal arms of this cannula were so shaped that they could be tied directly into the central and peripheral ends of the artery. A third vertical arm was expanded to form a small reservoir containing air and closed by a rubber cap through which injections could be made. Heparin was injected intravenously as an anticoagulant.

RESULTS

The results recorded in Table 2 summarize our observations on the sensitivity of various tests for adrenaline and noradrenaline. The figures given in column 3 represent the lowest dose of adrenaline likely to cause a definite effect in an average good test. These figures are liable to rather wide variations. For an accurate assay it is necessary to repeat this dose several times, and slightly larger doses would generally be more satisfactory. Special attention was paid to the effects of noradrenaline because it had become likely that this substance was liberated by certain nerves. Column 4 gives the estimates of the ratio of a dose of L-noradrenaline to the equivalent dose of L-adrenaline. In some cases these ratios were determined directly, and in others noradrenaline was used in

the racemic form and the observed ratio halved. This is thought to be justified by the fact that in a number of experiments of various kinds L-noradrenaline has always appeared to be approximately twice as active as racemic noradrenaline. This confirms previous evidence that D-noradrenaline has comparatively little pharmacological activity (Luduena, Ananenko, Siegmund & Miller, 1949). The ratios for the colorimetric and fluorescence tests are not adjusted in this way.

TABLE 2

Test		Dose adrenaline (mμg.)	Dose L-noradrenaline / Dose L-adrenaline
Isolated muscle in 2 c.c. bath			
(*a*) Inhibitor effects (de Jalon's method):			
Rat	Uterus	0·5	75–300
	Colon (or ileum)	15	0·2–1
Rabbit	Bladder	10	1
Guinea-pig	Pregnant uterus	20	40
	Colon	200	2
Rabbit	Ileum	200	3
	Colon	2,000	0·5
Rat	Bladder	20,000	2
(*b*) Excitor effects:			
Guinea-pig	Virgin uterus	3	2·5
Frog	Heart (Straub)	10	10–20
Rabbit	Splenic capsule	200	2·5
Rat	Vagina	200	—
	Splenic capsule	20,000	1
	Seminal vesicle	20,000	—
Perfusion with salt solutions			
Rabbit	Ear	2	1–3
Frog	Whole animal	50	2·5
Rat (or mouse)	Hind limbs	50	2·5
Rabbit	Kidney	100	2·5
	Hind limbs	500	2·5
Frog	Lung	10,000	*c.* 1
Other tests			
Cat	Spleen perfused *in situ*	30	0·5–1
	Nictitating membrane	50	1
Fluorescence		20	50
Colorimetric (Shaw)		40	16

Uterus. It is well known that the uterus may be excited or inhibited by adrenaline. The effect in cats is excitor in pregnancy and inhibitor in virgins or after ergot alkaloids. The effect of noradrenaline is usually in the same direction as that of adrenaline. When this effect is excitor, noradrenaline may be slightly more active than adrenaline, but when the effect is inhibitor it is much less active.

This failure to inhibit the uterus except in comparatively high concentrations is a characteristic feature of noradrenaline. This fact was discovered by Barger & Dale (1910) in experiments on cats, but West (1947*b*) found that the ratio of a dose of noradrenaline to the equivalent dose of adrenaline was particularly high in the rat's uterus, and we have also found a high ratio in the guinea-pig's uterus. In rabbits and pregnant cats these drugs stimulate the uterus and are about equally active.

The test on the rat's uterus is the most sensitive and specific of the known tests for adrenaline. It is the only one of the sensitive tests listed in Table 3 in which noradrenaline is comparatively inactive. Fig. 2 shows that in a good preparation the inhibitory effects of 1, 1·5 and 2 mμg. of adrenaline can be distinguished from one another.

In the experiment shown in Fig. 3 the sizes of successive responses were measured and plotted. In this experiment the ratio of the dose of noradrenaline to that of adrenaline was 200. In twelve experiments the ratio varied between 75 and 300 with a median value about 150.

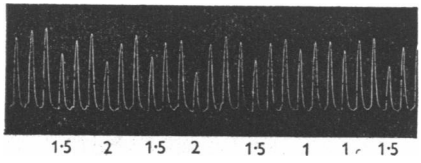

Fig. 2.

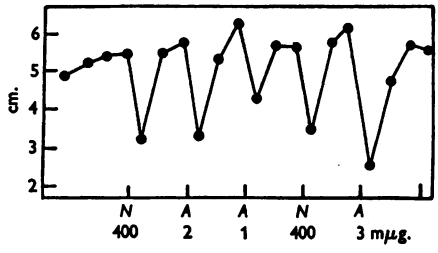

Fig. 3.

Fig. 2. Rat uterus in 2 c.c. bath. Responses to acetylcholine (1 μg. for 30 sec. every 2 min.). Inhibitory effects of adrenaline. Doses in mμg.

Fig. 3. Rat uterus in 2 c.c. bath. Inhibitory effects of adrenaline (*A*) and noradrenaline (*N*) on measured responses to 1 μg. of acetylcholine for 30 sec. every 2 min.

The effects of various other substances are shown in Table 3 and discussed later. The fact that histamine inhibits this tissue is well known. Its activity is not great, but it must be borne in mind as a possible complication of estimates of the adrenaline-concentration when this is low. Adenosine only showed an excitor effect. This is fortunate, since on other preparations the large amounts of adenosine compounds present in some tissues cause effects like those of adrenaline.

Dialysates of haemolysed cat's blood caused a slow contraction of the uterus. When all the precautions described above were taken it was generally possible to obtain control samples of plasma which had no effect on the uterus, but in some experiments, and particularly when only some of the precautions were taken, this was not achieved. In some experiments in which splenic blood was collected before and after stimulation of the splenic nerves, it was found that although the control sample might stimulate the uterus and increase the response to acetylcholine, the sample collected after stimulation of the nerves might have an inhibitory effect. In such cases it was sometimes possible to obtain an estimate of the adrenaline equivalent of the stimulus sample by adding different amounts of adrenaline to the control sample, and comparing the effects of such mixtures with those of the stimulus sample. Similar comparisons have been made with noradrenaline.

Intestine. Cat's intestine was used by Cannon & de la Paz (1911) to detect adrenaline in a conscious cat under the influence of emotion. They collected the blood from the inferior vena cava through a catheter in the femoral vein. Hoskins (1911) found that rabbit's intestine was more sensitive, and this tissue has been much used to detect adrenaline. Used in the conventional way it was not sensitive enough for our purpose. It is not very suitable for de Jalon's technique, since its spontaneous activity is difficult to suppress, but the rat's colon gives good results by this method for concentrations of adrenaline similar to those used by Hoskins. Small doses of adrenaline (such as 10 mμg.) caused a diminution of only one of the responses to acetylcholine. With larger doses the effect lasted longer and several responses were affected.

This test on the rat's colon is particularly sensitive to noradrenaline. No other tissue except rabbit's ear was so sensitive to this drug and no other drug was so active in this test. The threshold dose in the 2 c.c. bath was 5–20 mμg.

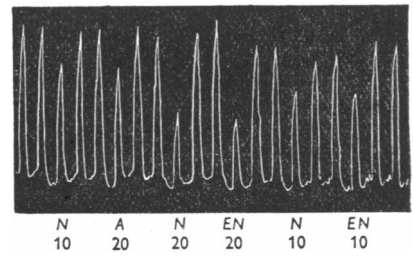

Fig. 4. Rat colon in 2 c.c. bath. Responses to acetylcholine (10 mμg. for 30 sec. every 2 min.) Inhibitory effects of adrenaline (*A*), noradrenaline (*N*) and α-ethylnoradrenaline (*EN*). Doses in mμg.

Adrenaline was generally slightly less active than noradrenaline in this test. For example, Fig. 4 shows an experiment in which it was about half as active. This agrees with West's finding that some preparations made from the intestine are inhibited by noradrenaline in smaller doses than adrenaline. In the early part of some experiments, both of these drugs caused contraction of the muscle, but this effect always disappeared with further doses. It was surprising to find that histamine in suitable concentrations inhibited the response to acetylcholine. In higher concentrations histamine has been found to stimulate the rat's intestine (Feldberg & Schilf, 1930).

The interfering substances in control plasma caused contraction of this preparation and interfered with this test more than with that on the uterus.

These two tests on pieces of plain muscle from rats are an interesting contrast. The uterus provides the most sensitive and specific test for adrenaline, and the colon provides the most sensitive and specific test for noradrenaline. It is not known why these two drugs should act so differently in these two tests, but this fortunate circumstance provides a convenient method of discriminating between them. The intestines of guinea-pigs and rabbits were comparatively insensitive when used in this way. The reason why the rabbit's intestine was so insensitive is probably that it had been exposed to very unphysiological conditions in order to reduce the spontaneous activity.

It is known that when isolated intestine is studied in the conventional way, blood serum causes an initial inhibition followed by contraction. The inhibition is partly due to adenosine. The contraction is partly due to histamine, but mainly due to a substance which has not yet been identified (Gaddum, 1936). This unknown contractor substance may be the same as the 'slow reacting substance' of Feldberg & Kellaway (Kellaway, 1947). It has been found in platelets, but haemolysed red cells seem to be a richer source. In our experiments with heparinized human blood haemolysed red cells had marked actions like those of serum. The plasma, haemolysed platelets and haemolysed white cells had much feebler actions of the same kind.

Frog's heart. Various attempts have been made to use the isolated frog's heart for the detection of adrenaline in blood (Schlossmann, 1927). It may be very sensitive to adrenaline, particularly if it is beating feebly, and it has been suggested that it should be deliberately depressed in order to sensitize it. Schlossmann used aconitine for this purpose, but eventually abandoned this method. West (1947*b*) found that perfused frogs' hearts were rather insensitive to noradrenaline compared with adrenaline.

Frogs' hearts were used in the early stages of the present work. Straub's preparation gave satisfactory results with pure solutions of adrenaline and noradrenaline, though the ratio of activities was very variable. Cocaine (10^{-5}–10^{-6}) increased the sensitivity to adrenaline two to three times, but not that to noradrenaline. Ergotoxine antagonized adrenaline more effectively than noradrenaline. The heart was found to beat well in cat's plasma (heparin) diluted with half its volume of water, but in these conditions it was not sensitive to adrenaline.

It is well known that various substances in blood may affect the frog's heart. Adenosine compounds may be present in sufficient quantities to inhibit the heart, but the main effect of serum is generally stimulation (Clark, 1913). The nature of the substances with this effect is not known, but it is not lecithin, as was once supposed (Eggleton, 1926).

We have found that lysed human red cells are a good source of stimulant substances. Plasma or lysed white cells or platelets were less active. Lysed whole blood from rabbits or cats or man was also active. These solutions were dialysed against water in Verney's (1926) dialyser, and the dialysate had a complex effect which appeared to be due to two stimulant substances, both of which increased the rate and force of the beat of the frog's heart. The first substance had an immediate effect which often disappeared in the later stages of an experiment. The effect of the second substance was like that of adrenaline. It appeared after the first effect was over and did not become less with successive doses. It was found that the substances responsible for these effects could be removed from dialysates of haemolysed whole blood (from men, cats or rabbits) by adsorption on alumina of the type used by Shaw (1938). In these

experiments 5 c.c. of blood were mixed with 5 c.c. of water and dialysed against 10 c.c. of water for 4 hr. The pH of the dialysate was adjusted to 5–6 with HCl, using chlorophenol red as an indicator. The suspension of alumina (0·3–0·5 c.c./c.c. of dialysate) was then added and the mixture shaken, and then centrifuged. This removed the interfering substances which stimulate the frog's heart, but not adrenaline, which is only adsorbed on alumina at more alkaline reactions. Another coarser type of alumina was ineffective when used in the same way. This technique is similar to that used by Jørgensen (1945) for separating adrenaline from fluorescent substances in plasma. By such methods it should be possible to use the frog's heart for the estimation of adrenaline in blood. This test was, however, abandoned because a simpler method of obtaining inactive control samples was sufficient for tests on other tissues.

Rabbit's ear. Various previous workers have come to the conclusion that the best of the known tests for adrenaline in blood is the perfused rabbit's ear (Schlossmann, 1927; Kahlson & Werz, 1930; Kuré, Okinaka, Ohshima, Shimamota & Okamura, 1936). We have also been successful with this preparation. It could be used about 30 min. after starting the perfusion, and small doses of adrenaline continued to produce a brief decrease in the outflow for periods up to 72 hr. The sensitivity usually increased as the perfusion continued, and reached a maximum on the second day, when the preparation was occasionally ten times as sensitive as it had been. It has often been found convenient to make such preparations the day before they were needed. When a series of doses of adrenaline were injected at regular intervals the sensitivity increased for 30–60 min., but this increase of sensitivity was lost when the series of injections ceased.

In the later stages of a perfusion the response appeared to depend on the concentration of calcium in the fluids injected. Small doses of adrenaline in saline became less effective than the same doses in Locke solution. The use of a perfusion fluid with a low calcium content caused an increase of sensitivity, but the effect was small and was not further investigated. It was hoped to eliminate possible interference from histamine by using neoantergan in the perfusion fluid (Dews & Graham, 1946). This was unsatisfactory since this drug inhibited the actions of adrenaline and noradrenaline, as well as those of histamine, though less potently. When small doses of ergotoxine ethane sulphonate (0·1 μg.) or dihydroergotamine (0·05 μg.) were injected into the cannula they caused a temporary inhibition of the effects of similar doses (by weight) of adrenaline and noradrenaline, both of which were equally affected. Noradrenaline was usually slightly less active than adrenaline. Fig. 5 shows an experiment in which the ratio of activities was between 1 and 2.

Cat's plasma in doses greater than 0·1 ml. caused vaso-constriction in this preparation if the cooling and centrifuging of the blood had been too slow. This

effect was not due to histamine since it was not antagonized by neoantergan. On the other hand, the surprising observation was made that suitable small doses of dihydroergotamine abolished this effect of plasma while leaving the effect of adrenaline unchanged.

The fact that ergot alkaloids inhibit the action of a vaso-constrictor substance which may be present in defibrinated blood was established by Heymans, Bouckaert & Moraes (1932). This substance is presumably different from the substance studied by Kahlson & Werz (1930) the effects of which were not antagonized by ergotoxine.

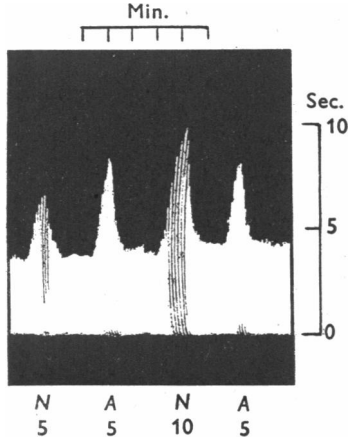

Fig. 5.

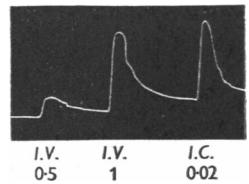

Fig. 6.

Fig. 5. Outflow from perfused rabbit's ear. Heights measure drop interval. Noradrenaline and adrenaline. Doses in mμg.

Fig. 6. Cat. Nictitating membrane. Intravenous (*I.V.*) and intracarotid (*I.C.*) injection of noradrenaline. Doses in μg.

Other perfused tissues. None of the other perfused tissues was as sensitive as the rabbit's ear. Whole frogs were slightly more sensitive than frogs' hind limbs, but too erratic. Dialysates of haemolysed whole blood from cats or men caused vaso-constriction in perfused frog's vessels (0·5 c.c. of 1/10 dilution). Plasma separated by rapid centrifugation usually had no effect even when undiluted.

Nictitating membrane. This tissue has been much used as an indicator of substances liberated by adrenergic nerves into the general circulation. The effective dose by intravenous injection has been found to be twenty-five to fifty times the effective dose by intra-arterial injection (Fig. 6), and the latter technique was therefore used. When heparinized whole blood, which had been standing in glass, was injected it usually caused a contraction of the membrane. Plasma which had been separated with the precautions recorded above usually

had little or no effect in doses of 0·5 c.c. injected rapidly, except in conditions where the effect could reasonably be attributed to adrenaline or noradrenaline.

Perfusion in situ. This technique provides a simple means of comparing the effects of drugs on different parts of the circulation. The cat's spleen responded to smaller doses of adrenaline than the tissues supplied by the superior or inferior mesenteric artery, the renal, hepatic or femoral artery. The activity of noradrenaline was usually about equal to that of adrenaline (Fig. 7) or slightly greater and sometimes as much as twice as great. When the femoral artery or the artery to the tail was perfused in cats anaesthetized with ether, adrenaline usually caused a fall of pressure while noradrenaline caused a rise.

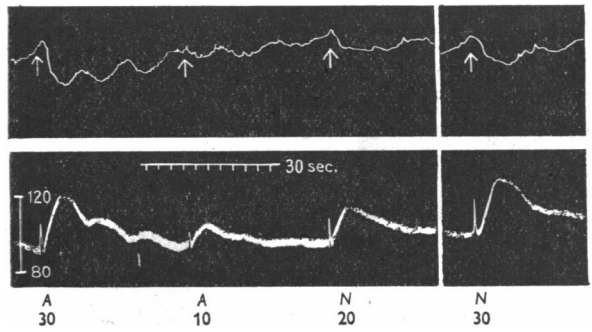

Fig. 7. Cat. Spleen perfused *in situ*. Splenic volume (above) and perfusion pressure (mm. Hg). Injection of adrenaline (*A*) and noradrenaline (*N*). Doses in mμg.

The difference between adrenaline and noradrenaline. In many tests the action of noradrenaline was about equal to that of adrenaline. It was generally slightly less active, but in some tests it was slightly more active. No tissue is known on which noradrenaline has much more effect than adrenaline, but various tissues are known on which it has much less action. Barger & Dale (1910) found that it had much less inhibitor action on the virgin cat's uterus and on the arterioles of cats. Its inhibitory action on the uteri of rats and guinea-pigs is also weak. These facts have suggested that the essential difference between these two drugs is that noradrenaline lacks inhibitory actions, but West (1947*b*) has drawn attention to various exceptions to this generalization. For example, the inhibitor action of noradrenaline on the intestine is often greater than that of adrenaline, and its excitor action on perfused frog's heart is much less.

Solutions of the pure substances can easily be distinguished by using a test in which noradrenaline is relatively active in parallel with one in which it is less active. The specific tests, in which noradrenaline is relatively inactive, are those showing high values in the last column of Table 2 such as the biological tests on the uterus and the perfused frog's heart, the fluorescence test (Gaddum & Schild, 1933) and the colorimetric test of Shaw (1938). If adrenaline and

noradrenaline are both present together in similar amounts any of these latter tests can be used to estimate adrenaline without much interference from noradrenaline. There is no simple way of estimating noradrenaline in the presence of similar amounts of adrenaline.

Other substances. Five sensitive tests were selected for more detailed investigation. The effects of a number of substances in these tests are summarized in Table 3 which shows the ratios of the doses of these substances to the equivalent doses of adrenaline. These ratios were found to vary fairly widely. This variation does not diminish the value of parallel quantitative assays for qualitative identification.

TABLE 3. Ratio of doses of various drugs to equivalent dose of L-adrenaline

Effect	Inhibitor			Excitor	
Animal	Rat		Rabbit	Cat	
Organ	Uterus	Colon	Ear	Spleen	Nictitating membrane
Dose adrenaline (mμg.)	0·5	2–5	2	30	10–50
Probable precursors of adrenaline:					
Hydroxytyramine	2000–6000	10^5	(3000)	75	100
L-Noradrenaline	75–300	0·2–1	1–3	0·5–1	1
α-Substituted noradrenalines:					
DL-corbasil	10	5	1000–5000	—	200
DL-α-ethylnoradrenaline	50, 60	0·5	20	1·5	7, 15
β-Substituted adrenalines:					
Epinine	120	100	20	10	1·3
Adrenalone	25	4, 20	130, 200	200	150
N-Substitution:					
DL-N-methyladrenaline	850	60	40	—	25
DL-N-ethylnoradrenaline	0·5, 1	1	12	Dilator	45
DL-N-isopropylnoradrenaline	0·5, 1	1	10^4	Dilator	Inhibitor
Other substances:					
Tyramine	3×10^5, 10^6	10^5	—	250	400
Ephedrine	5000	750	—	10^4	—
Histamine	5000	50	10	Dilator	10
Adenosine	Excitor	3000	—	—	—

On the colon hydroxytyramine is no more active than tyramine. On the other tissues it is somewhat more active, but still not really potent. The introduction of the β-OH converts it to noradrenaline and greatly increases its activity in all the tests and especially on the colon. The great effect of the β-OH is also shown by comparing adrenaline and epinine. Of both these comparisons it may be said that reduction of the OH to H decreases the activity. On the other hand, oxidation of this group in adrenaline to form the ketone adrenalone also decreases the activity. By comparing the figures for epinine and adrenalone it may be seen that reduction appears to affect the inhibitor actions more than the excitor actions and oxidation appears to affect the excitor actions more than the inhibitor actions.

The replacement of the *N*-methyl group in adrenaline by larger alkyl groups appears to have little effect on the inhibition of the uterus and colon, but diminishes or reverses the excitor effects. These compounds have been studied by Konzett (1940) and found to have powerful dilator effects on blood vessels and bronchi.

Discrimination between different substances. In the case of most of the substances in Table 3 the ratios shown in the different tests vary widely and there should be little difficulty in distinguishing these substances from adrenaline. In the case of the two *N*-mono-alkyl compounds the ratios on the rat's uterus and colon were about equal, but these compounds are distinguished by their lack of excitor effects.

α-Ethylnoradrenaline showed ratios not very different from those of noradrenaline, but the two substances could presumably be distinguished by using the rat's uterus or colon together with the rabbit's ear or the nictitating membrane.

A detailed comparison of the other figures shows that there should be no difficulty in distinguishing any one of these substances from any other.

DISCUSSION

If parallel quantitative assays of an unknown solution are made by the five tests shown in Table 2, using adrenaline as a standard, and if these tests agree with a maximum error ratio of not more than 2, it is reasonable to conclude that the effects of the unknown solution are due to adrenaline. It is quite certain that they are not due to any one of the other substances used in the present investigation. Any one of these other substances can also be identified in the same way, provided that only one pharmacologically active substance is present in the solution.

This principle of parallel quantitative assays was used by Cannon & Rosenblueth (1937) to prove that adrenaline is not the only substance liberated by adrenergic nerves. Such negative evidence is immediately convincing. Positive evidence identifying an unknown substance is only convincing when it is known that the tests used can distinguish closely allied substances from one another. The tests described here do appear to have this power. They have been applied to the study of the substances released by the adrenergic nerves in the cat's spleen and the results are published elsewhere (Peart, 1949).

SUMMARY

1. Various pharmacological methods of estimating adrenaline and allied substances have been compared and five sensitive methods selected for detailed study.

2. Closely allied sympathomimetic amines can be distinguished from one another by making parallel quantitative assays by these five methods.

These substances can therefore be identified by such assays in unknown solutions.

3. When these tests are applied directly to blood the result is vitiated by the release of interfering substances. This can be avoided if the blood is collected with much heparin in cooled tubes coated with silicone. If the cells are rapidly removed by centrifugation adrenaline (10^{-8}) or noradrenaline (10^{-7}) can be identified in the plasma, if present.

We are much indebted to Dr M. L. Tainter for samples of the various compounds made by Sterling Winthrop, and to Dr H. Blaschko for samples of hydroxytyramine and methyladrenaline.

REFERENCES

Bain, W. A., Gaunt, W. E. & Suffolk, S. F. (1937). *J. Physiol.* **91**, 233.
Barger, G. & Dale, H. H. (1910). *J. Physiol.* **41**, 19.
Barsoum, G. S. & Gaddum, J. H. (1935). *J. Physiol.* **85**, 1.
Cannon, W. B. & de la Paz, D. (1911). *Amer. J. Physiol.* **28**, 64.
Cannon, W. B. & Rosenblueth, A. (1937). *Autonomic Neuro-Effector Systems.* New York: The Macmillan Co.
Clark, A. J. (1913). *J. Physiol.* **47**, 66.
Dews, P. B. & Graham, J. D. P. (1946). *Brit. J. Pharmacol.* **1**, 278.
Eggleton, P. (1926). *Biochem. J.* **20**, 395.
Feldberg, W. & Schilf, E. (1930). *Histamin.* Berlin: Springer.
Gaddum, J. H. (1936). *Gefässerweiternde Stoffe der Gewebe.* Leipzig: Thième.
Gaddum, J. H. & Kwiatkowski, H. (1938). *J. Physiol.* **94**, 87.
Gaddum, J. H. & Schild, H. (1933). *J. Physiol.* **80**, 9*P*.
Heymans, C., Bouckaert, J. J. & Moraes, A. (1932). *Arch. int. Pharmacodyn.* **43**, 468.
Hoskins, R. G. (1911). *J. Pharmacol.* **3**, 93.
Isola, W. & Bacq, Z. M. (1946). *Arch. int. Physiol.* **54**, 30.
de Jalon, P. G., Bayo, J. B. & de Jalon, M. G. (1945). *Farmacoterap. Actual.* **2**, 313.
Jørgensen, K. S. (1945). *Acta pharmacol.* **1**, 225.
Kahlson, G. & v. Werz, R. (1930). *Arch. exp. Path. Pharmak.* **148**, 173.
Kellaway, C. H. (1947). *Edin. med. J.* **54**, 333.
Konzett, H. (1940). *Arch. exp. Path. Pharmak.* **197**, 27, 41.
Kuré, K., Okinaka, S., Ohshima, K., Shimamota, T. & Okamura, D. (1936). *Klin. Wschr.* **15**, 477.
Luduena, F. P., Ananenko, E., Siegmund, O. H. & Miller, L. C. (1949). *J. Pharmacol.* **95**, 155.
Peart, W. S. (1949). *J. Physiol.* **108**, 492.
Reid, G. & Bick, M. (1942). *Med. J. Aust.* **1**, 245.
Richards, A. N. & Plant, O. H. (1915). *J. Pharmacol.* **7**, 485.
Schlossmann, H. (1927). *Arch. exp. Path. Pharmak.* **121**, 160.
Shaw, F. H. (1938). *Biochem. J.* **32**, 19.
Verney, E. B. (1926). *J. Physiol.* **61**, 319.
West, G. B. (1947*a*). *Brit. J. Pharmacol.* **2**, 121.
West, G. B. (1947*b*). *J. Physiol.* **106**, 418, 426.
Zucker, M. B. (1944). *Amer. J. Physiol.* **142**, 12.