Brit. J. Pharmacol. (1964), **23**, 360–373.

THE USE OF ISOLATED ORGANS FOR DETECTING ACTIVE SUBSTANCES IN THE CIRCULATING BLOOD

BY

J. R. VANE

From the Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C.2

(*Received February* 7, 1964)

A method is described for the assay of circulating hormones after their injection or release into the circulation. The method is applicable to cats, dogs and rabbits, and consists of bathing or superfusing isolated smooth muscle preparations in a stream of heparinized arterial blood taken from and returned to the animal at a constant rate. The tone of the smooth muscle preparations was affected by small changes in the concentrations of various amines. Thus increases in blood concentrations of catechol amines can be assayed with the rat stomach strip and chick rectum preparations. The proportions of adrenaline and noradrenaline in a mixture can be determined. Circulating histamine can be assayed on the blood-bathed guinea-pig ileum and bradykinin on the rat duodenum preparations. The uses and limitations of the technique are discussed.

The estimation of hormones circulating in the blood stream of animals has always needed not only considerable skill but also a painstaking process of collection, purification and assay. Many of these lengthy procedures make use of an isolated organ for the final determination. The method to be described here eliminates the intermediate steps of collection and purification, by bathing the isolated assay organ directly in the circulating blood ; a brief account has already been published (Vane, 1958).

METHODS

The method depends upon establishing a secondary circulation of blood outside the animal. Blood is taken from a convenient artery, passed over the assay organ and then returned to a convenient vein. As this involves depleting the animal of 5 to 15 ml. of blood for the extracorporeal circuit, the technique has so far been confined to cats, rabbits and dogs. The results presented in this paper were all obtained with cats.

Cats were anaesthetized with ethyl chloride and ether. They were then either made spinal by the method of Kosterlitz, Krayer & Mattalana (1955) or anaesthetized with chloralose (80 mg/kg. intravenously). The trachea was cannulated to facilitate artificial ventilation. A brachial, femoral or carotid artery was cannulated for recording arterial blood pressure with a mercury manometer. Intravenous injections were made through a cannula in a femoral vein. Polyethylene cannulae were tied into either a brachial or a carotid artery to supply the extracorporeal circulation with blood and into a jugular vein to return the blood to the body.

The apparatus for the extracorporeal circulation was made from silicone tubing, polyethylene tubing and siliconed glass and is shown in Fig. 1*a*. The organ-bath was kept warm by circulating water at 38° C through a glass jacket. The blood overflowed from the inner chamber into a venous reservoir in which a second isolated organ was sometimes suspended. The blood superfused the second tissue and was then returned to the jugular vein by gravity.

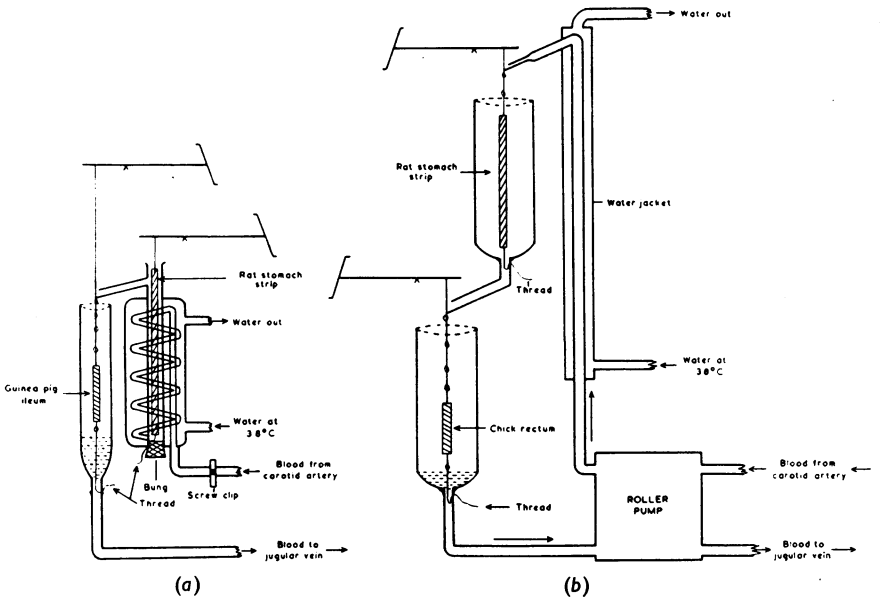

Fig. 1. Diagram of apparatus, not to scale. (*a*) Bathing one tissue in a bath of blood and superfusing another. (*b*) Superfusing two tissues with blood.

In the early experiments the blood was pumped through the external circuit by the heart, and the rate of flow was controlled by a screw-clip. As the rate of flow needed frequent adjustment, especially after the intravenous injection of a drug such as adrenaline, a pump was interposed to control the flow of blood through the outside circuit. In order not to add substantially to the external volume of blood either a roller pump or a " Sigmamotor " finger pump was used. These pumps had the advantage that a second tube could be added to pump the blood back into the cat.

In later experiments it was found that superfusion (Gaddum, 1953) of all the isolated organs worked equally well (Fig. 1, *b*). The organs were suspended in jackets made from polypropylene centrifuge tubes (100 or 130 ml.) of 38 mm internal diameter and the warm blood was made to trickle over the tissue. Superfusion had several advantages: less blood was removed from the cat ; up to three isolated organs could be included in the extracorporeal circulation, with the blood cascading down from one to the next ; and the sensitivity of the organs to drugs was higher.

The organ-baths and recording levers were clamped to a pair of rigid uprights and were carefully aligned before the experiment began. A vibrator attached to one of the uprights minimized friction between the levers and the smoked paper. The movements of the assay organs were recorded either by pendulum levers (Paton, 1957) or by isotonic levers. The magnification of the levers was 16:1 with a load on the tissues of 1 to 3 g.

After the animal had been prepared and the apparatus aligned the external circuit was connected with silicone or polyethylene tubing of narrow bore (1 to 2 mm internal diameter) to the arterial and venous cannulae. The animal was heparinized by the intravenous injection of Pularin (Evans ; 1,000U/kg). The external circuit was primed with about 15 ml. of heparinized 0.9% saline or Tyrode solution in the venous return reservoir.

Rat stomach strip preparation

This preparation, described by Vane (1957), was made either from normal rats or from rats which had been treated with reserpine (1 mg/kg, intramuscularly, 18 hr previously). The strip

was swirled around in Tyrode solution to remove any loose filaments and traces of stomach contents. It was then pulled into the organ-bath or the superfusion jacket by a previously threaded piece of cotton. When the stomach strip was in place, the cotton attached to the lower end was fixed. The roller pump was turned on to allow blood to flow into the bath. At the same time, saline followed by blood was pumped back into the jugular vein. The stomach strip was attached to the pendulum lever so that its movements were recorded.

Other isolated organ preparations

The following preparations were used: ileum, taenia coli and vas deferens of the guinea-pig; duodenum, uterus and seminal vesicle of the rat; and rectum of the chicken. The organs were taken from animals which had just been killed by stunning and exsanguination. The part to be used was dissected out and washed in Tyrode solution. It was suspended in the apparatus, connected to a frontal-writing lever, and bathing or superfusion with blood was started.

Drugs

The following drugs were used, and the doses of salts are given in terms of base: acetylcholine perchlorate, (−)-adrenaline hydrogen tartrate (B.D.H.), bradykinin (Parke Davis), choline chloride, Compound 48/80 (Burroughs Wellcome), histamine hydrogen phosphate (B.D.H.), 5-hydroxytryptamine creatinine sulphate (M & B), hyoscine hydrobromide (B.D.H.), (±)-isoprenaline sulphate, methysergide (Sandoz), nicotine hydrogen tartrate (B.D.H.), (−)-noradrenaline hydrogen tartrate (B.D.H.) and trypsin (Armour).

RESULTS

Effects of changing from Tyrode solution to blood

The sensitivities of the isolated organs were different when bathed in Tyrode solution and when bathed in blood. This is illustrated in Fig. 2 for the rat stomach strip. The tissue was first superfused with a constant flow (12 ml./min) of oxygenated Tyrode solution at 37° C. Injections of adrenaline and noradrenaline caused a relaxation of the strip, whereas histamine, acetylcholine, 5-hydroxytryptamine and choline all caused contractions (Fig. 2,*a*, *b* and *c*). An extracorporeal circulation from a cat was then started so that the strip was bathed, in place of

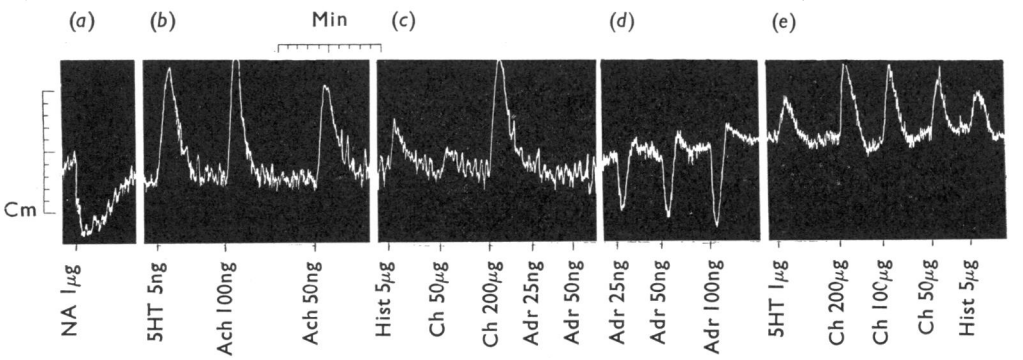

Fig. 2. Sensitivity to various drugs of the rat stomach strip preparation when superfused with Tyrode solution (*a*, *b* and *c*) and with blood (*d* and *e*). Noradrenaline (NA), 5-hydroxytryptamine (5HT), acetylcholine (Ach), histamine (Hist), choline (Ch) and adrenaline (Adr) were injected into the bathing fluid in the doses shown. When bathed in blood from a spinal cat, the strip was much more sensitive to catechol amines and much less sensitive to 5-hydroxytryptamine. Time in minutes. Vertical scale in cm on the kymograph.

Tyrode solution, in recirculating blood at the same rate of flow. As soon as the blood came into contact with the rat stomach strip its tone increased substantially, so much so that the position of the lever fulcrum had to be lowered to bring the writing point back on to the kymograph. The increase in tone represented a shortening of the muscle by about 20%. Thereafter, the preparation was much more sensitive to the relaxant effects of adrenaline and noradrenaline and much less sensitive to the contractor effect of 5-hydroxytryptamine. The sensitivities to choline and histamine were comparatively unchanged (Fig. 2,*e*). Acetylcholine was much less effective, probably because it was destroyed by plasma cholinesterases.

The tone of other isolated organs also increased when bathed in blood, and they also became less sensitive to acetylcholine and to 5-hydroxytryptamine. The increased tone of the tissues and the decreased sensitivity to some contractor drugs could be accounted for by a circulating contractor substance or a mixture of substances. Treatment of the tissues with hyoscine (10^{-7} g/l. added to the Tyrode solution for 1 hr) did not reduce the contractor effect of blood. Treatment of the tissues with methysergide (10^{-7} g/l. added to the Tyrode solution for 1 hr) partially reduced the contractor effect of blood, which suggested that circulating 5-hydroxytryptamine might contribute to the contraction. Since the rat duodenum preparation was also contracted by blood, but is relaxed by bradykinin, a circulating kinin was unlikely to account for the increase in tone.

Effects of rate of flow

The rate at which the blood was pumped through the bath or superfused affected the tone of the isolated organs. When the rate of flow was lowered from 10 ml./min the tone of the rat stomach strip decreased. When the rate of flow was raised from 10 to 20 ml./min there was little change in tone and in the sensitivity to drugs. Therefore a constant rate of flow of about 12 to 15 ml./min was used.

Effects of oxygen tension

Both the tone of the preparations and their responses to drugs were substantially affected by changes in the oxygen tension of the bathing blood. When the cat was ventilated artificially with air, the tone of the blood-bathed stomach strip was constant. When the animal was made to breathe 100% oxygen, the tone of the stomach strip rose considerably to a higher constant level; it was then more sensitive to adrenaline and to noradrenaline. This effect of oxygen tension will be the subject of a future paper (Smith & Vane).

In all the following experiments, to minimize variations in the tone of the organs, the cat was ventilated artificially with air at a fixed stroke volume.

Detection of circulating catechol amines

The rat stomach strip was generally the most sensitive tissue for detecting circulating catechol amine. However, in order to assay both noradrenaline and adrenaline in a mixture, at least two tissues were needed. The rat stomach strip gave an estimate of total amines, for its sensitivity to both adrenaline and noradrenaline was of the same order (adrenaline was twice as active as noradrenaline).

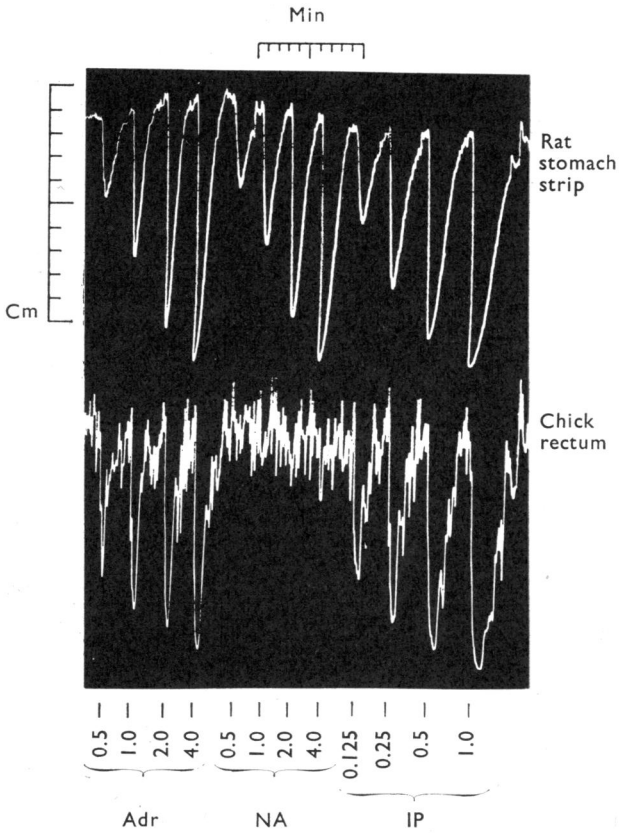

Fig. 3. Rat stomach strip (top) and chick rectum (bottom) preparations superfused with blood from a cat anaesthetized with chloralose. The records show the relaxations produced in the blood-bathed organs when doses of adrenaline (Adr), noradrenaline (NA) and isoprenaline (IP) were injected intravenously into the cat. All doses in μg. Time in minutes. Vertical scale in cm on the kymograph.

The chick rectum preparation, as shown by Mann & West (1950), responded primarily to adrenaline (25- to 100-times more active than noradrenaline).

The effects of intravenous injections of adrenaline, noradrenaline and isoprenaline are shown in Fig. 3. It can be seen that the combination of rat stomach strip and chick rectum preparations distinguished between adrenaline and noradrenaline, but not between adrenaline and isoprenaline. Quantitative estimates of the proportions of adrenaline and noradrenaline were obtained by plotting the isobols. In some experiments the guinea-pig vas deferens was used as a third test organ in an attempt to distinguish between adrenaline, noradrenaline and isoprenaline, but it was usually too insensitive in comparison with the other two organs.

Calibration by injection

The blood-bathed organs were calibrated either by injecting or by infusing drugs into the constant flow of bathing blood. An example is shown in Fig. 4,*a*. Nor-

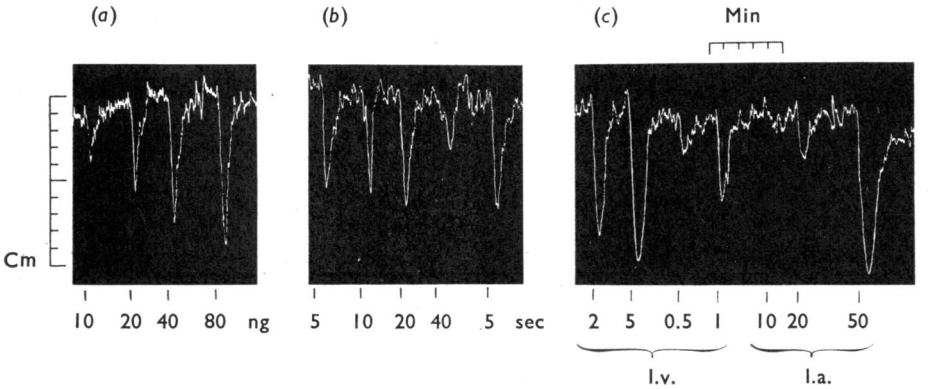

Fig. 4. Rat stomach strip preparation superfused with blood from a cat anaesthetised with chloralose (*a*) Sensitivity of the strip to noradrenaline injected in nanogram doses (given below) into the superfusing blood ; (*b*) reactions of the strip to a repeated dose of noradrenaline (30 ng) injected over the different time periods shown below ; (*c*) comparison of the effects of noradrenaline injected in microgram doses intravenously (i.v.) and intra-arterially (i.a.). Time in minutes. Vertical scale in cm on the kymograph.

adrenaline was injected into the bathing blood in increasing doses, giving a dose/response curve with good discrimination. Fig. 4,*c* shows the effects of injecting noradrenaline intravenously. The concentration of noradrenaline in the bathing blood after an intravenous injection of 1 μg was equivalent to the concentration when 20 ng were injected directly into the bathing blood. With all drugs, except those destroyed rapidly in the blood stream, an injection into the bathing blood of 1/20th to 1/50th of the intravenous dose gave approximately the same response as the intravenous dose.

On intravenous injection, a drug first has to pass through the heart and lungs before it reaches the arterial blood ; during this passage the drug will become distributed into a greater volume than when it is injected directly into the bathing blood. To determine whether such distribution might change the magnitude of the responses of the organ, a dose of noradrenaline was injected directly into the bathing blood over time periods of 5, 10, 20 and 40 sec (Fig. 4,*b*). As long as the injection was completed within less than 20 sec the relaxation of the stomach strip was substantially the same ; when the injection took more than 20 sec the relaxation was smaller. Thus, the possible spreading out of an injection by passage through a vascular bed would interfere with the assay only if the passage caused a delay of more than 20 sec.

Calibration of the monitoring organs by single injections gave only relative values for the concentrations of drugs in the blood. It was, however, valuable for comparing the concentrations after intravenous injection with those after intraportal or intra-arterial injections. Fig. 4,*c* shows that about forty-times the intravenous dose of noradrenaline had to be injected into the abdominal aorta of the cat (through a cannula tied into the inferior mesenteric artery) to give the same concentration in the carotid blood. Thus, in one circulation through the hind-quarters of a cat, more than 95% of the noradrenaline was either destroyed or removed from the circulation.

Calibration by infusion

To determine changes in blood levels of catechol amines quantitatively, the assay organs were calibrated by infusing the drugs directly into the bathing blood. Since the rate of flow of bathing blood was known the response of the tissue could be calibrated in terms of nanograms of drug per ml. of blood. It was fortunate that, with the rat stomach strip and with the chick rectum preparations, the change of tone produced by catechol amines remained constant as long as the catechol amines were present, in other words there was little or no tachyphylaxis (Fig. 5).

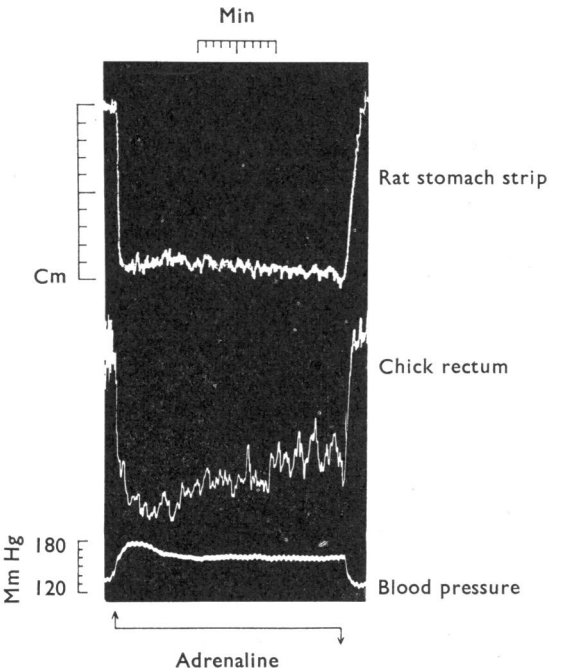

Fig. 5. Rat stomach strip (top) and chick rectum (middle) preparations superfused with blood from a cat anaesthetized with chloralose (bottom record is of the cat's blood pressure). Between the arrows adrenaline (2 μg/min) was infused intravenously. Time in minutes. Vertical scale in cm on the kymograph.

Determination of hormones released by other drugs

When the technique was used to detect catechol amines or other hormones released into the circulation by the injection of another drug, the effects on the test organs of the releasing drug had to be determined first. This is well illustrated by phenethylamine, which liberates noradrenaline from tissues (Burn, 1960; Vane, 1960). When the rat stomach strip was bathed in Tyrode or Krebs solution, phenethylamine caused a contraction (Vane, 1960). However, when the rat stomach strip was bathed in blood, an injection of phenethylamine directly into the bathing blood produced a relaxation, very similar to that produced by noradrenaline. Thus, a normal rat stomach strip preparation could not be used for detecting any circulating catechol amines released by phenethylamine. However, a stomach strip taken from a rat

previously treated with reserpine and bathed in blood responded to phenethylamine with a small contraction but still relaxed to catecholamine ; it could therefore be used for assay of catechol amines in the presence of phenethylamine (Vane, 1960).

Nicotine. Nicotine itself caused only a small contraction of the blood-bathed rat stomach strip or chick rectum, and the relaxation of the tissues produced by circulating catechol amines was not masked by the direct action of the nicotine. Nicotine (50 to 100 μg, intravenously) releases 0.3 to 1 μg of catechol amines.

Histamine. The rat stomach strip and chick rectum preparations are relatively insensitive to histamine and can therefore be used to detect catechol amines released

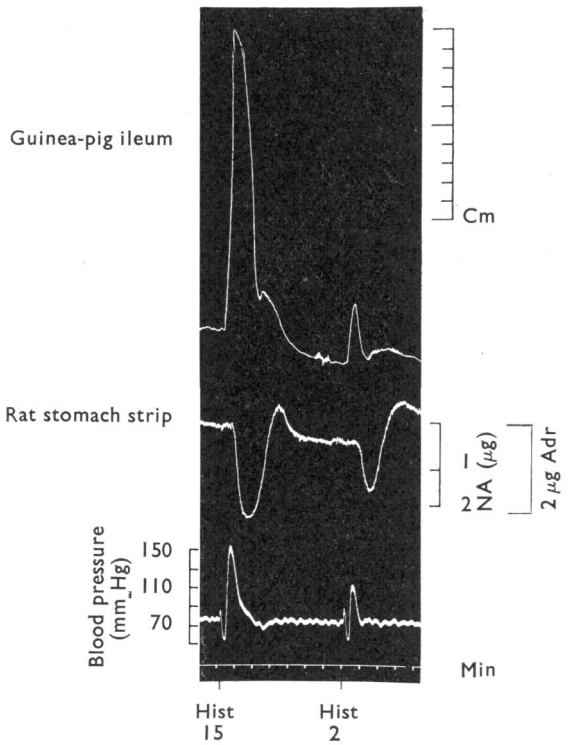

Fig. 6. Guinea-pig ileum (top) and rat stomach strip (middle) preparations bathed in blood from a spinal cat (bottom record is of the cat's blood pressure). After injections of histamine (Hist 15 and 2 μg, intravenously) the guinea-pig ileum was contracted by the circulating histamine ; 20 to 30 sec later, the rat stomach strip relaxed, due to catechol amines liberated into the circulation. Time in minutes. Vertical scales in cm on the kymograph and in equivalent intravenous doses of adrenaline (Adr) and noradrenaline (NA).

by histamine. Fig. 6 shows that the well-known liberation of catechol amines by histamine could be detected by this method. Hexamethonium did not prevent this release whereas mepyramine did. In this experiment a blood-bathed guinea-pig ileum was used to detect circulating histamine. By using both the rat stomach strip and the chick rectum as parallel assay preparations, it was possible to show that

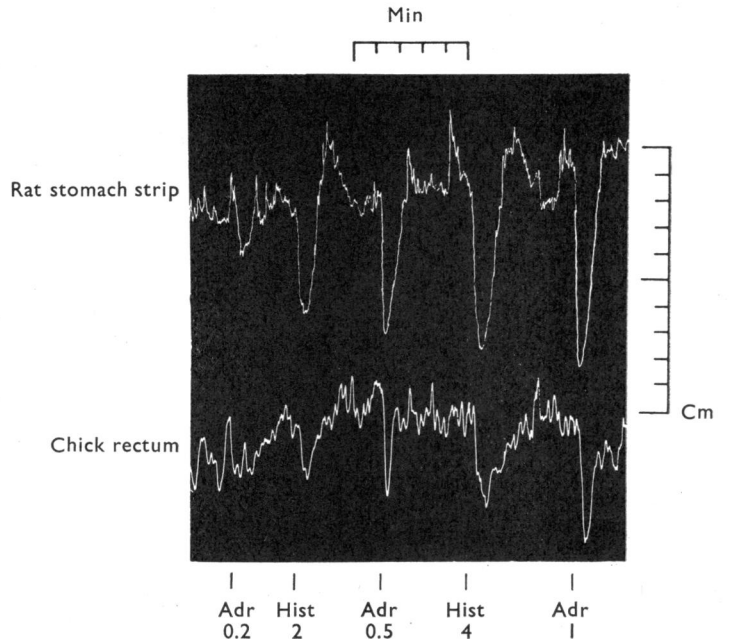

Fig. 7. Rat stomach strip (top) and chick rectum (bottom) preparations superfused with blood from a cat anaesthetized with chloralose. Comparison of the effects of catechol amines liberated by intravenous doses of histamine (Hist, 2 and 4 μg) with intravenous injections of adrenaline (Adr, 0·2, 0·5 and 1 μg). Time in minutes. Vertical scale in cm on the kymograph.

histamine liberated mainly adrenaline (Fig. 7). Only a small fraction of the histamine injected intravenously reached the adrenal glands; nevertheless, as little as 2 μg released about 0.4 μg of adrenaline (Fig. 7). The release of catechol amine was abolished by removing the adrenal glands.

Release of histamine

The release of histamine into the circulation can be readily demonstrated with the guinea-pig ileum as the assay organ. Fig. 8 shows the detection of circulating histamine after an intravenous injection of Compound 48/80 (50 μg), a substance shown to release histamine by Paton (1951). The effects of the histamine on the guinea-pig ileum were abolished by mepyramine.

Detection of 5-hydroxytryptamine

In Tyrode solution, the rat stomach strip preparation is sensitive to concentrations of 10^{-11} g/ml. of 5-hydroxytryptamine. When bathed in blood, however, it was much less sensitive, and the guinea-pig ileum, taenia coli and rat duodenum preparations gave better responses. However, none of these organs was sufficiently sensitive to detect the changes in concentration of 5-hydroxytryptamine in the blood stream produced by intravenous injections of 1 to 5 μg.

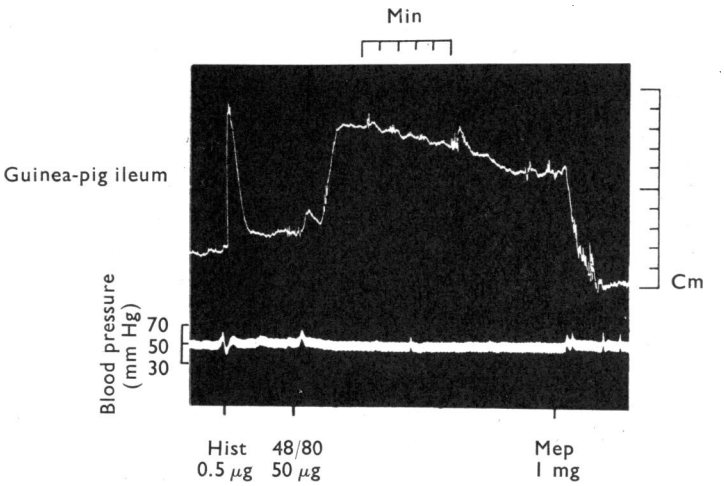

Fig. 8. Guinea-pig ileum (top) preparation bathed in blood from a spinal cat (bottom record is of the cat's blood pressure). The guinea-pig ileum responds to histamine (Hist, 0·5 μg, intravenously) with a transient contraction, but gives a prolonged contraction after the intravenous injection of the histamine liberator, Compound 48/80 (50 μg, intravenously). The contraction is abolished by mepyramine (Mep, 1 mg, intravenously). Time in minutes. Vertical scale in cm on the kymograph.

Detection of acetylcholine and choline-like substances

Acetylcholine is rapidly destroyed in blood and was difficult to detect after intravenous injections. Intravenous choline (100 μg) contracted the blood-bathed rat stomach strip and duodenum, and the guinea-pig ileum and taenia coli preparations.

Release of bradykinin

The rat duodenum relaxes in the presence of bradykinin (Horton, 1959), whereas most other isolated organs contract. A combination of rat duodenum with rat stomach strip was, therefore, especially useful for the identification of bradykinin. Bradykinin injected into the bathing blood relaxed the duodenum and contracted the stomach strip. Catechol amines relaxed both organs, whereas 5-hydroxytryptamine contracted both. Fig. 9 shows all these responses.

Trypsin is well known to release bradykinin (Rocha e Silva, 1960) and when injected intravenously it released sufficient bradykinin-like substance to be detected by this method. Similarly, when blood was withdrawn from the external circuit into a glass syringe for 1 min and then pushed back, the rat duodenum relaxed and the stomach strip contracted (Fig. 9,*b*); this showed that contact with the glass (Armstrong, Jepson, Keele & Stewart, 1957) had released a bradykinin-like substance.

Determination of hormones released by reflexes

The contribution of the adrenal glands to cardiovascular reactions could also be determined with this technique. Fig. 10 demonstrates the release of minute

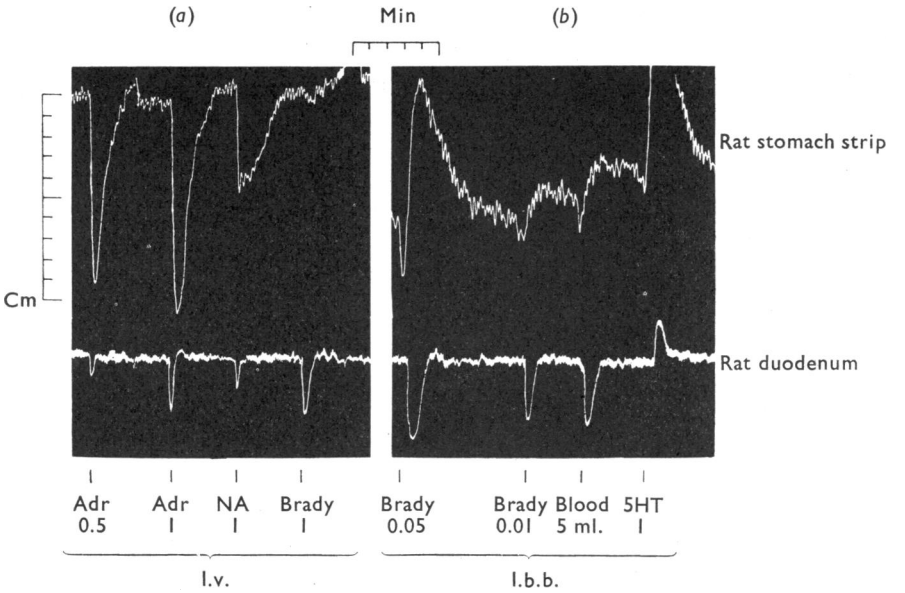

Fig. 9. Rat stomach strip (top) and rat duodenum (bottom) preparations superfused with blood from a cat anaesthetized with chloralose. (*a*) The effects of intravenous (I.v.) adrenaline (Adr) and noradrenaline (NA) on the two tissues, compared with those of bradykinin (Brady) ; (*b*) a comparison of the effects of bradykinin, 5-hyroxytryptamine (5 HT) and 5 ml. of blood reinjected into the superfusion circuit after contact with glass for 1 min. The reactions of the tissues indicate the liberation of about 10 μg of bradykinin. Time in minutes. Vertical scale in cm on the kymograph. All doses in μg.

amounts of catechol amines into the blood stream when the carotid artery occlusion response was elicited. These concentrations were negligible compared with those following the intravenous injection of sufficient adrenaline or noradrenaline to raise the blood pressure. Therefore, the rise in blood pressure induced by carotid occlusion was almost entirely due to sympathetic nerve stimulation with very little contribution due to the release of catechol amines from the adrenal glands.

DISCUSSION

The results show that blood-bathed organs can detect low concentrations of many hormones, whether they are injected into or released within the body. The method will detect concentrations of about 1 ng/ml. of adrenaline or of noradrenaline in blood and is therefore of the same order of sensitivity as, for instance, the denervated nictitating membrane preparation of the cat for adrenaline, or the pithed rat blood pressure response for noradrenaline. Furthermore, concentrations of amine can be determined quantitatively and immediately by suitable direct calibration of the assay organ.

During the development of the method, several interesting observations were made. When the organs were first bathed with blood instead of with Tyrode solution they contracted. Part of the contraction was probably due to a low

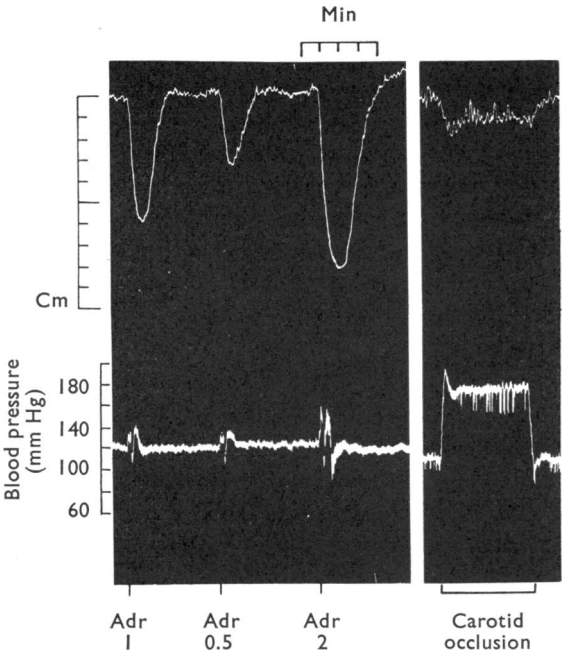

Fig. 10. Rat stomach strip preparation (top) superfused with blood from a cat anaesthetized with chloralose (bottom record is of the cat's blood pressure). (*a*), the effects of adrenaline (0·5, 1 and 2 μg, intravenously). (*b*), only small amounts of catechol amines were liberated into the circulation during the period of carotid arterial occlusion marked by the horizontal line. Time in minutes. Vertical scale in cm on the kymograph.

concentration of 5-hydroxytryptamine circulating in the blood since (a) the sensitivity of the blood-bathed stomach strip to 5-hydroxytryptamine was so much decreased, and (b) a specific antagonist of 5-hydroxytryptamine, methysergide, partially abolished the contractor effect of blood. The part of the contraction induced by blood which is resistant to methysergide was unlikely to be due to acetylcholine, bradykinin or histamine because (a) it was resistant to hyoscine, (b) the rat duodenum preparation is also contracted, but is relaxed by bradykinin, and (c) the rat stomach strip is insensitive to histamine. Part of the contractor effect of blood may be due to a natural hormone as yet unidentified ; or to the synthesis of a contractor substance within the isolated organ from a precursor carried in the blood ; or to a change in *p*H or osmotic pressure: these possibilities have still to be investigated. Increases in the oxygen tension of the bathing blood also lead to increases in the resting tone of the isolated organs (Smith & Vane, unpublished).

There are several limitations to the use of this method:

(i) It only detects *changes* in the concentrations of circulating hormones around those which may be present at the start of the experiment.

(ii) If a mixture of substances is injected or liberated, the individual components can only be distinguished by using more than one monitoring organ preparation. With a combination of chick rectum and rat stomach strip preparations it is possible

to detect and to estimate the amounts of adrenaline and noradrenaline in a mixture. Similarly, if two or three blood-bathed organs are chosen with care it is possible to identify a single circulating hormone with reasonable certainty. For instance, a substance which contracts guinea-pig ileum but has no effect on the rat stomach or duodenum preparation is most likely to be histamine. A substance which relaxes rat stomach and duodenum and has no effect on the guinea-pig ileum preparation is likely to be a catechol amine; and a substance which relaxes rat duodenum but contracts or has no effect on the guinea-pig ileum and rat stomach strip preparations is likely to be bradykinin. The time courses of the responses are also instructive; thus, with an injection of histamine there is first a contraction of the guinea-pig ileum; 20 to 30 sec later the stomach strip begins to relax due to a liberation of catechol amines from the adrenal glands.

The method has been used to detect increases in concentration of substances in the blood stream after several well-known procedures; the release of catechol amines by nicotine and histamine; the release of histamine by histamine-liberators, and the release of bradykinin by trypsin, and by contact of blood with glass. The method has also been used to demonstrate that tyramine-like substances do not liberate catechol amines into the general circulation (Vane, 1960); to measure the release of catechol amines from the adrenal gland (Marley, 1960, 1961; Marley & Paton, 1961; Marley & Prout, unpublished); and to estimate the amount of oxytocin released by intracarotid arterial injections of hypertonic saline (Saxby, Siddiqi & Walker, 1960). In its present form, the technique has been applied to cats, dogs and rabbits, mainly because these animals can easily supply the 10 to 15 ml. of blood needed to fill the external circuit. By reducing the capacity of the external circuit, or by priming it with blood from a second animal, it should be possible to use the method with smaller animals.

The applications of the method so far discussed involve the use of isolated organs to detect circulating hormones. The technique can also be used to study the reactions of isolated organs when they are bathed in blood instead of in an artificial salt solution. For instance, phenethylamine contracted the rat stomach strip preparation when it was bathed in Krebs solution (an action on tryptamine receptors: Vane, 1960) but relaxed the strip when it was bathed in blood. This is evidence for a *local* release of noradrenaline by phenethylamine-like substances. Apparently, this local release does not take place in an artificial salt solution, either because the stores of noradrenaline have been depleted or because they have, in some way, become unavailable.

I would like to thank the technical staff for their assistance, and Mr. O. B. Saxby, of the University Department of Pharmacology, Oxford, for designing and supplying the roller pumps.

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