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B. THE ESTIMATION OF CATECHOLAMINES BY BIOLOGICAL ASSAY

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Of all the aspects of research into catecholamines the field of bioassay has, perhaps, changed less than any other since the subject was reviewed in 1959 by the late Sir John Gaddum (9). He suggested then that, in comparison to chemical methods, "there is little doubt that as time goes on biological methods of assay will be less used, but they are still important and chemical methods will only inspire universal confidence if they are shown to give the same results as the biological methods." This prediction has been well substantiated in the last 7 years. Chemical methods have indeed been more generally used; both specificity and sensitivity have been increased and new methods to assay labelled catecholamines separate from their metabolic products have been developed, as have improved histochemical techniques. Perhaps as a consequence of these changes, few new techniques of bioassay for catecholamines have been developed (see (4)). Alternatively, it might be that present methods of bioassay are so satisfactory that there is no impetus to develop new ones. An example of the use of well established methods being used to solve new problems is given by Feldberg and Lewis (6, 7). They demonstrated that in the cat, both angiotensin and bradykinin released catecholamines when given as close arterial injections (8) to the adrenal glands. They used the denervated nictitating membrane to detect the release of catecholamines, a technique first devised over 30 years ago (5).

SENSITIVITY AND SPECIFICITY OF ASSAY METHODS

There are two main requirements for an assay: first, that it should be sensitive, and secondly that it should be specific. In most bioassay techniques the sensitivity cannot be questioned. For instance, Booker (2) suggested using the isolated perfused guinea pig heart as a biologic assay method for norepinephrine (NE) and epinephrine (E), and this preparation is sensitive to less than 1 ng. He says, however, that the method is "highly qualitative but only semi-quantitative." In a later paper Booker *et al.* (3) used the guinea pig heart to estimate NE and E in plasma. Little information was given on the specificity of the isolated heart. Indeed, Watson and Booker (31) described the same preparation as an assay for angiotensin, sensitive to 2 to 20 ng. Valette and Nguyn-ba-Muoi (26) have used segments of rat aorta for the assay of E. Here again the tissue is not specific for catecholamines, for vascular smooth muscle will react to many other amines and polypeptides.

EFFECTS OF INTERFERING SUBSTANCES

When extracts contain substances other than the catecholamines the contamination may affect the assay in several ways. The substances may them-

selves have a catecholamine-like action on the assay tissue, or they may potentiate the effects of catecholamines without themselves having such a direct action. Conversely, they may produce an effect opposite to that of the catecholamines, thereby diminishing their effect, or they may reduce it without giving an overt response.

Two examples of such interactions may be cited, one seen with intestinal and the other with vascular muscle. The rat stomach strip preparation (27) was developed as a bioassay for 5-hydroxytryptamine (5-HT), and at the time it was noted that trace amounts of catecholamines reduced the contraction caused by 5-HT. To cause an overt relaxation of the preparation, however, much larger doses (between 50 to 500 ng in a 5 ml bath) were required. Armitage and Vane (1) made use of these observations and added 5-HT (10 μg/1) to the bathing fluid, so that the rat stomach strip maintained a much higher than normal tone. It was then sensitive to 1 to 10 ng E. If the tissue was contracted to the same degree by acetylcholine or by amphetamine, the sensitivity to E increased but not as much as when the tissue was contracted with 5-HT. Therefore, the increase in sensitivity was associated with two factors. First, a contracted tissue showed relaxation better and secondly, the relaxation induced by E was potentiated by the presence of 5-HT. An incidental advantage of contracting the rat stomach with 5-HT was that contractor substances, especially 5-HT, present in an extract being assayed for catecholamines, interfered less with the assay, since the preparation was already almost maximally contracted.

Another example of interaction between the effects of E and 5-HT has been described for the central artery of the rabbit ear. This has been developed as an isolated preparation, sensitive enough to catecholamines for use as a bioassay (14). The artery was removed from the ear, cannulated and perfused with Krebs or McEwans solution. A roller pump supplied the perfusion fluid at a constant rate of 8 ml per min and the pressure generated (30 to 40 mm) was recorded with a mercury manometer. The fluid which had perfused the preparation was collected in a bath at 37°C, in which the artery also was suspended. Injections of NE or E caused a rise in perfusion pressure, graded according to the dose. The threshold to NE was 5 to 50 ng. 5-Hydroxytryptamine had little vasoconstrictor effect but its presence potentiated the actions of catecholamines so that the preparation became sensitive to 1 to 5 ng of NE. Presumably, with 5-HT in the perfusion fluid, this preparation also became relatively insensitive to traces of 5-HT contaminating the extracts.

DISCRIMINATION BETWEEN CATECHOLAMINES

In both of these examples, 5-HT was used to make the preparation more sensitive to catecholamines and at the same time more specific for them. However, neither the rat stomach strip nor the rabbit ear artery discriminates well between E and NE. To add discrimination to their method, Armitage and Vane (1) superfused the rat stomach strip in series with a chick rectum (15), which is about 100 times more sensitive to E than to NE. By comparing the reactions of the two tissues to the unknown mixture with the reactions to E and NE, the

proportions of each in the mixture can be calculated, either by solving simultaneous equations or by the use of isobols. Since the isolated central artery of the rabbit's ear has a sensitivity to catecholamines in the same range as the rat stomach strip and chick rectum, it should be possible to use all three preparations simultaneously, bathed in the same stream of Krebs solution containing 5-HT. If this were done it should theoretically be possible to determine the proportions of E, NE, and isoprenaline in a mixture of the three amines.

One of the best known preparations for discriminating between E and NE is the isolated uterus from a rat pretreated with stilboestrol (10). The uterus contracts in response to carbachol or 5-HT, and E selectively inhibits the contraction to a degree proportional to the concentration. Instead of causing contractions with carbachol, Harvey and Pennefather (11, 12) stimulated the uterus electrically for 10 to 20 sec with 4 to 8 v 50 c per sec AC; the resulting contraction was inhibited by E in concentrations as low as 0.1 pg per ml. This represented a 20-fold increase of sensitivity over the carbachol-contracted uterus. NE was 10,000 to 1,000,000 times less potent than E. Another modification of an older method has been to use a transducer manometer to record the blood pressure of the pithed rat. Although the sensitivity of the method is not increased Rand (19) has told me that it is easier to distinguish between the pressor effects of E, NF, and 5-HT.

ANTAGONISTS

The specificity of bioassays can also be increased by the use of antagonists. The effects of some of the possible interfering substances can be eliminated by the presence of antagonists to acetylcholine, histamine, and 5-HT; at the end of the assay, confirmation of catecholamine effects can be obtained by block with α- and β-receptor antagonists. Now that highly potent and specific β-receptor antagonists are available, it might be worth investigating whether the discrimination between the effects of E and NE in some of the conventional catecholamine assays can be increased by the presence of β-receptor antagonists. It should also be worthwhile investigating further the use of potentiating substances, such as cocaine or desmethylimipramine; assays may well be increased severalfold in sensitivity.

EXTRACTION AND PROCESSING

When assays lack specificity, the extracts to be assayed have to be processed very carefully in order to reduce the amounts of interfering substances that might be present. The following steps are usually necessary: 1) taking of samples of blood or tissue in sufficient quantity to permit estimation of the catecholamines after processing; 2) their extraction and purification to remove interfering substances; 3) concentration of the extract and, if necessary, separation of E and NE, followed by further adjustment of the volume; and 4) chemical or biologic assay using E and NE as standards of comparison. These procedures have several disadvantages, as follows: 1) They are time-consuming. 2) Losses of E and NE are inevitable. 3) Chemicals affecting a subsequent bioassay may

be introduced by the very process designed to eliminate them. Strips of paper chromatograms contained depressor substances which antagonized the actions of E and NE on the blood pressure of pithed rats (23). This depressor action on the blood pressure, which resembled that of isoprenaline, was probably due both to insufficient removal of the phenolic solvent from the paper and to the use of sodium dihydrogen phosphate in the eluting fluid (23). In an addendum to this paper Dr. M. Vogt recapitulated some of the pitfalls to be avoided when chromatographing catecholamines in phenol-HCl for subsequent bioassay. 4) Finally, a further disadvantage of some extraction procedures, especially when coupled with a relatively insensitive assay, is that they may require blood or tissue samples of such a size as to upset the homeostasis of the animal. The normal response of the body to hemorrhage includes the liberation of catecholamines into the blood stream; therefore, the process of taking too many or too large samples of blood for assay will by itself increase the amount of catecholamine in the samples.

BLOOD-BATHED ORGAN TECHNIQUE

Most of these disadvantages would be eliminated if blood could be removed from an animal, assayed without any processing, and immediately returned to it. Such a technique depending on the continuous superfusion of isolated organs with blood from an anaesthetized animal is now available (28, 30). With this "blood-bathed organ technique" the circulating blood of the animal is continuously monitored for changes in hormone level. For detection of E and NE, the most suitable organs are the rat stomach strip and the chick rectum. Hormones other than catecholamines can also be estimated, for example, with the rat duodenum (13) to detect bradykinin and the rat colon (20) to detect angiotensin.

For the assay of catecholamines, the isolated organs are more reliable, more sensitive, and longer-lasting if the blood superfusing them is arterial rather than venous. Carotid blood is pumped by a roller pump at a rate of 12 to 15 ml per min over a rat stomach strip, a chick rectum and, if necessary, a third tissue in series. The blood then collects in a reservoir from which it is returned to a jugular vein either by gravity or through a second channel of the pump (fig. 1). The tissues can be calibrated as follows: (a) By injections or infusions of catecholamines directly into the blood bathing the organs; this gives an estimate of their absolute sensitivity. Good responses are usually obtained with concentrations of less than 1 ng per ml. (b) By injections or infusions intravenously into the animal. In this way only a small proportion of the injection reaches the assay organs but the method allows a direct comparison to be made with the release, also intravenously, of catecholamines from the adrenal glands. To increase the sensitivity of the organs to catecholamines released by the adrenal medulla, Marley (16) took adrenal venous blood withdrawn (retrogradely) from the adrenolumbar vein and allowed it to mix with a much larger volume of carotid blood before it reached the assay tissues. This made it possible to detect the release of E in response to as little as two shocks to the sympathetic nerves of the medulla (17, 18).

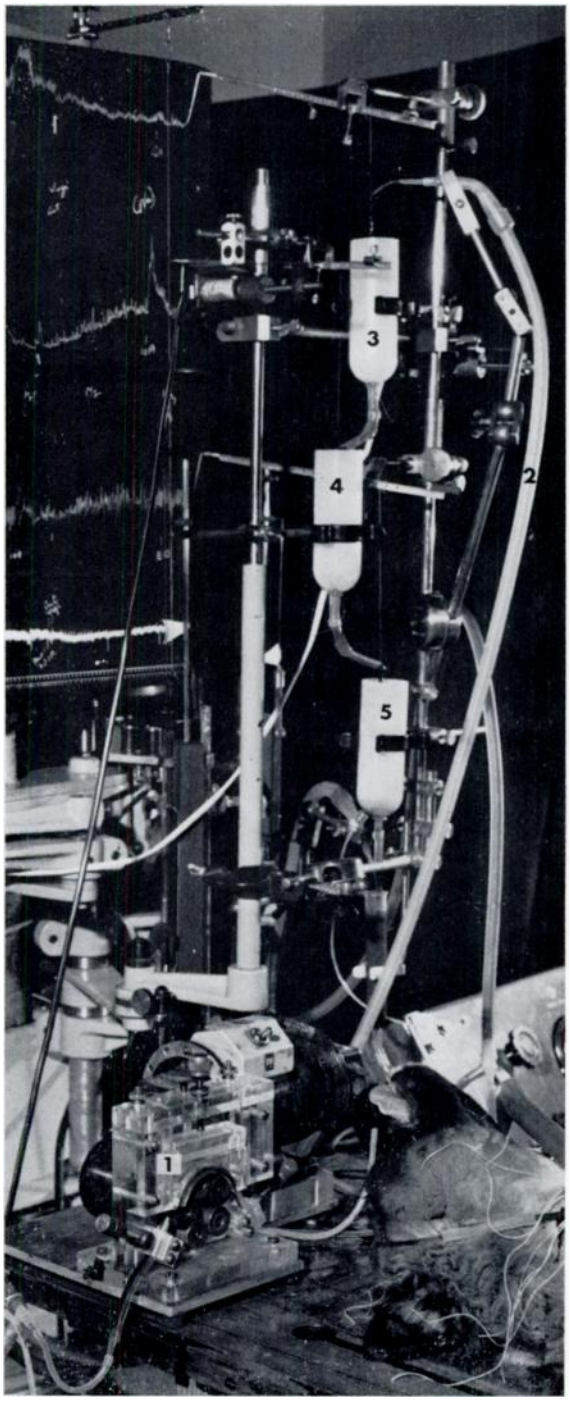

FIG. 1. Photograph of blood-bathed organ equipment. Blood from a carotid artery is pumped by the roller pump (1) up a water jacketed tube (2). It then cascades over three isolated organs contained in plastic jackets (3, 4 and 5), collects in a reservoir and is returned to the animal.

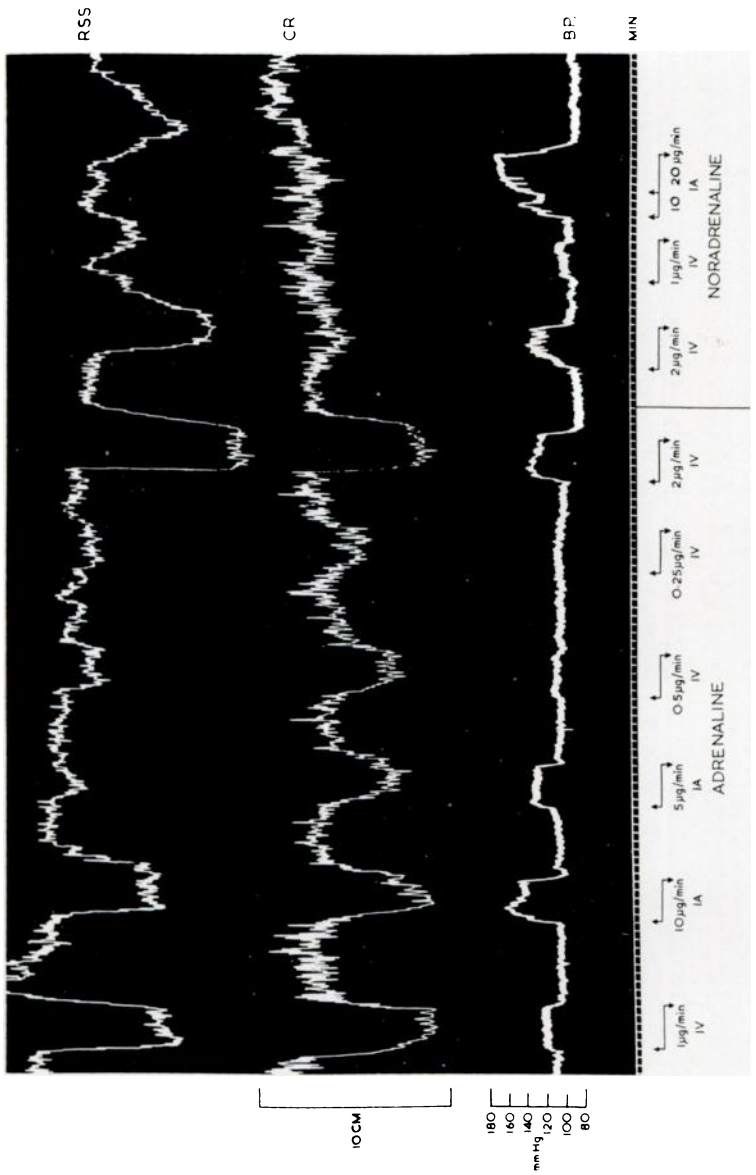

FIG. 2. Part of an experiment with a 3.4 kg chloralose cat. From top to bottom, movements of a rat stomach strip (RSS), a chick rectum (CR) and the blood pressure (BP). Time in minutes. The tracing shows responses to infusions of adrenaline intravenously (1 µg, 0.5 µg, 0.25 µg, and 2 µg per min) compared with intra-aortic infusions of 10 µg and 5 µg per min. The difference in effects between intravenous and intra-aortic infusions represents the amount of adrenaline which disappears in one transit through the lower parts of the cat. Thus, for the aortic infusions, 9 µg of the 10 µg per min and 4.5 µg of the 5 µg per min disappear. The last part of the tracing shows that more than 18 µg out of 20 µg of noradrenaline per min disappear in one circulation.

The tracing also illustrates the differential sensitivity of the chick rectum to adrenaline and noradrenaline.

DISAPPEARANCE OF CATECHOLAMINES FROM THE CIRCULATION

Calibration of the assay tissues by intravenous injections or infusions can also be used to estimate the disappearance of intra-arterial injections of catecholamines in a particular vascular bed. Figure 2 for example, is from an experiment in which E and NE were given as infusions to a cat. In the first part of the tracing, the effects of intravenous infusions of E are compared with the effects of infusions given intra-arterially through a fine polyethylene catheter inserted into a femoral artery and pushed up the aorta so that its tip was just below the renal arteries. An intra-aortic infusion of 5 μg of E per min almost completely disappeared in one circulation and gave a carotid blood concentration similar to that produced by 0.5 μg per min i.v. Similarly, an intra-aortic infusion of 10 μg of E per min gave a carotid blood concentration equivalent to that produced by 1 μg per min i.v. Thus, 90% of the aortic infusion of E disappeared before reaching the venous circulation. The last part of the tracing shows a similar experiment with NE infusions. During the infusion more than 18 out of the 20 μg of NE infused per min into the aorta disappeared in transit from the arterial to venous circulation. We are at present using the technique to measure the disappearance of infused E, NE, and angiotensin before and after various antagonists.

If the isolated organs could be suspended in venous blood the sensitivity and scope of the method would be considerably increased. In an attempt to achieve this, Dr. R. L. Hodge and I are using a small oxygenator to oxygenate venous blood before it reaches the assay tissues. We are also trying to apply the blood-bathed organ technique to man. With a slower rate of blood flow over the organs and, of course, without returning the blood to the donor, we hope to be able to estimate continuously the release of catecholamines in man, in response either to drugs or to the slow haemorrhage that the method involves.

SUMMARY

The blood-bathed organ technique has made it possible to estimate the release, distribution, and uptake of catecholamines directly and continuously without extracting and purifying them from samples of blood. The technique has been used to measure the release of catecholamines and of angiotensin during haemorrhage (21); the release of catecholamines induced by peptides (24), histamine (25), tyramine (29), and nerve stimulation (17, 18). The technique has the advantage that the results are immediate and continuous, so that they can be used to direct the further course of an experiment. Furthermore, it has the advantage, common to other bioassay procedures, of being relatively inexpensive.

It is difficult to foresee what place bioassay procedures will have in catecholamine research in 5 to 10 years. Certainly, the fact that they are relatively inexpensive will continue to make them attractive to some laboratories, just as the increasing number of pharmacologists primarily trained in biochemistry will encourage the use of chemical methods in others. These, however, are not the only factors influencing the choice of biological *vs.* chemical method of assay. With many people, psychological factors also are important. Some find a bio-

logical assay is more pleasing aesthetically. Furthermore, biological methods by their very nature, constantly remind the investigator of the subtleties and intricacies of living organisms, whereas chemical methods may well allow him to forget them.

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