BIOASSAY PROCEDURES

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In 1856 Vulpian (35) found that watery extracts of the adrenal glands of many different species of animal, unlike extracts of other glands, contained a substance which gave a remarkable rose-carmine colour with iodine, alkali, sunlight and various other reagents. Acid inhibited this reaction but did not destroy the original substance; extracts of cortex inhibited it. Vulpian suggested that this substance was liberated into the blood during life and obtained evidence of its presence in the blood in the adrenal veins after death. We now know that these effects must have been largely due to adrenaline, but the importance of this work was not realized until the pharmacological properties of the extracts were discovered 40 years later. This soon led to the introduction of biological methods of estimating quantitatively the amount of adrenaline in a solution, and most of our knowledge of the physiological properties of adrenaline and noradrenaline still depends on these pharmacological methods.

I have been much impressed with the value of these pharmacological methods, but I have not been prejudiced against other methods. One of the most used colorimetric methods was developed by Shaw (32) in my laboratory, and Schild and I (18) described a fluorimetric method in 1934, and discovered that noradrenaline was less readily converted into a fluorescent compound than adrenaline. New chemical methods have been described in recent years which are a great improvement on older methods, and 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.

Bioassay methods

1. *Blood pressure.* One of the first enthusiasts for these techniques was Elliott (10) who worked out an accurate method dependent on the rise of blood pressure which adrenaline causes in cats. He destroyed the spinal cord down to the fourth cervical segment, in order to avoid vasomotor reflexes, and injected a series of doses of synthetic adrenaline, turning the kymograph back so that the curves were superimposed. He injected a constant volume of drug, washed in with a constant volume of saline from a burette at constant speed, which was timed with a metronome. He then injected his unknown solutions and was not satisfied if the resulting curves were not the correct slope throughout their whole course. He calculated the exact results by interpolation. If all these things are done, he said, "the circulation then will respond with the accuracy of a chemical balance to any dose of adrenaline." He expected to test about 8 or 10 extracts in each experiment.

Most of the details of this technique are still important today; the main difference is that nowadays we generally give standard and unknown solutions al-

ternately and depend on a comparison between neighbouring effects. A tracing given by Burn (4) shows that it is quite easy by this method to distinguish doses differing by 10%. This method has been used to control adrenal extracts but is not sensitive enough for most purposes.

When a similar technique is applied to rats it provides a sensitive method of estimating both adrenaline and noradrenaline (21) and has been much used.

2. *Rat uterus.* This tissue provides one of the most sensitive and specific tests for adrenaline. De Jalon *et al.* (22) described a test for adrenaline in which the spontaneous movements were abolished by suspending the organ in a special solution, and adrenaline was assayed by its effect in diminishing the response to a small dose of acetylcholine.

This was the basis of the method developed by Gaddum, Peart and Vogt (17) and Gaddum and Lembeck (16). The organ is suspended in a small bath, the contents of which are changed automatically at regular intervals by solutions which run in from below and overflow. In this way, carbachol (10^{-6}, w/v) is applied and allowed to act for about 30 seconds and then washed out; this process is repeated every 2 minutes and a regular series of contractions of the uterus is thus produced. If adrenaline is applied 1 minute before carbachol the response of the uterus is reduced and this reduction gives a measure of the dose of adrenaline. In favourable conditions less than 0.1 ng[1] (0.0001 μg) of adrenaline can be detected in this way. The sensitivity of insensitive uteri can be increased by adding drugs such as phenoxybenzamine (Dibenzyline) to the bath (20).

In the simplest form of the test, standard and unknown solutions are given alternately and the doses adjusted until they produce equal effects. When more accurate results are required, two doses of each preparation are given in random order and the experiment is repeated several times. The responses are then measured and the results interpreted by a formula which has the same effect as fitting two parallel log-dose-response lines to the average results. In an experiment lasting about 2 hours it is generally possible in this way to repeat each dose 4 times and to obtain a result with a standard deviation of less than 10% (16).

In order to have effects like those of adrenaline, noradrenaline must be given in about 100 to 200 times the dose. This ratio of activities is higher than in any other test.

3. *Blood vessels of the rabbit ear.* It has long been known that the blood vessels in the rabbit ear are particularly sensitive to vasoconstrictor drugs. Schlossmann (30) studied various methods of estimating adrenaline and recommended the use of the rabbit ear perfused at constant pressure with a solution containing serum and citrate. The solutions to be tested were perfused for a few minutes from a Mariotte bottle containing 40 ml and controlled by a three-way tap; the rate of flow of drops was recorded. This preparation was clearly very sensitive and has never been adequately exploited. In over two-thirds of the experiments it responded to a concentration of 10^{-11}, w/v, adrenaline, and sometimes to much

[1] 1 ng (nanogram) = 0.001 μg.

less. Blood was tested directly after dilution so that its concentration was the same as that of the serum in the perfusion fluid. This precaution seems to have eliminated complications due to other vasomotor substances in the blood. It is now known that the most important of these substances is 5-hydroxytryptamine and it might be thought that the success of the experiments depended on the serum having the same concentration of this substance as the samples of blood tested. This does not appear to be so, because Schlossmann found that blood from the carotid artery of a lightly anaesthetized cat or rabbit had comparatively little action, and when the preparation was sensitive he was able to say that the concentration of adrenaline in the blood was certainly less than 10^{-12}, w/v. When the adrenals were stimulated by nicotine or asphyxia the adrenaline content of the carotid blood rose, but never exceeded 2×10^{-8}, w/v.

In recent years, more convenient methods of recording the effects have been devised, but the preparations have generally been less sensitive than Schlossmann's, and it might be worth following his technique more closely; however, Schlossmann only made very approximate estimates of concentrations.

One convenient way of recording the outflow is to use a drop timer, which records the interval between successive drops as a series of vertical lines on a drum. Savini (29) using the solution of Page and Green (25) obtained good effects with about 1 ng of adrenaline or 2 ng of noradrenaline dissolved in 0.1 ml of solution. These effects can be repeated at intervals of 10 min so that it is possible to make quantitative assays. This is one of the most sensitive tests for noradrenaline.

Armin and Grant (1) have also described a very sensitive test depending on the blood vessels in the rabbit ear. Injections are made through the peripheral end of the artery in a conscious, but tranquil, rabbit and the diameter of the artery is observed microscopically. This method will detect a concentration of 10^{-11}, w/v, of adrenaline and uses only 0.25 ml of solution.

4. *Intestinal muscle.* The inhibitory action of adrenaline on the intestine has been the basis of many assays. The rabbit intestine was much used by Stewart and Rogoff (33) and by Satake (28). Burn *et al.* (5) give a record of an experiment by this method.

The hen rectal caecum (2) is inhibited by low concentrations of adrenaline (10^{-9}, w/v) and has been used because it is much less sensitive to noradrenaline (11).

The rat colon can be used as the rat uterus and stimulated with carbachol. It provides a sensitive test for noradrenaline (17).

5. *Other tissues.* A number of other tissues have been used to detect adrenaline. The isolated frog heart was used by Loewi (24) in his first experiments on the release of substances by adrenergic nerves. It is sensitive, but responds to too many other substances (17). An accurate method of assay using the perfused heart of a frog was described by West (36).

The cat nictitating membrane, the uterus, the heart and various other tissues have been used to record the release of adrenaline and noradrenaline with the circulation intact (8). Tissues may be sensitized by preliminary aseptic section

TABLE 1

Sensitivity of biological tests

Species	System	Adrenaline	Noradrenaline
		ng*	ng*
Cat	Blood pressure	200	100
Rat	Blood pressure	2	2
	Uterus	0.1	15
Rabbit	Perfused ear	0.5	1
	Ear†	0.002	
	Intestine	40	40
Fowl	Rectal caecum	2	50

* Amount (ng = nanogram) for each test. Accurate assays require 5 to 10 times these amounts.

† See Reference 1 (Armin and Grant).

of the nerve supply or by the injection of cocaine. Solutions may be assayed by injecting them intraarterially (17, 27). The difference in sensitivity of biological tests to adrenaline and noradrenaline is illustrated in Table 1.

Specificity

It is sometimes easy to decide that the active substance in the unknown extract is different from the standard substance because the time relations of the effects are different or because the dose-effect curves are different. Tests are generally applied for such things as stability; the unknown substance should be as rapidly inactivated as adrenaline in alkaline solutions.

Some of the effects of adrenaline can be antagonized by certain drugs and appropriate tests should be applied to the unknown solution. Tests with antagonists are, however, not very specific. The best known antagonists, such as the ergot alkaloids and phenoxybenzamine, antagonize most of the sympathomimetic amines, but it has been realized in the last few years that they are also even more powerful antagonists of 5-hydroxytryptamine than of adrenaline. Hermann *et al.* (19) recommend the use of phentolamine (Regitine) or F883 (diethylaminoethylbenzodioxane) which, like other similar drugs, reduce the action of noradrenaline but actually reverse that of adrenaline on the dog blood pressure.

Some of the effects of adrenaline are increased by cocaine and this drug may be used either to increase the sensitivity or as a test of identity.

Criteria such as these may show that two substances are different but cannot prove that they are the same. There are, however, two important tests which provide more specific evidence and yet can be applied when the amount of adrenaline available is too small to be detected by chemical methods. These are the methods of paper chromatography and of parallel assays.

The rate of flow of very small amounts of active substances in paper chromatography can be determined by eluting them from the paper and then testing them biologically. The rate of flow provides evidence which helps to identify the active substance, particularly if the experiment is repeated with several different

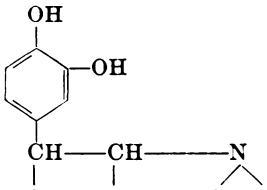
solvent systems chosen for their ability to separate catecholamines. The rates of flow of known catecholamines are determined simultaneously, preferably by adding them to a sample of the extract, since impurities in the extract may affect the result. In practice, however, there is often more than one active substance and paper chromatography is used to separate them before testing. This technique will be discussed later. When chromatography is used to identify substances the evidence may be misleading if the paper is divided into strips on the assumption, for example, that only adrenaline and noradrenaline are present. Dopamine may lie between them and be confused with one or the other unless the number of samples tested is sufficient to define the position of the activity on the paper independently of the position of the control spots.

The strongest evidence of identification depends on parallel pharmacological assays. If an unknown solution contains noradrenaline it is almost always possible to find a dose of adrenaline which produces just the same effect in any single test, but if another test is used the equivalent dose of adrenaline may be 10 or 100 times smaller. The value of any two tests for distinguishing between adrenaline and noradrenaline depends on the ratio of the results when these two substances are compared by the two tests. This ratio has been called the index of discrimination (15). For example, adrenaline may be 150 times as active as noradrenaline when tested on the rat uterus, but only twice as active on the perfused rabbit ear. In this case, the index of discrimination for adrenaline against noradrenaline on the rat uterus over the rabbit ear would be 150/2, or 75. It would be quite easy to distinguish the two substances by using these two tests.

By an appropriate choice of tests it is possible to distinguish closely allied sympathomimetic amines from one another. This is illustrated in Table 2, which shows estimates of indices of discrimination for various amines against noradrenaline on two different pairs of tissues, calculated from the results given by Gaddum, Peart and Vogt (17). For adrenaline the index in the first column is

TABLE 2

Indices of discrimination



				Activity of Amines/Noradrenaline on	
				Rat uterus / Rat colon	Rat uterus / Rabbit ear
OH	H	H	H	1	1
OH	H	H	CH_3	300	75
OH	H	H	C_2H_5	300	900
OH	H	H	$CH(CH_3)$	300	10^6
OH	CH_3	H	H	150	15000
OH	C_2H_5	H	H	3	30
OH	H	CH_3	CH_3	20	4
H	H	H	H	10000	75
H	H	H	CH_3	250	12

300 because adrenaline is about 300 times as active, compared with noradrenaline, on the uterus as it is on the colon. In practice, of course, this figure would vary, but it is always large. There is never any difficulty in distinguishing between adrenaline and noradrenaline by testing them on these two tissues. The same applies to most of the other substances in the table although in two cases the index was only 3. It might be difficult to distinguish these amines from noradrenaline using these two tissues, but if an additional test were done using the rabbit ear there would be no difficulty.

It will be seen that there are two other substances besides adrenaline for which the index for rat uterus over rat colon was 300. These differ from adrenaline only in the fact that they have different alkyl groups attached to the nitrogen in the side chain—one of them is isopropylnoradrenaline. It would be easy to distinguish these substances from noradrenaline, but the fact that the index was 300 shows that it would be difficult to distinguish them from adrenaline. Here again an experiment on the perfused rabbit ear would provide clear evidence identifying the active substance since the three indices of discrimination were 75, 900 and one million. The last figure represents isopropylnoradrenaline, which is very active on the uterus and does not constrict the vessels in the rabbit ear except in enormous doses. Its main effect on blood vessels is, of course, dilator, but under the conditions of these experiments the vessels were already dilated and the only effect observed was vasoconstriction after a very large dose. It will be seen that the actual doses used in these tests have not been mentioned; the evidence depends entirely on a comparison of ratios.

This method of identifying catecholamines by parallel tests is by no means new. It was used by Cannon and Rosenblueth (7, 8) to show that the substance liberated by the hepatic nerves in a cat is not adrenaline; it was used by von Euler (11) to show that the main active substance in extracts of adrenergic nerves is *l*-noradrenaline; and it was used by Peart (26) to show that the main substance liberated when adrenergic nerves are stimulated is noradrenaline.

The methods discussed so far give satisfactory results with solutions which are pharmacologically pure, but not when more than one active substance is present. If it is safe to assume that the unknown solution contains no active substances except adrenaline and noradrenaline it is sometimes possible to obtain an approximate estimate of the concentration of each substance by using one of them as a standard in each of two tests, and by measuring the ratio of equivalent doses of noradrenaline and adrenaline in each test. The result is calculated on the assumption that the effects of the two drugs are additive. This method of calculation depends on the use of one preparation which is much more sensitive to adrenaline than to noradrenaline and another preparation which should if possible be especially sensitive to noradrenaline, although no tissue is known which is much more sensitive to noradrenaline than to adrenaline. Bülbring (3) used the rat uterus and the cat blood pressure; Gaddum and Lembeck (16) used the rat uterus and the rat colon; von Euler (13) used the fowl rectal caecum and the cat blood pressure. Table 3 shows an example given by von Euler (14).

In this case the percentage of adrenaline was 1.25 and its estimation depended on the difference between 2.4 and 1.6. It is possible to make such estimates

TABLE 3

Method of identifying catecholamines by parallel tests

Concentrations	Adrenaline (x)	Noradrenaline (y)
	Fowl rectal caecum	Cat blood pressure
Dose ratio (Nora/Ad)	Q (40)	q (0.27)
Noradrenaline equivalents	A (2.4)	a (1.6)

$$A = y + xQ$$
$$a = y + xq$$

$$A - a = x(Q - q)$$
$$x = \frac{A - a}{Q - q} = \frac{2.4 - 1.6}{40 - 0.27} = 0.0202$$
$$y = A - xQ = 2.4 - 0.0202 \times 40 = 1.59$$

Calculated results, assuming total catecholamines 1.6

Noradrenaline	Adrenaline equivalents	
%	Rectal caecum	Cat B.P.
1.25	1.58	1.65
5	1.52	1.82
10	1.44	1.99
20	1.28	2.47

fairly accurately and the results are therefore fairly reliable. When the percentage of adrenaline is high the method is less reliable. In order to get evidence about a small proportion of noradrenaline mixed with a large amount of adrenaline it is necessary to estimate the adrenaline equivalents, by both tests. The lowest part of Table 3 shows calculations based on the data of the upper part, assuming the same total content of amines and the same tests, but with an excess of adrenaline. The results would depend on the difference between the two adrenaline equivalents shown in the table. It would clearly be impossible to detect less than about 5% of noradrenaline and difficult to measure less than 10%. These calculations are based on a pair of tests which are particularly suitable for the estimation of small amounts of noradrenaline, since the value of q was 0.27, which means that the cat blood pressure was about 4 times more sensitive to noradrenaline than to adrenaline. Most of the other tests would be even less suitable for this purpose. This method of determination has been widely used because of its convenience, but it is not very accurate.

Burn *et al.* (6) have described a method in which the effects on the blood pressure and the nictitating membrane of a spinal cat are simultaneously recorded. Their calculations are based on the observed fact that the ratio of the heights of these two effects was linearly related to the percentage of adrenaline in a mixture of the two main catecholamines.

The final identification of active substances by pharmacological methods depends on obtaining a solution which is pharmacologically pure, and this means that there is only one active substance present and no substances which alter the sensitivity of the tissue. The chemist might object to calling a preparation

pure when it may contain 99% of inert matter; on the other hand, the pharmacologist can sometimes detect small amounts of histamine in preparations which the chemist considers pure.

In collecting blood for bioassay, precautions are taken to avoid the unnecessary release of 5-hydroxytryptamine from platelets (17).

Catecholamines can be concentrated by absorption on $Al(OH)_3$ (32) and then eluted for bioassay (12), but the best method of purifying them involves separation on a paper chromatogram.

This technique was first applied to catecholamines by James (23) who used phenol and water and detected the drugs as coloured spots after treatment with potassium ferricyanide. The R_f for adrenaline is about 0.5 and that for noradrenaline about 0.2; dopamine and histamine lie between them. If butanol and dilute HCl are used, dopamine runs faster than either adrenaline or noradrenaline (31). The smallest amount of these drugs that can be detected as coloured spots on the paper is about 1 μg; but smaller quantities can be estimated after elution. If suitable precautions are taken to avoid oxidation it is possible to extract the catecholamines quantitatively from tissues with acid alcohol and to apply the extract to paper. After chromatographic separation the amines can be eluted and estimated pharmacologically. The position of the catecholamine on the paper is determined by a simultaneous experiment with a larger amount of a standard preparation. With this method it is possible to estimate about 1 nanogram of adrenaline or a rather larger amount of noradrenaline in a mixture. The two amines can be clearly separated so that the presence of one does not affect the estimate of the other (9, 34). If doubt remains about the identity of the substances present, parallel pharmacological assays can be made on the eluted material. Schümann (31) used this method to identify dopamine in extracts of adrenergic nerves. This substance causes a rise of blood pressure in rats and a fall in guinea pigs. The material eluted from the appropriate part of the paper had the same two effects.

If properly carried out, pharmacological methods of estimation are very sensitive and very specific. Their main disadvantages are that they are not very accurate and comparatively slow; it is generally not possible to make more than about 10 estimates in one day. Another disadvantage is that there are fewer pharmacologists in the world than chemists so that it is more difficult to find some one else who will do the tests for you.

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DISCUSSION

POINTS TO BE CONSIDERED IN RUNNING CHROMATOGRAMS OF TISSUE EXTRACTS

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Figure 1 illustrates some of the practical points which must be considered when chromatograms of tissue extracts are run in phenol-HCl.