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ASSAY OF SUBSTANCE P ON THE FOWL RECTAL CAECUM

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The optimum conditions for the assay of substance P on fowl rectal caecum have been studied. Effective concentrations vary from 0.01 to 0.5 u./ml. ; it is remarkably insensitive to other polypeptides, such as bradykinin. The test can be made more specific by using a bath fluid containing antagonists for known interfering substances. A suitable antagonist for acetylcholine is atropine or hyoscine, for 5-hydroxytryptamine, methysergide and for catecholamines, ephedrine. The effects of histamine and adenosine compounds can be abolished by specific tachyphylaxis, in which the bath fluid contains an excess of the active substance itself. The assay is reasonably accurate. Woolf's index of precision (L) was estimated as 15.5 with a range (9) of 9.2 to 29.4. Simplified methods of calculating L, and the fiducial range, are described.

The effects of drugs on the muscle of the fowl rectal caecum were first studied by Barsoum & Gaddum (1935). This tissue has been used by Euler (1948) to discriminate between catecholamines, since it is about 40 times more sensitive to adrenaline than to noradrenaline. It is sensitive to the active polypeptide known as substance P, but remarkably insensitive to bradykinin and other kinins (Pernow & Rocha e Silva, 1955 ; Gomes, 1955). It can therefore be used to detect and estimate small amounts of substance P in the presence of these other polypeptides, but it is sensitive to many pharmacologically active substances which may be present in tissue extracts.

The effects of these other substances can sometimes be excluded by using suitable methods of extraction. Amin, Crawford & Gaddum (1954) used 95% acetone to extract acetylcholine, catecholamines, 5-hydroxytryptamine and histamine, leaving substance P in the insoluble residue, together with adenosine compounds and possibly other substances. It is, however, difficult to be sure that such methods will always be effective. Amin *et al.* concluded that two extractions with acetone were sufficient, since no 5-hydroxytryptamine was detected in the third extract. We have made similar extracts and compared the acetone-insoluble fraction with substance P, using the guinea-pig ileum ; when the experiment was repeated after the ileum had been desensitized to 5-hydroxytryptamine by adding tryptamine to the bath, the substance P equivalent of the extract was decreased. This result suggests that some of the 5-hydroxytryptamine had not been removed by the acetone, either because it was present in a combined form or for some other reason. Other evidence of this is described below.

An alternative method of eliminating the effects of other pharmacologically active substances is to add suitable antagonists to the bath fluid, so as to make the test more specific. The experiments described below were undertaken to find the best conditions for using the rectal caecum as a specific test for substance P.

METHODS

Fowl rectal caecum. The narrow part of the rectal caecum was removed from the hen and stored in modified Tyrode solution at 4° C for 2 to 40 hr. The optimum duration of storage was about 18 hr; after storage the muscle had less spontaneous activity but was sometimes less sensitive to drugs. A length of about 4 to 5 cm was suspended in a bath (3 to 5 ml.). The movements of the longitudinal muscle were recorded on a smoked drum, with an amplification of 15 and a tension of 1 to 5 *g*.

The bath fluid was that used by Barsoum & Gaddum (1935). Its composition is as follows: sodium chloride 8, potassium chloride 0.2, calcium chloride 0.2, magnesium chloride 0.01, sodium bicarbonate 1, sodium dihydrogen phosphate 0.05, glucose 1 g/l. It is identical with Tyrode solution, but contains only 10% of the usual amount of magnesium chloride.

In some experiments, in order to eliminate the effect of the accumulation of active substances in the small bath, a slow flow of fluid was allowed to pass through the bath from below, between the additions of drugs, and sucked out from above (Schild, 1947; Fastier & Reid, 1949). This flow was stopped 15 sec before the drugs were added and the drugs were usually left in contact with the tissue for 30 sec. The fluid was then allowed to run in quickly from below until the bath contents were changed. The whole cycle usually occupied 4 min.

In the first experiments, air was bubbled through the bath, but when this was replaced by 95% oxygen with 5% carbon dioxide the spontaneous contractions decreased and the responses to substance P and acetylcholine increased. This mixture of gases was therefore used in the later experiments.

Estimation of purine (adenine and guanine) compounds. The extracts (20 mg acetone powder/ml.) were treated with perchloric acid to give a final concentration of 5% and allowed to stand for at least 1 hr at 4° C. After centrifuging the mixture, the supernatant was decanted and neutralized with 3N potassium hydroxide. The mixture was centrifuged again, the supernatant was diluted 10 times and used for determination of specific absorption at 260 mμ (Kalckar, 1947). Extracts prepared from intestine had a specific absorption equivalent to 16 nM/mg or 5.3 μg/mg acetone powder (calculated as adenosine monophosphate). Extracts prepared from brain usually contained the equivalent of 18 nM/mg or 6 μg/mg acetone-dried power. These figures are comparable with those of other workers (McIlwain, 1959).

On a few occasions the adenosine triphosphate was measured with the firefly luminescence method (Strehler & Totter, 1954), and amounts varying from 10% to 25% of the total purine were found.

Tissue extracts. Acetone-dried powders of fresh tissue were prepared by three extractions with 20 vol. of dry acetone. In some experiments the powders were then ground in a mortar with 2 vol. *n*-butanol and the mixture heated at 75° C for 30 min. The butanol was removed by evaporation at 60° C under reduced pressure. This treatment with butanol was found by Leach (1959) to increase the activity by 35%, but the explanation of this phenomenon is still unknown. The dry powder was extracted with 0.02 M acetate (or citrate) buffer *p*H 5.5 containing 0.9% (w/v) sodium chloride, by heating for 10 min in a boiling water bath. The extract was either used the same day or dried for storage. The buffer solution alone had no effect on the caecum.

Drugs used. In many of the experiments the preparation of substance P was one made by Hoffmann La Roche & Co. from horse intestine by the methods used by Pernow (1953). It contained 75 Euler u./mg, and has been used as a standard preparation in many laboratories. In other experiments a laboratory standard was used. This was prepared from cattle brain using similar methods. A solution of the ammonium sulphate precipitate was passed through

one alumina column and freeze-dried. It was compared with the first preparation on guinea-pig ileum and estimated to contain 12 u./mg. It was also tested on rat uterus, fowl rectal caecum and rabbit jejunum, and similar results were obtained. It lost more than 90% of activity on incubation with chymotrypsin. These results suggest that the two preparations owe their activity to the same substance.

A preparation of synthetic bradykinin was kindly supplied by Parke, Davis & Co. Preparations of lysergic acid diethylamide, 2-bromolysergic acid diethylamide, 1-methyl-lysergic acid butanolamide (methysergide) and dihydroergotamine were presented by Sandoz & Co.

Phenoxybenzamine (Dibenzyline) and dibenamine (*NN*-dibenzyl-β-chloroethylamine) were presented by Smith, Kline & French Co. Triprolidine (295C51 ; trans-1-(4′-methylphenyl)-1-(2′-pyridyl)-3-pyrrolidinoprop-1-ene) was presented by Dr A. F. Green.

RESULTS

Sensitive preparations often had much spontaneous activity, which obscured the record. Attempts have therefore been made to reduce spontaneous movements without reducing sensitivity ; storage at 4° C or the addition of ephedrine or adenosine monophosphate had some effect (see below).

Inorganic salts. Some of the effects of these were described by Barsoum & Gaddum (1935). A few experiments were undertaken in the hope of finding a more suitable bath fluid. When the concentration of magnesium chloride was increased to that present in Tyrode solution (0.1 g/l.) the spontaneous movements decreased, but the response to drugs also decreased.

Reduction of the concentration of potassium chloride to one-half had the same two effects. These changes in inorganic salts conferred no apparent benefit.

Temperature. The optimum temperature appeared to be 34 to 35° C. At 30° C the spontaneous movements were less, but so also was the response to substance P. Increasing the temperature to 38° C sometimes increased the response to substance P, but also increased spontaneous movements and the effect of adrenaline.

Age and sex of fowl. Preparations made from chickens 10 days old responded to substance P, but the results were not satisfactory when the age was less than 9 weeks ; the optimum age seemed to be 14 to 20 weeks. Cocks and hens were equally satisfactory.

Substance P. The concentration of substance P which caused a reasonable contraction of the fowl rectal caecum was 0.1 to 0.5 u./ml. and was not significantly different from the concentrations causing a similar contraction of isolated preparations of rabbit jejunum, guinea-pig ileum, guinea-pig rectum or rat uterus. However, in the presence of adenine nucleotides the caecum often responded to 0.01 u./ml. (see below). The fowl rectal caecum is relatively insensitive to bradykinin, which causes a slower contraction and is difficult to compare accurately with substance P (Pernow & Rocha e Silva, 1955). It was estimated that 1 u. of substance P was equivalent to more than 10 μg of bradykinin.

The rat uterus, on the other hand, is about 1,000 times more sensitive to bradykinin and can be used to detect small amounts of bradykinin present as an impurity in preparations of substance P. The guinea-pig ileum is intermediate between these two preparations, being more sensitive to bradykinin than the fowl rectal caecum and less sensitive than the rat uterus (Gaddum, 1955).

The most important known substances likely to be present in tissue extracts are given in the following list, together with the concentrations (μg/l.) found to cause a small contraction of the rectal caecum: acetylcholine 5 ; histamine 10 or more ; 5-hydroxytryptamine 100 to 1,000 ; bradykinin 10,000. Adrenaline (1), noradrenaline (40), adenosine (100), adenosine monophosphate (200) or adenosine triphosphate (400) caused inhibition.

Antagonists

Atropine (10^{-7}) decreased the sensitivity to acetylcholine by 200 times. Hyoscine is effective in the same concentration and was used in many of the tests.

Antagonists of catecholamines

Ephedrine. A concentration of $1–5 \times 10^{-6}$ in the reservoir usually relaxed the muscle and inhibited spontaneous movements. The effects of histamine, substance P and 5-hydroxytryptamine were often increased, and the inhibitory effect of adrenaline or noradrenaline was decreased.

In Fig. 1 it will be seen that the effect of 0.2 u. substance P was decreased when it was mixed with 1 ng of adrenaline. The addition of ephedrine (10^{-6}) to the bath fluid inhibited the tone of the muscle and the spontaneous movements.

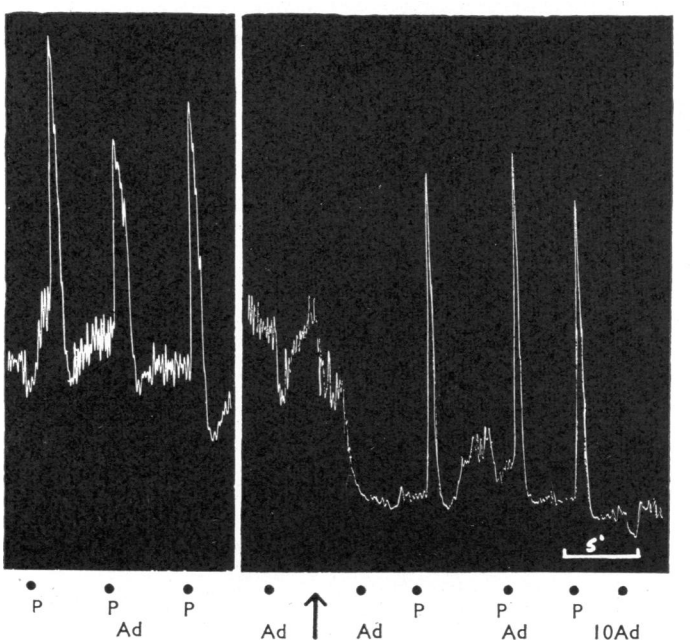

Fig. 1. Fowl rectal caecum suspended in a 5 ml. bath containing Tyrode solution (prepared with 10% of the usual amount of magnesium chloride). Drugs were added at 4 min intervals and allowed to act for 30 sec. P=substance P (0.2 u.); Ad=adrenaline (1 ng). From the arrow (↑) until the end of the tracing ephedrine (10^{-6}) was present in the bath.

Adrenaline now had no inhibitory effect by itself and did not diminish the response to substance P when the two substances were mixed. Higher concentrations of ephedrine (10^{-5}) eventually made the preparation less sensitive to substance P. Lower concentrations ($<10^{-6}$) were less effective in diminishing the response to adrenaline.

The noradrenaline content of extracts of parts of the dog brain was estimated by Vogt (1954), and her figures were compared with estimates of substance P by Amin *et al.* (1954). It will be seen from their Table 6 that in different tissues 1 u. substance P was associated with 0.25 to 25 ng noradrenaline, or approximately 1/10th of this quantity of adrenaline. The ratio for the intestine probably lies within this range. In these tissues the amount of adrenaline is thus likely to be less than 2.5 ng/u. substance P, which is slightly less than the amount which interferes with assays on the fowl rectal caecum (Fig. 1). Noradrenaline is less likely to interfere, though its concentration in the brain is about 10 times that of adrenaline, since its activity on the rectal caecum is about 1/40. Extraction with acetone removes most of the catecholamines which are then even less likely to interfere with the assay.

The presence of ephedrine in the bath is thus perhaps unnecessary for many purposes, but it does have some antiadrenaline action and appeared to be beneficial in other ways; it was, therefore, often present in the bath in experiments with other drugs.

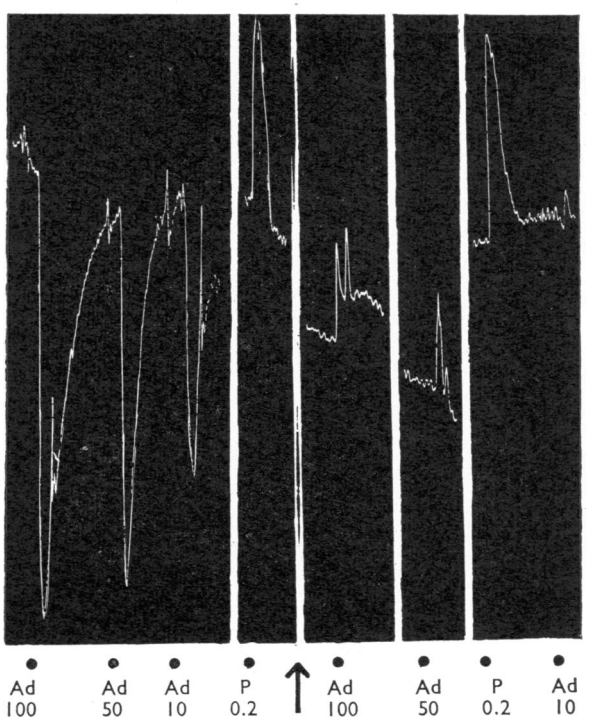

Fig. 2. As Fig. 1. Doses of adrenaline in ng and of substance P in u. From the arrow (↑) until the end of the tracing dichloroisoproterenol (10^{-6}) was present in the bath.

Dichloroisoproterenol (1-(3'4'dichlorophenyl)-2-isopropylaminoethanol). This substance antagonizes many of the inhibitory effects of adrenaline (Powell & Slater, 1958). It is thought to act on β-receptors (Ahlquist & Levy, 1959). Fig. 2 shows that it blocks the inhibitory effect of adrenaline on the rectal caecum. The muscle was inhibited by doses of 5 to 100 ng of adrenaline. When dichloroisoproterenol (10^{-6}) was added to the bath, this effect was reversed. Large doses of adrenaline now caused a brief contraction of the muscle, while small doses had practically no effect. In other experiments, similar to that shown in Fig. 1, substance P was mixed with adrenaline. Initially, this reduced the response, but when dichloroisoproterenol (10^{-6}) was added to the bath it increased the response. This reversal of the effect of adrenaline on the response to substance P occurred when the dose of adrenaline was only 10 ng. This drug is therefore not an ideal routine antagonist for adrenaline, but could be used to test for the presence of adrenaline in extracts.

Mushroom extracts. Mushroom extracts were used by Garven (1956) to inactivate adrenaline in the assay of 5-hydroxytryptamine. They have been found to destroy substance P.

Antagonists of histamine

Phenoxybenzamine (N-benzyl-N-phenoxyisopropyl-β-chloroethylamine). This substance irreversibly blocks the action of various drugs. It blocks the motor actions of adrenaline, but does not block the inhibitor actions (Nickerson, 1949). It was therefore not surprising that it did not antagonize the inhibitor action of adrenaline on the rectal caecum. Phenoxybenzamine has been found to be a particularly powerful antagonist of histamine on the rectal caecum. Fig. 3 shows an experiment in which 0.05 μg histamine in the presence of ephedrine caused an effect similar to that of

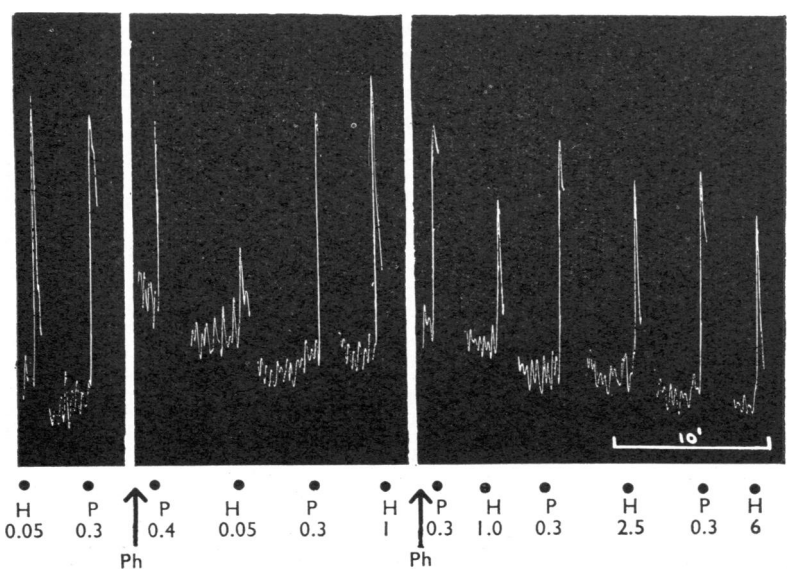

Fig. 3. As Fig. 1. Doses of substance P in u. and of histamine in μg. At the arrows phenoxybenzamine (10 μg) was added to the bath for 10 min.

0.3 u. substance P. A dose of 10 μg phenoxybenzamine was then added to the bath for 10 min and washed out again. This had no effect on the response to substance P, but decreased the response to histamine. The treatment with phenoxybenzamine was then repeated, and the effect of histamine was reduced still further, so that 6 μg now had less effect than 0.3 u. substance P. The relative potency of histamine had thus been reduced more than 120 times.

Other histamine antagonists. Dibenamine had a comparatively small effect on the action of histamine and reduced that of substance P. Mepyramine (10^{-6}) or promethazine (10^{-7}) reduced the effect of histamine, but did not abolish it. Higher concentrations reduced the effects of both histamine and substance P. Triprolidine (Green, 1953) is a particularly active and specific antihistamine on other tissues. A concentration of 10^{-6} (in the presence of ephedrine) reduced the sensitivity to histamine 3 times without altering the response to substance P. A concentration of 10^{-5} reduced the sensitivity to histamine 30 times, but reduced also the response to substance P.

A high concentration of histamine itself in the bath was found by Barsoum & Gaddum (1935) to cause a contraction lasting only a few min, in spite of the continued presence of the drug in the bath. This observation has been confirmed; a concentration of 0.5 to 2×10^{-6} of histamine reduced the sensitivity to histamine by 50 to 200 times. The response to substance P was in some cases unaffected, but in others it was reduced. In many of the experiments on extracts histamine (2×10^{-6}) was present in the bath.

Antagonists of 5-hydroxytryptamine

Many preparations were rather insensitive to 5-hydroxytryptamine, but a concentration in the bath of 0.1 to 2×10^{-6} often produced a biphasic contraction. An excess of tryptamine in the bath has been found to desensitize some plain muscle to both the tryptamine itself and to 5-hydroxytryptamine (Gaddum, 1953a); but this was not effective with fowl rectal caecum; a concentration of 5×10^{-6} caused a prolonged contraction with little or no tachyphylaxis.

Phenoxybenzamine, in a concentration of 2×10^{-6} for 10 min, had no effect, but double this concentration for the same time made the muscle 10 times less sensitive to 5-hydroxytryptamine.

Various derivatives of lysergic acid acted as specific antagonists of 5-hydroxytryptamine when added to the bathing fluid, so as to be indefinitely in contact with the muscle; lysergic acid diethylamide, in a concentration of 10^{-7}, made the muscle 3 to 10 times less sensitive. The 2 bromo-derivative and dihydroergotamine had a similar effect. The most satisfactory results were obtained with methysergide. In a concentration of 5×10^{-8} this reduced the sensitivity of the muscle to 5-hydroxytryptamine by more than 100 times and had little or no effect on the response to substance P. According to Amin *et al.* (1954), extracts of horse intestine contain less than 20 ng of 5-hydroxytryptamine/u. substance P, and extracts of various parts of dog brain all contained less than this. A dose of 0.3 u. of substance P is thus likely to be mixed in an extract with not more than 6 ng of 5-hydroxytryptamine,

which would give a concentration in the bath of less than 2×10^{-9}, and this would have no effect, even in the absence of antagonists. An example is given below, however, of an experiment where 5-hydroxytryptamine was present in just sufficient concentration to affect the result when no antagonist was used. It is thus generally not necessary to use an antagonist for 5-hydroxytryptamine in experiments with the rectal caecum, but it is advisable to check the sensitivity to 5-hydroxytryptamine on each occasion.

Purine compounds

Contractions of the fowl rectal caecum are inhibited by low concentrations of adenosine compounds. The most active is adenosine itself, which has an effect in a concentration of 10^{-7} as was first observed by Barsoum & Gaddum (1935). We have found that when this same concentration was mixed with substance P (about 0.25 μg of adenosine/u. substance P) it caused a small diminution of the response ; smaller doses of adenosine had no effect. The threshold doses of adenosine monophosphate, adenosine diphosphate and adenosine triphosphate were about 0.5 μg, 1 μg and 1 μg respectively/u. substance P. Adenine had negligible activity. The guinea-pig ileum is sometimes less sensitive to these adenosine compounds, but results are variable. In experiments on the fowl rectal caecum it is particularly important to overcome the inhibition or to separate substance P from the adenosine compounds before the assay. These substances are all likely to be present in the acetone-insoluble fraction of tissue extracts, the proportions varying with the conditions of preparation. The autolytic breakdown of adenosine triphosphate in nervous tissue produces a mixture of adenosine diphosphate, adenosine monophosphate, adenosine and adenine (Kerr, 1942). In our extracts the total purine and the adenosine triphosphate were measured, and from the low proportion of adenosine triphosphate it could be assumed that some breakdown of adenosine monophosphate to adenosine and adenine had occurred.

About 14% of the specific absorption from which the total purine was estimated is due to guanine derivatives. Therefore, guanosine, guanosine monophosphate and guanosine triphosphate were tested for pharmacological activity. Guanosine was the most active, but since it was only from 1 to 25% as active as adenosine it is not likely to be present in great enough concentrations to interfere with the assays. In any case, the responses to guanosine were abolished when the tissue was desensitized to adenosine.

The results of Thorn, Scholl, Pfleiderer & Mueldener (1958) suggest that the concentration of adenosine diphosphate would be about half that of adenosine triphosphate, but Kratzing & Narayanaswami (1953) found no adenosine diphosphate. Calculations based on the tables of Kerr (1942) suggest that significant quantities of adenosine may be present in our extracts, but the evidence is indirect. The probable concentrations of these compounds in our extracts are shown in Table 1. It can be seen that various adenosine derivatives may be present in high enough concentrations to interfere with substance P assays. It must be pointed out that these estimates refer only to our extracts. In particular, as adenosine triphosphate

TABLE 1

ESTIMATED AMOUNTS OF ADENINE AND GUANINE DERIVATIVES IN 1 MG OF ACETONE POWDERS OF BRAIN, AND IN TERMS OF SUBSTANCE P CONCENTRATIONS ASSUMING 0·2 U. SUBSTANCE P/MG ACETONE POWDER

AMP=adenosine monophosphate; ADP=adenosine diphosphate; ATP=adenosine triphosphate

Compound	Method	mole/mg	μg/u. substance P: Present	Test dose	Threshold
ATP	Firefly method	1·5–3	5	5	1
ADP	Kratzing & Narayanaswami (1953); Thorn *et al.* (1958)	0–1·5	5	5	1
AMP	Calculated from proportion of ATP using data by Kerr (1942)	12	17	20	0·5
Adenosine				10	0·25
Adenine				10	—
Guanine derivatives	Heald (1960)	2·5	7·5	5	—
Total	Absorption 260 mμ	17·5	—	—	—

decreases (during further autolysis) the concentration of the other adenosine compounds would be expected to rise.

At first we attempted to separate substance P from these substances by various techniques (paper chromatography, paper electrophoresis, barium and alcohol precipitation), but the recovery of substance P was not quantitative. Another method recently proposed by Laszlo (1960) involves the inactivation of adenosine monophosphate with adenylic deaminase. When adenosine monophosphate is responsible for most of the inhibitory activity of extracts this method is satisfactory, but our extracts contained sufficient adenosine triphosphate to affect the fowl rectal caecum and adenosine triphosphate is not destroyed by the enzyme.

The method adopted in our experiments was that of specific inhibition of response by high doses (specific tachyphylaxis) (Gaddum, 1953a). In general it was found that a large dose (200 μg to 2 mg) of adenosine monophosphate always desensitized the tissue to adenosine and to adenosine monophosphate, whereas adenosine triphosphate (100 μg to 200 μg) desensitized the tissue to adenosine triphosphate and adenosine diphosphate. The most certain method of abolishing the responses to all four adenosine compounds was to use a mixture of adenosine monophosphate (100 μg to 200 μg) and adenosine triphosphate (50 μg to 100 μg). However, this was not always necessary, since on many occasions adenosine monophosphate or adenosine triphosphate alone were effective against all the test substances. The large doses were put into the 4 ml. bath each time the Tyrode was renewed, and the criterion of satisfactory desensitization was that a dose of substance P should produce identical contractions when given alone and with the test substances in at least the proportions (test doses) shown in Table 1. This is illustrated in Fig 4. In this experiment addition of 1 μg adenosine triphosphate abolished the response to the test dose of substance P. The response to a mixture of the four adenosine compounds and substance P was a relaxation so large that it was not recorded. After desensitizing with adenosine monophosphate, the contractions in response to substance P were the same height whether the adenosine derivatives were added or not. This

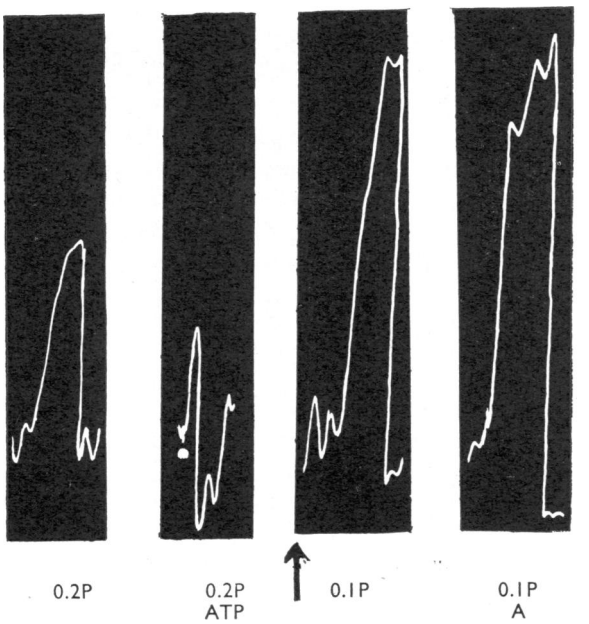

Fig. 4. As Fig. 1. Dose of substance P in u. ATP, 1 μg adenosine triphosphate was added to the bath with substance P. At the arrow adenosine monophosphate 5×10^{-5} was added and the concentration maintained throughout. A, The following mixture was added with substance P: adenosine (1 μg), adenosine monophosphate (2 μg), adenosine diphosphate (0.5 μg) and adenosine triphosphate (0.5 μg).

result is typical of about half the experiments with adenosine monophosphate. On other occasions adenosine triphosphate caused a contraction in the presence of adenosine monophosphate, and it was then necessary to use a mixture of adenosine monophosphate and adenosine triphosphate for desensitization.

Similar results were obtained with adenosine triphosphate alone. Usually the responses to adenosine monophosphate and adenosine were abolished as well as those to adenosine triphosphate and adenosine diphosphate, but in some experiments adenosine monophosphate and adenosine still caused slight inhibition in the presence of adenosine triphosphate. Addition of adenosine monophosphate to the desensitizing dose of adenosine triphosphate then abolished responses to all the adenosine compounds. Large doses of adenosine (100 μg) were as effective as adenosine monophosphate against adenosine itself and usually also against adenosine monophosphate. However, the responses to adenosine triphosphate and occasionally also to adenosine monophosphate were reversed so that a small dose produced a contraction in the presence of adenosine. Therefore adenosine could not be used alone for assay of extracts. Mixtures of adenosine (100 μg) and adenosine triphosphate (100 μg), however, were effective against all four compounds.

From these results it can be concluded that it is possible to desensitize the tissue to adenosine and its phosphates by specific tachyphylaxis. The composition of our extracts necessitated testing for sensitivity to both adenosine monophosphate and adenosine triphosphate. It would have simplified the experimental procedure

if no breakdown of adenosine triphosphate had occurred or alternatively if autolysis had proceeded to the point where no adenosine triphosphate or adenosine diphosphate remained.

The use of adenosine compounds for specific tachyphylaxis had the additional advantage of facilitating assays by inhibiting spontaneous contractions and increasing the sensitivity to substance P. When adenosine, adenosine monophosphate or adenosine triphosphate was added to the bath the caecum relaxed and remained quiescent at the greater length. It was stable for several hours. The responses to substance P from this new baseline were usually greater than before adding the nucleotide as illustrated in Fig. 4.

Assays

The approximate strength of an unknown solution was first determined by matching contractions produced by standard substance P. When the amount of material was sufficient, a 6-point assay design (Burn, Finney & Goodwin, 1950) was used. Solutions were prepared, 3 each of test and standard, so that the different doses were contained in the same vol. (usually 0.1 ml.). This was a necessary precaution, because the height of contraction varied slightly when the same dose was given in different vol. The 3 doses of each were equally spaced on a log. scale and were in the ratio 1:1.5:2.25 or 1:2:4. The 6 solutions were then given in random order according to a 6×6 Latin square. A strict time schedule was found to reduce the error of the assay. The cycle occupied from 2.5 to 4 min and substance P was allowed to act for 30 to 40 sec on different occasions. The heights of contraction from the baseline were measured and analysed according to Burn *et al.* (1950).

The dose-response relationship was investigated using 7 doses of substance P, each given 6 times in random order. The mean response was related to the log-dose by a sigmoid curve which was approximately linear over the range 0.1 u. to 1.6 u. substance P.

Two mixtures of substance P and adenosine monophosphate in the proportion 5 u. substance P to 100 μg adenosine monophosphate were made up by an assistant so that their strength was unknown to the tester. The results were calculated after statistical analysis (see Table 2). In Expt. 3 the correct value of the potency ratio

TABLE 2

RESULTS OF SUBSTANCE P ASSAYS

X indicates deviation from linearity ($P=0{\cdot}05$); + indicates deviation from parallelism ($P=0{\cdot}05$). The "result" for Expts. 3 and 4 represents the estimated relative potency (true figures 0·667 and 1·0 respectively), and for Expts. 5 to 9 represents substance P u./mg powder

Expt. no.		Doses	Groups	Deviations	Result	Fiducial limits $P=0{\cdot}05$
1	Standard	7	6	X	—	—
2	Standard	4	8	X	—	—
3	P and AMP mixture	6	6	X+	0·63	(0·56–0·70)
4	P and AMP mixture	6	6	X	1·02	(0·94–1·10)
5	Sheep brain	6	4	—	1·0	(0·89–1·14)
6	Sheep brain	6	6	—	1·39	(1·18–1·64)
7	Rabbit gut	6	6	X+	0·34	(0·29–0·40)
8	Rabbit brain	6	6	—	0·31	(0·26–0·37)
9	Rabbit brain	6	5	X	0·28	(0·25–0·31)

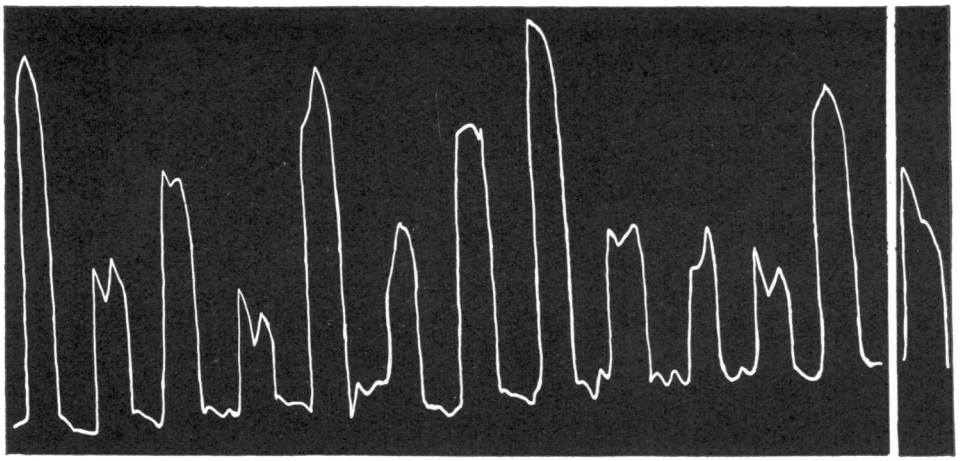

Fig. 5. As Fig. 1. E=an extract of rabbit intestine (mg). HT=5-hydroxytryptamine (ng). Doses of substance P in u.

was 0.667, so that the error was −5.5%, and in Expt. no. 4 the correct value was 1 and the error +2%. These results are well within the fiducial limits. This shows that it is possible to assay substance P in the presence of adenosine monophosphate.

Six-point assays were also carried out with extracts from sheep brain, rabbit brain and rabbit intestine. One of these assays is illustrated in Fig. 5, where it can be seen that the shape of the contractions in response to extract and substance P is the same. No methysergide was used in this experiment and the caecum was unusually sensitive to 5-hydroxytryptamine, which caused a contraction in a dose of 43 ng. It can be seen that the response to 5-hydroxytryptamine was a different shape from the response to substance P and to the extract. Nevertheless, it was thought necessary to determine the 5-hydroxytryptamine content of the extract. This was done with the rat fundal strip (Vane, 1957). The extract was assayed against 5-hydroxytryptamine, substance P and a mixture of 5-hydroxytryptamine and adenosine monophosphate to allow for the depressing effect of adenosine monophosphate in the extract. The concentrations of adenosine triphosphate and adrenaline in the extract were shown to be too low to affect the assay.

It was found that the extract contained an exceptionally high concentration of 5-hydroxytryptamine (10 ng/mg). In spite of this and the unusual sensitivity of the rectal caecum to 5-hydroxytryptamine the maximum contribution of 5-hydroxytryptamine was 4.6% of the total activity on the rectal caecum. This is within the limits of experimental error. In most experiments the rectal caecum was much less sensitive to 5-hydroxytryptamine, and the effect of the 5-hydroxytryptamine even in this extract would have been negligible.

The results of these assays are given in Table 2. It can be seen that the two assays of extracts from the same rabbit brain powder agree well. In these assays the caecum was insensitive to both adenosine monophosphate and triphosphate. The agreement between the assays for the sheep brain powder is not so good. The

discrepancy between them is just large enough to suggest that the calculation of the error of the test does not take all relevant factors into consideration. If the correct result was the geometric mean of the two estimates (1.18), both of these would be near the limits of their fiducial range ($P=0.05$). This could be accounted for by pipetting errors. On the other hand, these assays, which were the first in the series, were done without checking that the tissue had been desensitized to adenosine triphosphate, since at that time it was not realized that adenosine triphosphate effects were not always abolished by adenosine monophosphate alone. It is possible that this contributed to the discrepancy in the results.

The index of precision (λ) is estimated from the ratio s/b (Gaddum, 1931, 1953b), where s is the standard deviation of a single response, calculated from the analysis of variance after eliminating components due to differences between groups of results, preparations, regression, curvature, etc. It gives an absolute estimate of the precision of the method, which is independent of the number of responses measured, and the units in which they are measured.

Woolf has pointed out that, for many purposes, it is more convenient to calculate L ($=b/s$), which is the reciprocal of λ, and this is shown in Table 3. This

TABLE 3

ESTIMATES OF ERRORS

The figures in brackets give approximate estimates made by the simple method described in the text. b=Slope (mm on drum for 10-fold dose range); R=potency ratio (estimated/assumed); s=standard deviation of a single response (mm); L=b/s=1/λ=1/index of precision; FR=fiducial ratio (upper fiducial limit/R, $P=0.05$)

Expt. no.	b	R	s	L	FR
1	49	—	5·34 (4·6)	9·2 (10·6)	—
2	130	—	4·41 (4·3)	29·4 (30·3)	—
3	80	0·63	4·68 (5·3)	17·2 (15·3)	1·12 (1·11)
4	99	1·02	4·89 (5·8)	20·2 (17·1)	1·08 (1·09)
5	101	1·00	6·90 (6·6)	14·6 (15·0)	1·14 (1·14)
6	71	1·74	5·51 (5·1)	12·8 (14·1)	1·17 (1·12)
7	79	1·48	7·69 (7·8)	10·2 (10·0)	1·18 (1·17)
8	77	1·30	8·28 (7·8)	9·3 (9·8)	1·19 (1·17)
9	123	0·94	7·35 (7·9)	16·8 (15·6)	1·11 (1·11)

suggestion was mentioned by Gaddum (1953b), but Woolf's paper on the subject was not published. In a uniform series of tests L is normally distributed, and its deviation from the true L is due only to sampling error. Since the standard deviation of $b=s_b=\frac{s}{\sqrt{S(x-\bar{x})^2}}$ and $L=\frac{b}{s}$, the standard deviation of $L=s_L=\frac{1}{\sqrt{S(x-\bar{x})^2}}$. This quantity depends only on the design of the test and is independent of b and s.

For ease of calculation, it may be assumed, as an approximation, that the tests shown in Table 2 were all 6-point assays, based on 36 results, with a dose interval of log. 2. In such tests there are 24 results for which $(x-\bar{x})$ is log. 2, and 12 for which it is zero.

$$\therefore s_L = 1\sqrt{24(\log. 2)^2}$$
$$= 0.68.$$

The standard deviation of the observed values of L was much larger, namely, 6.5. This shows that the true value of L varied from test to test and that the variation

was not all due to the sampling error. Some experiments were thus actually less reliable than others, as was perhaps fairly obvious.

The mean value of L was 15.5 and the corresponding value of λ is 0.066. This result can be regarded as fairly satisfactory. In the table given by Gaddum (1953b) for experiments on isolated tissues, L varies from 5, for assays of oxytocin on the guinea-pig uterus, to 30 for assays of histamine on the guinea-pig ileum. In some of the assays by Mogey, Trevan & Young (1949) of D-tubocurarine on rat diaphragm, the value of L was as high as 63, but the value for the assay of adrenaline on rat uterus (Gaddum & Lembeck, 1949) was 16, which is similar to the mean value given by the results described here.

In the present work it was not always easy to discover the linear part of the dose-response curve, and several assays showed significant deviations from linearity. This was probably because the lower end of the curve was used in an attempt to obtain high sensitivity. The departure from strict linearity has little effect on the estimated strength of the unknown and is relatively unimportant for practical purposes. It does, however, affect the fiducial limits. Deviation from parallelism is bound to occur when there is deviation from linearity unless the doses of unknown and standard are equal. In none of the experiments was there any significant difference in curvature of the dose-response relationships for standard and unknown.

The error of these tests has been estimated on the assumption that the dose-response relation is linear and is shown in the last column of Table 3 in the form of the fiducial ratio ($P=0.05$), which is equal to the ratio of the upper fiducial limit to the result of the test (R).

The figures in brackets give estimates made by much simpler methods, which provide a useful check on the complicated calculations required for the ideal solution. Each value of s was estimated by dividing the range of the responses to each dose by the appropriate factor. With small groups this estimate is almost as reliable as the conventional method (Gaddum, 1953c). The main disadvantage of it for the present purpose is that it includes error due to differences between groups of results.

The approximate value of the fiducial ratio was calculated from the approximate value of L by the formula: Log (fiducial ratio)$=2t/L\sqrt{N}$ (where t is Student's t and N is the total number of observations). This formula, which was given in a slightly different form by Gaddum (1953b), is generally applicable to symmetrical parallel line assays. It gives a minimal estimate and is only exactly true when the mean effects of the standard and unknown are equal and when g is negligible.

The true value of the fiducial ratio will be greater than this approximation, but, in the experiments described here, the difference was negligible, except in Expt. no. 6, where R was particularly high (1.74).

The accurate method is laborious and gives scope for error. These approximate methods provide a check on the results.

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