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ASSAY OF SUBSTANCE P ON GOLDFISH INTESTINE IN A MICROBATH

BY

J. H. GADDUM AND J. C. SZERB*

From the A.R.C. Institute of Animal Physiology, Babraham, Cambridge

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A technique is described for testing the effects of drugs on the intestine of goldfish in a microbath (0.05 ml.). This preparation may be used for the assay of substance P, which causes contractions of the muscle in doses as small as 1 m-u. Other substances in tissue extracts also act on this muscle, but the effects of most of these can be abolished by antagonists. Some tissues, however, contain an unknown substance which causes powerful contractions of the muscle and interferes with the assay of substance P. An attempt to demonstrate the release of substance P in the central nervous system was unsuccessful.

The object of the experiments recorded here was to find a sensitive and specific method for the assay of substance P. After preliminary experiments with the intestine of crayfish, frogs, pigeons and budgerigars, it was discovered that isolated pieces of the intestine of the goldfish contract in the presence of small amounts of substance P. The action of substance P on fish intestine has previously been described (Euler & Östlund, 1956). This paper describes a method of assay using this muscle in a microbath.

METHODS

Microbath. The microbath described by Gaddum & Stephenson (1958) has been modified in various ways for the present experiments (Fig. 1). The muscle is contained in a horizontal hole in a 2 cm cube of perspex at room temperature, through which the bath solution runs continuously at a rate of about 1 ml./min. Drugs are applied by stopping the flow by means of a relay and running a known volume of a solution containing the drug into one end of the bath. The volume of the bath is about 0.05 ml. and the volume of fluid containing the drug is also 0.05 ml. This volume is delivered from a small, clean glass tube into which it is sucked by withdrawing the plunger of an air-filled 0.25 ml. syringe by the appropriate amount.

The drug is left in contact with the intestine for a constant time (for example, 45 sec) and then the flow is restarted. The final concentration of the drug in the bath will be less than in the solution added, but results are reasonably constant. Previously drugs were driven in through a small hole. It has been found difficult to avoid direct mechanical effects on the record with this method, and drugs now are added to a vertical well from which they run slowly into the bath. The bathing fluid also runs through this vertical well and may accumulate to a height which depends on the rate of flow, the diameter of the hole connecting

* Present address: Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada.

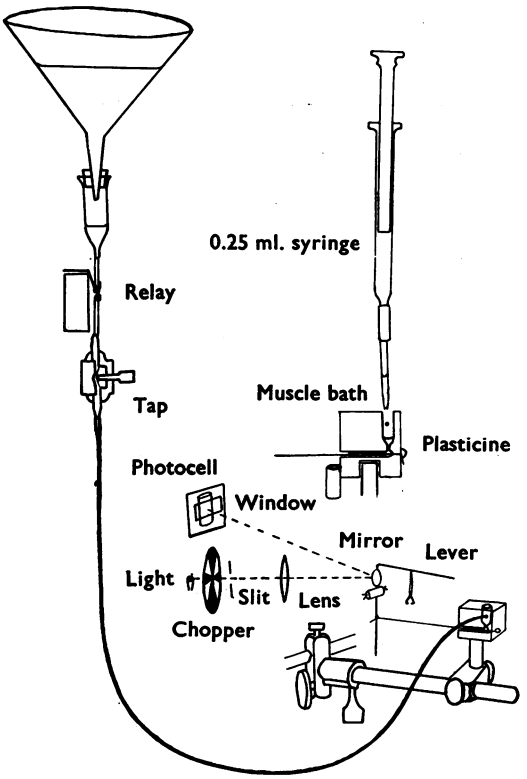

Fig. 1. Apparatus for perfusing the microbath (also shown in the inset). Diluted Locke solution runs from the funnel at a controlled rate of about 1 ml./min. At intervals the flow is stopped and 0.05 ml. of drug solution added to the well and allowed to run slowly into the microbath where it is left for a standard time.

the well with the bath and the rate of outflow from the bath. When the flow is stopped the fluid level in the well falls and the drug is not added until the well is empty. The bathing fluid accumulating in the well after the flow has been restarted will wash away any contamination from the previous application of a drug.

The bath fluid runs from a funnel through a small glass tube where the flow is measured by the formation of drops, and from there through a plastic tap (obtained from Townson & Mercer, Ltd., Croydon, England), designed for controlling slow flows. When the flow is stopped it is important that it should be completely stopped; this is achieved by the use of a relay which compresses a short piece of rubber tubing. All other parts of the system are made of glass or polythene—otherwise it is difficult to avoid complications due to the uptake and release of various substances (known or unknown) in the tubing.

Recording apparatus. In earlier work a torsion lever was used to record the contractions of the muscle. It has been found more satisfactory for the present purpose to use a simple isotonic lever. This is made of aluminium wire and consists of a horizontal arm, to which weights are attached, and a vertical arm, to which the thread from the muscle is attached. A small mirror is attached near the fulcrum and the liver is counterbalanced so that the tension depends solely on the attached weight. The tension in each muscle is adjusted and is generally about 100 mg weight. If it is too low, relaxation is poor and the tone continues to increase; if it is too high, drugs have little or no action.

A beam of light is chopped by a rotating sector and focused on the mirror. The image of a slit near the light is focused on a photocell. The output of the photocell is amplified by an AC amplifier and fed to an Elliott pen-recorder. The gain on the amplifier is adjusted so that full illumination of the photocell will give a full excursion of the pen-recorder. With a 3 cm photocell the movements of the muscle were magnified about 100 times. When greater magnification was desired a window was placed to reduce the effective height of the photocell to 1 or 0.5 cm and the gain increased accordingly. The greatest magnification was about 600 times. The object of the window was to prevent overloading of the pen recorder.

Isolated goldfish intestine. The goldfish (*Carassius auratus*) were generally 5 to 6 cm long. A piece of intestine about 5 mm long (unstretched) was removed from a point 2 to 5 cm from the anus. A fine terylene thread was tied round the intestine to attach it to the lever, and a second thread, attached to the other end of the intestine, was led through a very small hole at the bottom of the bath and fixed with a lump of plasticine.

Drugs used. In many of the experiments substance P was used in the form of a preparation prepared by Messrs. Hoffmann La Roche. This was compared with earlier standard preparations in this laboratory and in U. S. von Euler's laboratory and estimated to contain 75 Euler u./mg. It has been used in various laboratories as a provisional standard. In other experiments another preparation prepared in this laboratory was used. This preparation was estimated to contain 40 u./mg.

We are grateful to Messrs Sandoz for a sample of methysergide (UML 491, 1-methylysergic acid butanolamide) and to Messrs Parke, Davis & Co. for a sample of synthetic bradykinin.

Other drugs were obtained from the following sources: Dichloroisoprenaline—Eli Lilly; tryptamine hydrochloride—British Drug Houses; soyabean trypsin inhibitor—Light & Co.; chymotrypsin (crystallized) and trypsin (crystallized)—Armour & Co.; adenosine triphosphate—Sigma Chemical Co. The composition of the Locke solution in g/l. was the following: sodium chloride 9.0, potassium chloride 0.42, calcium chloride 0.24, glucose 2.0, sodium bicarbonate 0.2.

RESULTS

When the bath fluid was Locke solution the muscles generally gave repeated large contractions and were quite unsuitable for use. Occasionally they remained quiescent and appeared sensitive to substance P. Much time was therefore spent in trying to abolish these spontaneous movements. Various changes were made in the salt composition of the bath fluid, and drugs such as atropine, cocaine, alcohol, nitrates and adenosine were added, but none of these measures were effective. One evening when no drugs were available for trial, water was added to the Locke solution; the muscle contracted and then relaxed and remained quiescent, but sensitive to drugs. This procedure has been found regularly to provide good preparations; the muscle is first suspended in Locke solution, and after about 10 min, when spontaneous contractions start, the Locke solution is diluted by adding 40 ml. of distilled water for each 100 ml. of Locke solution. This causes a brief contraction followed by more regular spontaneous movements. After about 10 min this fluid is then replaced by Locke solution diluted with an equal volume of distilled water (1:2 Locke solution). This causes an immediate contraction followed by a slow relaxation; the spontaneous movements cease, but the muscle is still sensitive to drugs (Fig. 2). This stepwise dilution was found to produce more sensitive preparations than those obtained by omitting either of the preliminary stages.

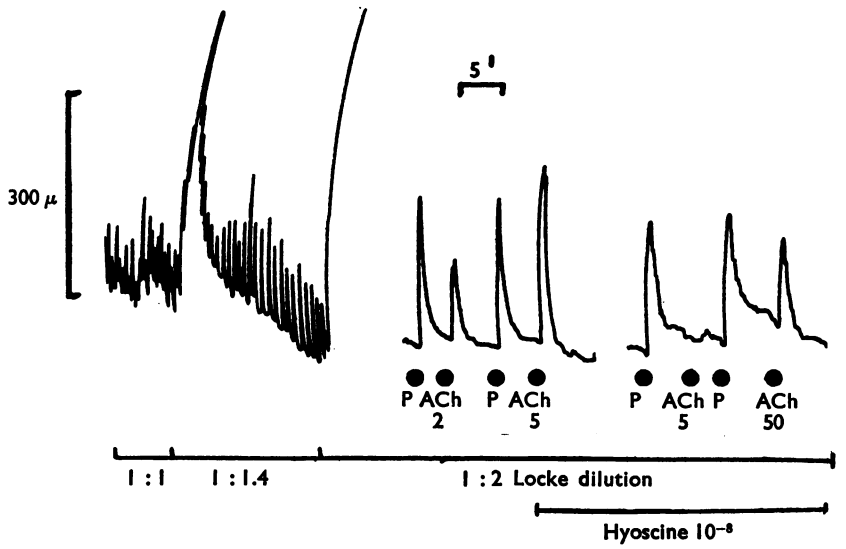

Fig. 2. Goldfish intestine in the microbath. Calibration shows actual shortening of the muscle. Dilution of the Locke solution caused disappearance of the spontaneous movements. P= substance P, 0.1 u./ml. ACh=acetylcholine (10^{-9}). Hyoscine (10^{-8}) antagonized acetylcholine but not substance P.

This effect of water appeared to be due to the hypotonicity of the solution. When the concentration of potassium chloride or calcium chloride was halved, the spontaneous contractions continued unchanged. When the concentration of sodium chloride was reduced to 50% or even 20% of the concentration in Locke solution, and the tonicity maintained with sucrose, the contractions also continued, but when it was reduced to 10% they ceased. Such preparations in which 90% of the sodium chloride had been replaced by sucrose remained quiescent and were, for a time, sensitive to drugs, but their sensitivity was not well maintained. These reactions have not been studied in detail. The tonicity of the solutions used appears to be much lower than the tonicity of goldfish blood ; there is some evidence that quiescent but sensitive preparations of other kinds of muscle can also be obtained by using hypotonic solutions (Szerb, 1961).

Since, with each administration of a drug, the entire bath fluid is replaced, small changes in the composition of this fluid may have an effect on the record. In order to know whether a reaction following the application of a small dose of drug is due to the drug itself, the bath fluid itself is applied as a control. In the absence of contamination, this should have little effect, but, with sensitive preparations, this effect may be appreciable.

The *p*H of the bath fluid was normally 6.9 to 7.1. A small increase (8.5) caused contraction of the muscle and a small decrease (5.5) relaxed it, but *p*H 2 caused contraction.

Substance P. Small doses of substance P caused a contraction starting 10 to 20 sec after its application, and disappearing in 1 to 2 min, when the time of

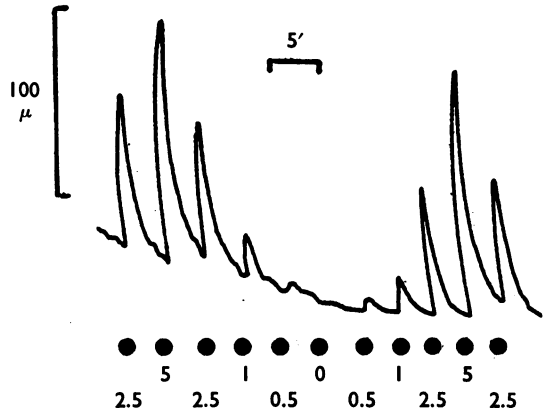

Fig. 3. As Fig. 2. Diluted Locke solution (1 : 2) containing hyoscine (10^{-8}), adenosine triphosphate (10^{-5}), and dichloroisoprenaline (5×10^{-8}). Contractions caused by substance P. Doses in milliunits contained in 0.05 ml.

contact with the tissue was 45 sec. The smallest concentration to have an effect significantly greater than control doses of the bathing fluid was generally 0.05 to 0.1 u./ml., but some preparations were more sensitive than this (see Fig. 3). The effect increased with the concentration over a 10-fold range of concentrations or more (Fig. 3) and application of the substance every 3 to 4 min did not lead to any appreciable decrease of the response.

The effect was fairly constant over a long period of time and satisfactory assays could be carried out. An extract of horse intestine was compared with the standard preparation in a (2+2) dose assay in the presence of atropine (10^{-8}) and adenosine diphosphate (10^{-5}). The concentrations of the standard were 0.25 and 0.5 u./ml. given every 4 min, each dose being repeated 4 times (Fig. 4). Since the volumes were 0.05 ml. the total amount of the standard used was 150 m-u.

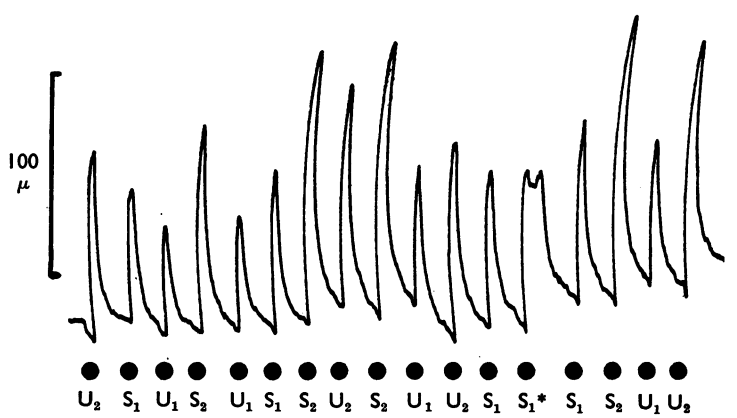

Fig. 4. As Fig. 2. Diluted Locke solution containing atropine (10^{-8}) and adenosine triphosphate (10^{-5}) (2+2) dose assay. S_1=0.25 u. standard substance P/ml. U_1=6.25 μg of an extract of horse intestine (P_3B). S_2 and U_2=double these doses. * Accidentally left in bath for 90 sec instead of 45 sec.

The analysis of variance showed that there was no significant deviation from parallelism, but the variance due to repetitions was highly significant, since the responses became larger during the test. The potency ratio was 76.4% and the index of precision ($\lambda = s/b$) was estimated as 0.0435. The corresponding value of Woolf's index of precision (Gaddum, 1953b) is 23, so that the test is reasonably precise. The extract of horse intestine was estimated in this experiment to contain 30 ± 3.4 ($P = 0.05$) u./mg. The mean results obtained by other methods were 42 (guinea-pig ileum), 37 (hen rectal caecum) and 31 (rat uterus). The differences between these results are of doubtful significance.

When substance P was left in the bath for longer times the contraction lasted 3 to 6 min and then disappeared, although the drug was not washed out (Fig. 5). The solution in the bath was then replaced with a fresh solution containing substance P. This caused another contraction so that the substance P in the bath must have

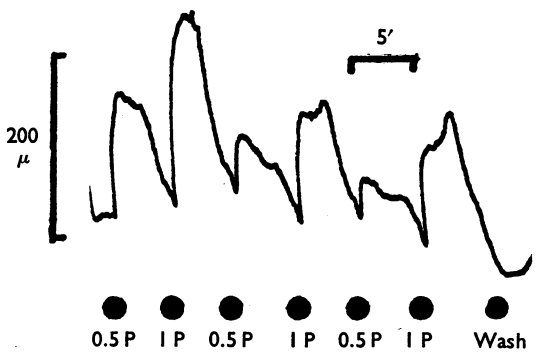

Fig. 5. As Fig. 2. Diluted Locke solution. No antagonist. Substance P was applied in alternate doses of 0.5 and 1.0 u./ml. without washing until the end.

been inactivated by the intestine. The second contraction was, however, smaller than the first, and successive contractions became smaller. This experiment suggests that there was also some tachyphylaxis.

Acetylcholine. Concentrations of acetylcholine between 10^{-9} and 10^{-8} caused a rapid contraction. This action was abolished by hyoscine or atropine (10^{-8}) so that the dose of acetylcholine had to be increased about 100 times to get similar effects (Fig. 2). This concentration of atropine reduced the effect of substance P; hyoscine was preferred since it appeared to be more specific.

Catecholamines. The muscle was inhibited by adrenaline (10^{-8}) or by noradrenaline (5×10^{-7}). The ratio of the concentrations of these two drugs causing equal effects was generally about 50 to 200. This ratio thus appears to be similar to that for the rat uterus and larger than that for the hen rectal caecum or for most other tests.

The effect of adrenaline was diminished or abolished when ephedrine (5×10^{-6}) was present in the bath, but the effect of noradrenaline was little, if at all, affected by this dose. Larger concentrations of ephedrine caused contractions of the muscle.

Ephedrine can thus be used to distinguish between effects due to adrenaline and effects due to noradrenaline, but cannot be used to exclude effects due to catecholamines when other substances are studied.

The effects of both adrenaline and noradrenaline were abolished by dichloroisoprenaline. When this substance was present in the bath in a concentration of 5×10^{-6} the relaxing effect of adrenaline was abolished and higher concentrations of adrenaline caused contraction. The relaxing effect of noradrenaline was also abolished by dichloroisoprenaline, but, in its presence, large doses of noradrenaline still caused relaxation (Fig. 6). Phenoxybenzamine (10^{-6}) did not affect the response to adrenaline or noradrenaline.

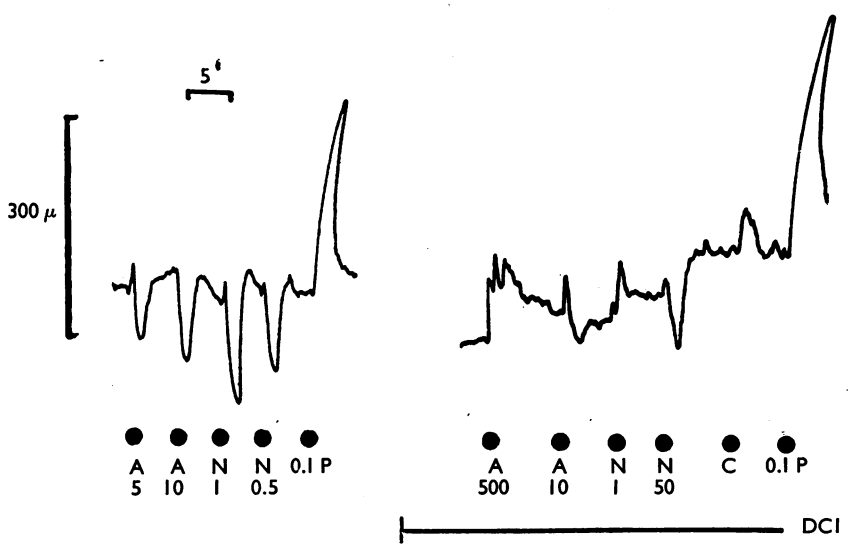

Fig. 6. As Fig. 2. Diluted Locke solution containing hyoscine (10^{-8}), adenosine triphosphate (10^{-5}) and methysergide (5×10^{-8}); A=adrenaline (ng/ml.); N=noradrenaline (μg/ml.); P=substance P u./ml.; C=control (no drug); DCI=dichloroisoprenaline, 5 μg/ml. (antagonizes A and N).

These results suggest that the inhibitory effect of the catecholamines depends on an action on β receptors. It is possible that high concentrations of ephedrine inhibit this action on β receptors. The effect of adrenaline was more easily inhibited than that of the much larger amounts of noradrenaline.

5-Hydroxytryptamine. The threshold concentration of 5-hydroxytryptamine causing contraction of the muscle was between 10^{-9} and 5×10^{-8}. A concentration of 1 to 2×10^{-8} was about equivalent to substance P 0.1 u./ml.

Tryptamine in larger doses also caused a contraction followed by relaxation in spite of the continued presence of tryptamine in the bath. Muscles which had been desensitized to tryptamine in this way were also insensitive to 5-hydroxytryptamine. This muscle thus resembled guinea-pig ileum in its response to these drugs

(Gaddum, 1953a). However, the relaxation of the goldfish intestine was often not complete in the presence of tryptamine and responses to other drugs became erratic.

Methysergide proved a more active and reliable antagonist than tryptamine. When it was present in the bath fluid in a concentration of 5×10^{-8} the muscle was specifically desensitized to 5-hydroxytryptamine so that the dose had to be increased 25 to 50 times (Fig. 7). This effect of methysergide developed in the course of about 1 hr.

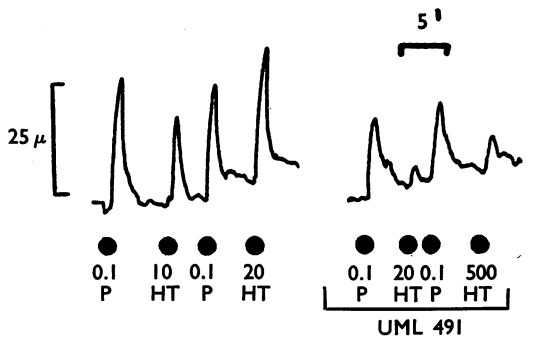

Fig. 7. As Fig. 2. Diluted Locke solution containing hyoscine (10^{-8}), adenosine triphosphate (10^{-5}) and dichloroisoprenaline (5×10^{-6}). HT=5-hydroxytryptamine (10^{-6}); UML 491= methysergide (5×10^{-8}). Antagonizes 5-hydroxytryptamine.

Histamine. The goldfish intestine is comparatively insensitive to histamine. A concentration of 10^{-6} sometimes caused a small contraction which was reversed by mepyramine. A concentration of 10^{-5} caused relaxation of the muscle. These small effects of large doses are unlikely to interfere with assays of other substances.

Adenosine compounds. The main effect of adenosine, adenosine 5-monophosphate and adenosine triphosphate was a contraction, sometimes following a brief relaxation. There was no marked and consistent difference between the effective concentrations of the three substances (about 10^{-6}), though adenosine triphosphate was generally less effective than the other two and its action was quicker.

The action of adenosine was abolished by atropine or hyoscine (10^{-8}). When adenosine (10^{-6}) was present in the bath fluid, the muscle was less sensitive to additional adenosine, but still sensitive to adenosine triphosphate.

The action of adenosine monophosphate was only reduced 5 times by hyoscine (10^{-8}). When adenosine triphosphate (10^{-5}) was also present in the bath fluid the effect was further reduced so that in the presence of both hyoscine and adenosine triphosphate the effective dose was increased 25 times.

The action of adenosine triphosphate (10^{-6}) was not affected by atropine. A concentration of 10^{-5} of adenosine triphosphate in the bath fluid caused an immediate powerful contraction followed by partial relaxation in the presence of the drug. The muscle was now 10 times less sensitive to additional doses of adenosine triphosphate (Fig. 8).

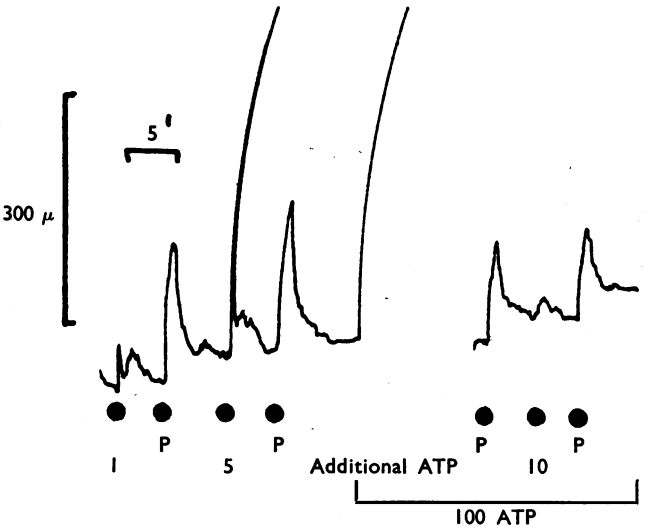

Fig. 8. As Fig. 2. Diluted Locke solution containing hyoscine (10^{-8}). P=substance P, 0.1 u./ml.; ATP=adenosine triphosphate 10^{-7}. Additional doses less effective in the presence of excess.

The presence of these concentrations of adenosine compounds did not diminish the effect of substance P. In some experiments it appeared to increase it.

Other polypeptides. Synthetic bradykinin has no action on the goldfish intestine except in high concentrations (2 to 10×10^{-6}). Oxytocin was without any effect in the concentrations tested (up to 0.1 u./ml.). Vasopressin caused a contraction when the concentration was 0.02 u./ml. and is thus the only one of these natural polypeptides which might possibly complicate assays of substance P.

Trypsin and chymotrypsin. Low concentrations of trypsin (0.2×10^{-6}) caused a contraction of the intestine, and this effect was antagonized by the soyabean trypsin inhibitor. In the experiment shown in Fig. 9 the intestine contracted in the presence of trypsin, but gave no response when a 250 times larger dose of trypsin was mixed with trypsin inhibitor before adding it to the bath. The response to trypsin reappeared within a few minutes when the inhibitor was removed from the bath.

The trypsin inhibitor had little or no effect on the response to bradykinin or substance P, so that it is possible to use it in experiments on the effect of trypsin on these substances which are present in tissue extracts. The extract is first incubated with trypsin, and the inhibitor is then added and the solution assayed for residual activity. The best method of carrying out this assay is probably to compare this incubated solution with another solution which is identical with it in all respects except that the inhibitor was added before incubation. The interpretation of such experiments is not complicated by the possible effect of the inhibitor on the gut, or by unspecific losses during incubation, or by other substances which may be present in the extract and alter the response of the intestine. It is known that trypsin destroys substance P, but not bradykinin, so that this test may be used to distinguish between the two substances. In one experiment, for example, incubation for 30 min

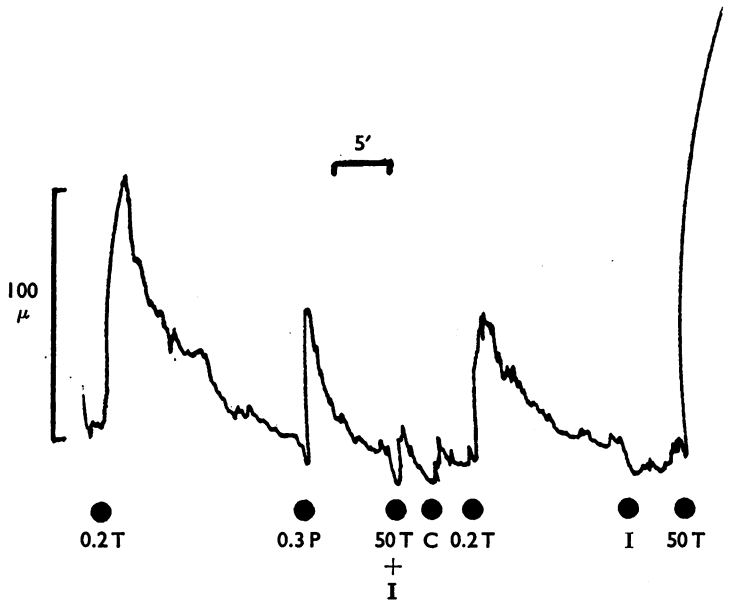

Fig. 9. As Fig. 2. Diluted Locke solution containing hyoscine (10^{-8}), adenosine triphosphate (10^{-5}) and methysergide (5×10^{-8}). P=substance P, u./ml.; T=trypsin, μg/ml.; I=soyabean trypsin inhibitor (5×10^{-4}). Antagonizes trypsin.

at 37° in the presence of trypsin (5×10^{-5}) almost completely abolished the effect of a preparation of substance P and only caused a slight loss of that of bradykinin. This slight loss might possibly have been due to a small trace of chymotrypsin in the preparation of trypsin, though there was no other evidence of this.

Chymotrypsin (5×10^{-6}) had no direct effect on the intestine, and reduced the effect of both substance P and bradykinin by 80% when incubated for 30 min at 37° C.

Unidentified substance. Simple watery extracts of guinea-pig brain contained some other unknown substance which caused a contraction of goldfish intestine even when much diluted. The effect of this substance overshadowed the effect of all the other substances known to be present in these extracts, so that a response was obtained with a dose corresponding to as little as 10 μg of fresh tissue. Similar effects were obtained with an extract of guinea-pig liver. The properties of this unknown substance are being studied in the hope of finding a method of separating it quantitatively from substance P.

Experiments with push-pull cannulae. The methods described in this paper are suitable for detecting active substances in the small volume of fluid which is obtained from a push-pull cannula (Gaddum, 1961). In this apparatus Locke solution runs through a fine needle into a tissue and is then collected through a tube surrounding the needle, so that a small volume of tissue is continually washed and substances released can be collected. Some experiments have been done with the object of detecting the release of substance P in the nuclei gracilis and cuneatus of a dog,

in the hope of obtaining evidence in favour of the theory of Lembeck (1953) that substance P is the chemical transmitter liberated by the first sensory neurone. In most of the experiments there was no evidence of the release of any substance acting on the goldfish intestine, and it is possible that the few positive results were artefacts, but the methods used will be briefly described.

The dogs were anaesthetized with allobarbitone (Dial) and the medulla oblongata was exposed by removing a small area of bone at the back of the skull. Push-pull cannulae were inserted through the pia to a depth of 1 to 5 mm at various points both above and below the obex and at different distances from the midline. Locke solution was run through the cannulae at a rate of 0.5 to 2 ml. in 10 min and collected in a centrifuge tube standing in ice. The samples collected sometimes contained a visible trace of blood, but were usually quite clear. These samples were diluted with an equal volume of a diluting fluid, which consisted of distilled water, containing double concentrations of any antagonists which were present in the fluid bathing the intestine. It was usually possible in this way to avoid artefacts, so that fluids which had run through the cannula before it was placed in the tissue had no action on the intestine. In most experiments the diluting fluid contained hyoscine 2×10^{-8} and dichloroisoprenaline 10^{-5}. In some of the earlier experiments stimulation of sensory nerves in the fore and hind limbs appeared to release small quantities of a substance which caused contraction of the goldfish intestine, but this substance differed from substance P in the fact that it was unstable and acetone soluble, and in the last 6 experiments no activity was detected at all.

DISCUSSION

The approximate concentrations of various substances which cause a small effect in 4 tests for substance P are shown in Table 1. The sensitivity of these tissues varies widely and the figures only give a rough indication of average values. All 4 tests are sensitive to about the same concentration of substance P, though the

TABLE 1

APPROXIMATE CONCENTRATIONS (NG/ML.) OF VARIOUS SUBSTANCES REQUIRED TO PRODUCE SMALL EFFECTS ON DIFFERENT TISSUES

Brackets denote inhibition

	Rat uterus	Guinea-pig ileum	Hen rectal caecum	Goldfish intestine
Substance P (u./ml.):	0·1–0·5	0·1–0·5	0·1–0·5	0·05–0·1
Polypeptides:				
Bradykinin	0·1	1	>10,000	2,000–10,000
Angiotensin	0·2	1	100	$>10^4$
Oxytocin	0·2	>500	>400	>200
Vasopressin	4	500	360	40
Other substances:				
Acetylcholine	250	10	5	1–10
Histamine	(10^4)	1	10	1,000
5-Hydroxytryptamine	10	40	100–1,000	10–50
Adrenaline	(0·05)	(10)	(1)	(10)
Noradrenaline	(10)	(20)	(40)	(500)
Adenosine triphosphate	1,000	(100)	(400)	1,000

goldfish intestine is slightly more sensitive and has the advantage that it can easily be used in a microbath with a volume of 0.05 ml. There is no other test which will detect 1 m-u. of substance P. This corresponds to about 30 pg of the preparation described by Franz, Boissonnas & Stürmer (1961).

The data given in Table 1 for other substances are taken from various sources. The figures for the rat uterus are mostly from Gaddum, Peart & Vogt (1949). The doses of bradykinin for rat uterus and guinea-pig ileum are from Elliott, Horton & Lewis (1960); in our experience the guinea-pig ileum is about 10 times less sensitive than this. The figures for angiotensin on these two tissues are from Gross & Turrian (1960). The figures for hen rectal caecum were mostly obtained by Cleugh (1961). Using pure synthetic bradykinin the dose of bradykinin was about 10,000 ng/ml. It may be calculated from the data of Van Arman & Miller (1961), who used an impure preparation made from trypsin, that the hen rectal caecum was about 1,000 times more sensitive than this. This calculation agrees with other evidence which suggests that partially purified preparations of bradykinin may contain a second active substance with an action on hen rectal caecum.

The goldfish intestine is comparatively insensitive to histamine and to the other polypeptides likely to be present in tissue extracts. If suitable antagonists are added, it may be made insensitive to other substances (Table 2), but it will be seen

TABLE 2

CONCENTRATIONS (μG/ML.) OF VARIOUS SUBSTANCES REQUIRED TO PRODUCE SMALL EFFECTS ON THE GOLDFISH INTESTINE IN THE ABSENCE AND PRESENCE OF ANTAGONISTS

Concentrations of antagonists in μg/ml. Dichloroisoprenaline=D.C.I. Adenosine triphosphate=A.T.P.

Agonist		Antagonist		
	Without antagonist	With antagonist		
Acetylcholine	0·01	1	Hyoscine	0·01
5-Hydroxytryptamine	0·015	0·4	Methysergide	0·05
Adrenaline	0·01	0·05–0·1	D.C.I.	5
Noradrenaline	0·5	100	D.C.I.	5
Adenosine	2	>1,000	Hyoscine	0·01
Adenosine mono-phosphate	2	50	Hyoscine +A.T.P.	0·01 10
Adenosine triphosphate	0·2–1	10	A.T.P.	10

that the effects of all these antagonists can be surmounted by increasing the dose of the agonist. In the presence of the antagonists these substances are unlikely to affect the results unless they are present in very high concentrations, but it would clearly be unwise to conclude that any effects were due to substance P without confirmatory evidence.

The possibility that the effects are due to acetylcholine can sometimes be excluded by estimating the concentration of this substance by some other method such as that based on the leech (Szerb, 1961). Another way is to compare unknown solutions on the goldfish intestine with standard substance P, both in the absence and presence of hyoscine. Similar tests may be used to exclude 5-hydroxytryptamine or adenosine compounds.

The active substance may also be tested for solubility and stability and by paper chromatography or electrophoresis in comparison with authentic substance P. It should be destroyed by trypsin, and a test for this is shown in Fig. 9.

When extracts of guinea-pig brain were tested, the apparent concentration of substance P measured with the goldfish was so large that it should have been easily detected by various other methods. It is clear that this effect was not due to substance P, but to some other substance. It will not be possible to get reliable estimates of substance P with the goldfish until the effects of this unknown substance can be excluded.

We are indebted to J. Lucas, who did the first experiments with the intestines of various species of animal and gave patient help later.

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