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SUBSTANCE P IN BRAIN EXTRACTS

By JOAN CLEUGH, J. H. GADDUM, A. A. MITCHELL, M. W. SMITH* and V. P. WHITTAKER

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

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Von Euler & Gaddum (1931) found that extracts of intestine or brain caused a contraction of rabbit intestine and a fall of rabbit blood pressure, both of which were only partially abolished by atropine. These authors concluded that both tissues contained an unknown substance having both effects. Gaddum & Schild (1934) studied this substance further and proposed that it should be called substance P (SP). Later work has been reviewed by Haefely & Hürlimann (1962).

During recent years SP has been estimated by its effect on guinea-pig ileum in the presence of atropine and an antihistamine drug (Douglas, Feldberg, Paton & Schachter, 1951; Pernow, 1951). 5-Hydroxytryptamine (5-HT) may be present in extracts and cause contraction of guinea-pig ileum, but this complication can be avoided by desensitizing with tryptamine (Gaddum, 1952). This causes a temporary contraction of the muscle, after which it is insensitive to 5-HT. This method has been used in surveys of the distribution of SP in the brain (Zetler & Schlosser, 1955) and we used it in 1960, as reported by Gaddum (1961) and Whittaker (1963) to survey the distribution of SP among particles separated by centrifugation from homogenates of guinea-pig brain by the methods described by Hebb & Whittaker (1958) and Gray & Whittaker (1962). The distribution of SP appeared to be similar to that of acetylcholine; the same result has been obtained by others (Lembeck & Holasek, 1960; Kataoka, 1962; Ryall, 1962*a*, *b*).

We tried to confirm that the substance measured by these tests was in fact SP by parallel assays on other test objects, and soon obtained results which threw doubt on the estimates and caused us to withhold publication. This method of parallel assays has given satisfactory results, when applied to comparatively pure preparations of SP. It provided part of the evidence that SP from brain is identical with SP from intestine (Amin, Crawford & Gaddum, 1954; Eliasson, Lie & Pernow, 1956). When Franz, Boissonnas & Stürmer (1961) made a highly purified preparation, they

* Wellcome Research Fellow.

estimated its activity as 30,000–35,000 units/mg, in terms of the standard IS (see Methods) by its effect on guinea-pig ileum, fowl rectal caecum and rabbit's blood pressure. The fact that these tests agreed quantitatively with one another suggested that the effects were all due to one substance. Cleugh & Gaddum (1963) compared another purified preparation with IS and found that the results with guinea-pig intestine, fowl rectal caecum and goldfish intestine were not widely different.

When attempts were made to apply the same technique to crude tissue extracts, the results varied widely and it became clear that tissues contain unknown substances which complicate the results. It should be possible to devise tests which would not be affected by these substances, but this has proved difficult.

METHODS

Assays

The four tests used in this work were:

(1) Guinea-pig ileum in a 2 ml. bath in Tyrode solution containing atropine (10^{-7}) to antagonize acetylcholine, mepyramine (10^{-6}) to antagonize histamine and tryptamine (10^{-5}) to antagonize 5-HT. In some experiments lysergic acid diethylamide (LSD) (5×10^{-8}) was used instead of tryptamine; this causes partial block.

(2) Fowl rectal caecum (Cleugh, Gaddum, Holton & Leach, 1961) in a 5 ml. bath of Tyrode solution with atropine (10^{-7}), methylsergide (UML 491, 1-methyl-lysergic acid butanolamide) (5×10^{-8}) to antagonize 5-HT, ephedrine (5×10^{-6}) to antagonize adrenaline and noradrenaline, and usually adenosine monophosphate (AMP) to antagonize adenosine compounds.

(3) Goldfish intestine (Gaddum & Szerb, 1961) in a 0·05 ml. bath of Locke's solution diluted 1:2, containing hyoscine (10^{-8}), methylsergide (5×10^{-8}), dichloro-isoprenaline (DCI) (5×10^{-8}) to antagonize adrenaline and noradrenaline and ATP (10^{-5}).

(4) Rat uterus in a 2 ml. bath containing atropine (10^{-6}) and LSD (10^{-8}). In some experiments the volume of the bath was 0·05 ml.

Subcellular fractionation

A homogenate (*H*) of guinea-pig's brain in sucrose was prepared and fractionated as described by Gray & Whittaker (1962). In some of the present experiments the distribution of bound acetylcholine and succinate dehydrogenase was determined in the fractions as described by Whittaker (1959). Fractions were deproteinized before being assayed for SP by adjusting to pH 4 with N/3-HCl, heating at 100° C for 10 min and centrifuging to remove denatured protein.

Fractions P_1, P_2 and P_3 were precipitates at increasing speeds of centrifugation and S_3 was the supernatant. It is unfortunate for the present discussion that the same letter (P) is used as a name for the substance studied and also for the fractions into which brain homogenates are separated by centrifugation. P_1 consists largely of nuclei and P_3 of microsomes. P_2 is subdivided in density gradients into fraction *A*, which floats on 0·8 M sucrose, fraction *B*, which lies between 0·8 and 1·2 M sucrose, and fraction *C*, which sinks in 1·2 M sucrose. *A* contains myelin fragments and *C* contains mitochondria. Fraction *B* consists largely of torn-off nerve endings and has been shown to be rich in acetylcholine, choline acetylase, 5-HT (Hebb & Whittaker, 1958; Whittaker, 1959) and noradrenaline (Chruściel, 1960).

When these fractions were run through chromatographic columns they were spun again

at 40,000 rev/min and the supernatant was discarded so as to get rid of most of the sucrose. The precipitate was suspended in water made acid (pH 4) and placed in boiling water for 10 min to liberate the active substances. They were then centrifuged and the supernatants were neutralized before testing.

Anion exchange columns

The columns measured $10 \times 0{\cdot}2$ cm and contained the resin Dowex 1 in the chloride form (Cohn, 1950). The resin, which was 10% cross-linked, had been prepared by washing with N-HCl, followed by distilled water, until the effluent no longer gave a positive test for chloride ions with $AgNO_3$ solution. Slightly acid solutions (pH 5–6) were passed through the columns at a rate of about 0·2 ml./min. This rate of flow was obtained by attaching a short length of polythene tubing below the columns. Each column was washed with 0·5 ml. of distilled water and the final volume of the effluent was measured.

Standards

One cause of error in assays of SP has been the lack of a pure preparation to use as a standard. The standard which is most widely accepted at the present time is one made by Messrs Hoffmann La Roche from horse intestine by the methods described by Pernow (1953). These involve precipitation of SP with ammonium sulphate from a crude concentrated extract (von Euler, 1942), followed by adsorption on alumina from 70% methanol and elution by water. On drying an active and stable preparation results, which is comparatively free from simple known substances such as histamine and adenosine compounds which are likely to interfere with assays, but which contains more than one active polypeptide (Gaddum, 1961; Zetler, 1963). This preparation was adopted by von Euler & Gaddum in 1959 as an unofficial standard containing 75 u./mg and will be referred to as IS. Some IS has been fractionated further on alumina as described in the Results section, to give two preparations, IS_a and IS_b (previously referred to as *B* and *D* respectively by Gaddum, 1961).

A local standard (LS) made from horse intestine by the same procedure has been used extensively in this work. During the preparation of a second local standard the ammonium sulphate precipitate, taken up in 70% methanol, was eluted from alumina with different concentrations of methanol to give 4 fractions referred to as F_1, F_2, F_3, and F_4.

Recently we were given two highly purified preparations of SP. One was prepared from horse intestine and is called PP_1; the second was made from cattle brain and is called PP_2.

PP_1. This consisted of 1 mg of an extract of horse intestine made in the laboratories of Messrs Hoffmann-La Roche. It was given to us by W. Haefely, who said that it had been estimated to contain 50,000 u./mg. It was used by Cleugh & Gaddum (1963) in experiments which confirmed that these purified preparations are unstable in simple watery solutions. They came to the conclusion that this was due to adsorption on glass and found that the preparation was much more stable in solutions containing gelatin. The maximum activity found at that time was 6500 u./mg; it will be seen that higher estimates were obtained in an experiment described below.

PP_2. This consisted of 0·5 mg of an extract (PA XLIII 2) made from cattle brain by H. Zuber of the Ciba Laboratories and given to us by R. Jaques. In April 1962 it was estimated to contain 40,000 u./mg when diluted with Tween 80 (50 μg/ml.) to increase the stability of SP.

RESULTS

Homogeneity of the standards

Extracts of intestine or brain may be fractionated by running different concentrations of methanol or ethanol through columns of alumina (Pernow, 1953). When the concentration of alcohol is over 70% SP

remains on the column, but when it falls to 40–50 % SP is eluted. Zetler (1961, 1963) has used this method to make SP and found that extracts of intestine contained a number of substances, causing contraction of guinea-pig ileum, but with different rates of flow in paper chromatography.

We have found that crude preparations of SP made from intestine commonly contain a second substance with a relatively large effect on rat uterus. The first indication of this came from further fractionation of IS and the results have already been briefly discussed (Gaddum, 1961). The material from which this standard was made was dissolved in 80 % ethanol and run through a column of alumina. The active fraction was eluted with 70, 60 and 50 % ethanol, and with water. IS_a consisted mainly of material which had passed through the column in 80 % ethanol and not been adsorbed. IS_b contained substances eluted by 50 % ethanol. Assays of these preparations are shown in Table 1.

TABLE 1. Assays (u./mg) of IS_a and IS_b by comparison with IS which contained 75 u./mg

Method	IS_a	IS_b
Rat uterus	110	180
Guinea-pig ileum	37	340
Fowl rectal caecum	28	375

If IS and the fractions IS_a and IS_b obtained from it had contained only one active substance, estimates of its concentration in IS_a (or IS_b) by these three methods should have agreed with one another within the error of the methods. In actual fact, in terms of IS the estimate for IS_a with the rat uterus was about four times the estimate with the fowl rectal caecum. This ratio is too large to be due to error, and there is thus evidence that IS_a differs qualitatively from IS. With preparation IS_b the estimates also differed among themselves more than could be accounted for by error. For example, the results obtained with the rat uterus in different experiments were 188, 188 and 163 u./mg and the results obtained with the fowl rectal caecum were 288, 349, 352 and 375. These figures differ among themselves, but it is clear without further calculation that there is a significant difference between the two groups. The simplest explanation of these results is that the fraction IS_a, obtained by elution with a high concentration of alcohol, contained a substance especially active on rat uterus, and fraction IS_b contained a substance especially active in the other two tests.

This conclusion was confirmed in an experiment in which 6·1 kg of horse ileum was extracted to make a local standard by the usual method. The material precipitated by ammonium sulphate was dissolved in 70 % methanol and passed through a column of alumina. Four fractions F_1–F_4

were obtained by elution with different concentrations of methanol and assayed by four methods in comparison with LS (see Table 2). Fraction F_1 is especially active on rat uterus, which gave an estimate 31 times higher than the estimate with fowl rectal caecum. Fraction F_2 was small in bulk and high in activity in three of the four tests. It presumably contained a particularly high concentration of SP. Fraction F_4 was made by the same process as IS. The four estimates cannot be said to agree completely, but they show better agreement than the results with the other fractions, and F_4 presumably contained approximately the same mixture of substances as the standard. According to tests on the rat uterus, 68 % of the total activity was in F_1 and only 12 % in F_4, and, according to tests on the fowl rectal caecum, 56 % of the total activity was in F_4 and only 6 % in F_1. If the rat uterus had been used to monitor the purification of SP, it is possible that a different peptide would have been isolated.

TABLE 2. Estimates by four methods of the activity of fractions of extract of horse intestine obtained by elution with methanol from a column of alumina

	F_1	F_2	F_3	F_4
Methanol (%)	70	60	50	0
Dry weight (mg)	855	153	1225	885
Activity (u./mg)				
Rat uterus	80	20	20	15
Guinea-pig ileum	25	75	11	12
Goldfish intestine	15	80	25	25
Fowl rectal caecum	2·5	67	4	25

The nature of the active substances in F_1 has not been studied in detail. It might contain bradykinin, which also has a large effect on rat uterus and little or no effect on fowl rectal caecum. It was compared with synthetic bradykinin in parallel assays. According to tests on the guinea-pig ileum, it contained 322 ng of bradykinin/mg and, according to tests on the rat uterus, it contained 60 ng. This discrepancy shows that bradykinin was not the only active substance in F_1, but does not exclude the possibility that it may have been present.

The conclusion that IS contains a second substance with a powerful effect on rat uterus was confirmed by Cleugh & Gaddum (1963) in the experiments with PP_1 mentioned above, which showed that this preparation had comparatively little effect on rat uterus. This conclusion has been confirmed in a more extensive experiment.

PP_1 made from horse intestine and PP_2 made from cattle brain were compared directly with one another and also with IS by four different tests on the same day. Both preparations were dissolved in water, containing 0·5 % gelatin, in order to stabilize SP, and all the diluting fluids contained the same concentration of gelatin. This amount of gelatin had

no effect by itself, when present in the solutions added to the baths containing guinea-pig intestine or fowl rectal caecum. It also had no effect on goldfish intestine or rat uterus when present as a 0·5 % solution in the fluid actually present in the bath. The same concentrations were present in the standard solutions, since proteins have been found to increase the effects of SP in certain conditions (Cleugh & Gaddum, 1963).

The results of this experiment are shown in Table 3. The ratios of activity (PP_1:PP_2) on the first three tissues varied between 2·52 and 4·16. It seems probable that the true ratio was about 3, and that the variations about this figure have no meaning. This observation confirms the evidence that SP from brain is identical with SP from intestine (Amin *et al.* 1954; Eliasson *et al.* 1956). The ratio for rat uterus was higher, but

TABLE 3. Assays of purified preparations PP_1 (horse intestine) and PP_2 (cattle brain) against IS and against one another

	Guinea-pig ileum	Goldfish intestine	Fowl rectal caecum	Rat uterus
Estimated activity* (u./mg)				
PP_1	37,000	27,000	13,300	(1,200)
PP_2	14,700	10,700	4,400	150
Calculated as percentages				
PP_1	100	73	36	3·2
PP_2	100	73	30	1
Ratio of activity PP_1:PP_2				
Calculated	2·59	2·52	3	—
Direct comparison	3	2·46	4·16	8

* In terms of IS

this fact is of doubtful significance. Owing to the insensitivity of the rat uterus and the shortage of material, the assays were done in a small bath (0·05 ml.), but in spite of this the assay was not accurate. PP_1 in a concentration of 1·5 μg/ml. caused a maximum contraction, while 1 μg/ml. had no effect. PP_2 in a concentration of 10 μg/ml. had an intermediate effect. This suggests that the ratio was between 6·7 and 10.

The results obtained by comparing preparations PP_1 and PP_2 with IS were surprising and not always in good agreement with previous evidence. They confirm that the standard contains some other pharmacologically active substance besides SP. This is most clearly shown by the low figures obtained with the rat uterus, which show that the second substance, active on the uterus and assumed to be present in IS, has a comparatively large effect on this tissue. On the other hand, the other figures show unexpected variations between the results obtained with the other tissues. Franz *et al.* (1961) and Cleugh & Gaddum (1963) both obtained approximate agreement with these tissues when they compared highly active preparations with less active ones. Presumably the differences shown in Table 1 did not appear, either because the substances causing them were

not present, or because the particular tissues used in these experiments were not sensitive to them.

The highest estimates of activity were obtained with guinea-pig ileum, which suggests that this tissue is particularly sensitive to SP and insensitive to other substances present in IS. The figure of 37,000 is the highest we have obtained with any preparation of SP. We do not know why it is so much higher than the estimate of Cleugh & Gaddum (1963), but there were minor differences in the experimental conditions.

The assays on the goldfish intestine were each based on 16 responses, while those on the guinea-pig ileum and the fowl rectal caecum were each based on 8 responses. The error ($P = 0{\cdot}05$) was estimated to be between 15 and 25 % in these three tests. The results obtained on the three test organs were significantly different—the difference between 27,000 and 37,000 was just significant ($P = 0{\cdot}05$).

The figures given as percentages in Table 3 are based on the assumption that the action of IS on guinea-pig ileum was entirely due to SP itself. They show what proportion of the effect of this standard in the other tests was due to SP. For example, according to the guinea-pig intestine, one unit of IS was equivalent to 27 ng of preparation PP_1 and it is assumed that it actually contained this amount of SP. According to experiments on the goldfish intestine, one unit of IS was equivalent to 37 ng of preparation PP_1, of which 27 ng or 73 % was due to SP and the rest due to some other substance. It is remarkable that the two figures calculated in this way from experiments with PP_1 and PP_2 agreed with one another. Both were obtained from the same goldfish intestine; it is probable that another goldfish intestine would give different results, because the relative activities of SP and the impurities would be different. Similar considerations apply to the other test organs. These results confirm other evidence that guinea-pig intestine is particularly suitable for the assay of SP.

Rat colon as a test organ

Bisset & Lewis (1962) found that this tissue was especially sensitive to a preparation of SP made from ox intestine (15 u./mg) and comparatively insensitive to bradykinin, angiotensin, oxytocin and vasopressin. They suggest that it will provide a specific test for SP. In their experiments the concentration causing contractions was about 0·05 u./ml. In our experiments with the preparation F_4 (Table 2), the dose varied from 0·05 to 0·25 u./ml. The rat colon was, however, much less sensitive to preparation PP_1, the dose of which was about 1 u./ml. In one experiment the potency of F_4, in terms of the pure P, was over 20 times higher when tested on rat colon than when tested on guinea-pig ileum. A similar result was obtained by Jaques (1963), who found that SP from brain had a

comparatively feeble effect on the rat colon. The rat colon does not seem, therefore, to be sensitive to SP itself but could, perhaps, be used to assay some other substance present as an impurity in certain preparations of SP.

Interfering substances in extracts of brain

The estimation of SP is complicated by the fact that the tissues sensitive to it are also sensitive to the following substances which may be present in tissue extracts:

Acetylcholine acts like SP on smooth muscle, but its effect can be abolished by atropine.

5-*HT* also has a similar action, which can be abolished by antagonists such as methylsergide and other lysergic acid derivatives. Guinea-pig ileum may also be desensitized with tryptamine, which causes a temporary contraction, followed by specific desensitization to both tryptamine and 5-HT.

Adrenaline and noradrenaline cause inhibition of intestinal muscle or rat uterus. Their action on goldfish intestine can be antagonized by DCI. Their action on fowl rectal caecum can be diminished by ephedrine.

Adenosine compounds. The importance of these compounds in assays of SP was discussed by von Euler & Gaddum (1931) and has recently been emphasized by Laszlo (1960, 1963). Extracts of tissues may contain adenosine triphosphate (ATP), adenosine diphosphate (ADP), AMP or adenosine, all of which have pharmacological actions and may interfere with assays. Phosphate groups are liberated during autolysis, and the proportion of ATP depends on the speed at which the extract is made. This does not make much difference to the pharmacological effect, since these adenosine compounds generally have similar actions, though one or other of them may be slightly more active in any one test.

The effect of these compounds on the fowl rectal caecum can be abolished by maintaining a high concentration of AMP or ATP or both in the bath fluid (specific tachyphylaxis (Cleugh *et al.* 1961)). Their effect on the goldfish intestine can also be reduced by maintaining ATP in the bath (Gaddum & Szerb, 1961). When this has been done, adenosine compounds are unlikely to interfere with the assay, unless present in unusually high concentrations compared with SP.

Laszlo (1960, 1963) showed that the interfering substance in his extracts was AMP and that this substance could be removed from extracts by incubating them with AMP deaminase. We have tested the effect of this enzyme on our extracts and found, in some experiments, that AMP was destroyed by the enzyme and that SP was not, and that the activity of homogenates was raised after treatment with the enzyme. In other experiments the enzyme had no effect, possibly because these extracts contained

ATP, which is not inactivated by this enzyme. It is clear that the adenosine compounds in brain may complicate assays, but we came to the conclusion that AMP deaminase does not provide a reliable method of avoiding errors due to this cause. The use of anion-exchange columns, as described below, provides another method of avoiding complications due to adenine nucleotides.

Uridine diphosphate (UDP) and uridine triphosphate (UTP) in small doses both cause contraction of goldfish intestine, so as to obscure the effect of SP. These nucleotides must be removed from extracts if this tissue is used to assay SP (Gaddum & Smith, 1963). Their effect on the other tissues is comparatively small, but must be considered as a possible source of error.

Histamine is present in brain extracts in concentrations up to about 1 μg/g brain (Adam, 1961). It has little action on goldfish intestine or rat uterus and its action on guinea-pig ileum and fowl rectal caecum can be abolished by antagonists.

Prostaglandin. Physiologically active lipids are present in various tissues (Vogt, 1958). One of the most active is the fatty acid prostaglandin, the formula of which was determined by Bjergström & Samuelson (1962). Darmstoff, irin, the menstrual stimulant, and other substances, whose formulae are not known, also belong to this group.

Ambache, Reynolds & Whiting (1963) found a substance similar to irin (originally found in iris) in rabbit brain. Dr Ambache gave us several extracts to test on goldfish intestine. The action of the acetone extracts might be due to various substances, but one extract of iris, obtained by partition into ether at pH 3 gave interesting results. It caused a contraction similar to that due to 0·1 u. SP/ml. in a concentration corresponding to 2 mg of iris/ml. This result suggests that this tissue is as sensitive to irin as the most sensitive tissue used by Ambache, the hamster colon. The total dose in the 0·05 ml. bath came from only 0·1 mg of iris. These active fatty acids might interfere with the assay of SP.

Gangliosides. According to Bogoch, Paasonen & Trendelenburg (1962) brain gangliosides stimulate guinea-pig intestine and have other pharmacological actions.

Brain extracts. Toh (1963) has shown that chloroform-methanol extracts of brain contain three different unknown substances which stimulate plain muscle. There is thus no lack of potential interfering substances.

Subcellular fractions

The nerve-ending particles in Fraction *B* are easily broken mechanically or by weak acids, organic solvents, hypotonic solutions, or freezing and thawing. They are only stable when kept at a temperature near 0° C.

When they disintegrate acetylcholine is liberated and rapidly destroyed, unless an anticholinesterase is present. The acetylcholine in these particles is said to be present in a bound form, from which it must be liberated before it is estimated. SP is also present in a bound form in guinea-pig's brain, since (*a*) the activity of the high-speed supernatant is low and (*b*) particulate fractions have little action unless deproteinized by heating for 10 min at pH 4 and 100° C. All such fractions were treated in this way before testing. SP could also be released from particulate fractions by converting them into acetone powders and extracting with N-HCl, but this method had no advantage over heating at pH 4 and was more time-consuming.

TABLE 4. Subcellular distribution of SP in guinea-pig brain (u./g of brain)

Expt.	*H*	P_1	P_2	P_3	S_3	*A*	*B*	*C*
1	41·5	4·5	29·4	3·2	< 9·5	—	—	—
2	37·5	3·9	21·3	3·2	< 17	< 4·3	10·4	2·5
3	50	3·3	21·7	1·7	< 9·6	9·6	10·8	0·6
4	20	< 1·5	33·4	3·2	< 13	18·9	9·9	< 1
5	26	1·0	10·3	1·3	< 2·8	—	—	—
	(50)	(4·8)	(29·6)	(3·7)	—	—	—	—
6	71·9	4·5	21·0	1·0	5·2	7·7	21·6	0·2
	(71·9)	(5·1)	(47·5)	(1·0)	—	(13·0)	(25·2)	(0·7)
7	44	0·9	7·1	1·5	< 5·3	1·8	2·5	0·5
8	18·7	< 0·7	6·5	1·3	< 4·5	2·0	3·4	0·6
	(66·7)	(4·7)	(22·6)	(5·8)	(8·9)	(5·6)	(17·9)	(1·8)

Most of the results were obtained with guinea-pig ileum; those in brackets with fowl rectal caecum. For nomenclature of subcellular fractions, see Methods.

Table 4 shows the results of testing sucrose suspensions, which had been adjusted to pH 4 and boiled for 10 min to liberate SP. In most cases the assays were made on guinea-pig ileum by comparing the extracts with LS. In experiments 1–4 the distribution of bound acetylcholine and in experiment 2 the distribution of the mitochondrial marker succinate dehydrogenase were also followed. The distribution in experiments 1–3 of these two substances was similar to those already reported (Whittaker, 1959). In experiment 4 there was a delay in dividing the density gradient after centrifuging and some remixing of Fractions *A* and *B* must have occurred; both the acetylcholine and SP contents of fraction *A* were about twice as high as those of Fraction *B*. There was a close similarity between the acetylcholine and SP distributions. Counting fractions with activity below threshold as zero, the average percentage distribution of recovered activity in experiments 1–3 was, for acetylcholine; P_1, 15; P_2, 75; P_3, 6; S_3, 4; *A*, 9; *B*, 85; *C*, 6; the corresponding figures for SP were: 13, 78, 9, 0, 9, 81 and 10.

These results suggested that SP is concentrated in the nerve endings, like acetylcholine. However, the possibility must be borne in mind that SP, like lactate dehydrogenase (Johnson & Whittaker, 1963), is present in the nerve-ending fraction by virtue of the presence in this fraction of entrapped cytoplasm, and that the high concentration in S_3 which would be expected of a soluble cytoplasmic component not specifically localized in nerve-ending particles, is not attained owing to the destruction of free SP during the initial homogenization. This is unlikely, because in 5 determinations with the classical extraction procedure (extraction of an acetone powder with N-HCl) the SP content of guinea-pig brain was found to vary between 21·2 and 42·5 u./g (mean 29·6), which is within the range for sucrose homogenates reported in Table 4 with the same assay method (guinea-pig ileum).

In three experiments the same extracts were also tested on fowl rectal caecum and the estimates of activity were generally 2–3 times larger than estimates on guinea-pig ileum. This difference is probably explained by the presence of other pharmacologically active substances in the brain extracts.

Anion-exchange columns

Of the substances listed as likely to interfere with assays of SP in brain extracts the adenine and uridine nucleotides, the physiologically active lipids, gangliosides, and the substances (A_2, B and C) described by Toh (1963) are all acidic. It should be possible to remove these substances by passing brain extracts through an anion-exchange column leaving SP, which behaves as a base at pH 5·5, to pass through such a column. In five experiments 1 ml. of a solution containing 5–20 u. of IS in water was passed through a column and then assayed by its action on guinea-pig ileum. The mean recovery was 89 % (range 74–100), which was regarded as satisfactory.

Nucleotides are adsorbed from solutions of low ionic strength, but not from solutions containing high concentrations of salt. Solutions (2 ml.) containing AMP (100 μg/ml.) or UDP (50 μg/ml.) dissolved in distilled water or NaCl solution (0·5–7 % 1 g/100 ml.) were run through columns of Dowex 1 (10 × 0·2 cm). The percentage of nucleotide passing through the column was calculated by measuring the optical density of the effluent. Table 5 shows that virtually all the AMP or UDP was adsorbed when the concentration of NaCl was not higher than 1g/100 ml. In higher concentrations of NaCl adsorption was incomplete. Adenosine would not be expected to be adsorbed by such columns, and an experiment showed little or no loss on the column, even when the concentration of NaCl was only 0·5 g/100 ml.

The combined molar concentration of Na and K in a subcellular brain

fraction (P_2) derived from 1 g of brain/ml. was estimated with a flame photometer as 0·0165, which is equivalent to about 0·1 g NaCl/100 ml. It was therefore expected that these columns would remove nucleotides from P_2 fractions. This was tested in the experiment shown in Fig. 1.

TABLE 5. Adsorption of nucleotides on anion exchange resin under conditions of varying ionic strengths, based on the optical density measured at a wave-length of 260 mμ

NaCl (g/100 ml.)	Percentage of nucleotides detected in the perfusate	
	AMP	UDP
0	0·6	—
0·5	0·6	—
1·0	0·2	0·9
1·5	0·6	11·5
2·0	14·0	41·0
2·5	27·5	46·2
3·0	43·2	55·7
3·5	56·2	70·6
5·0	70·6	67·6
6·5	83·0	—
7·0	—	74·8

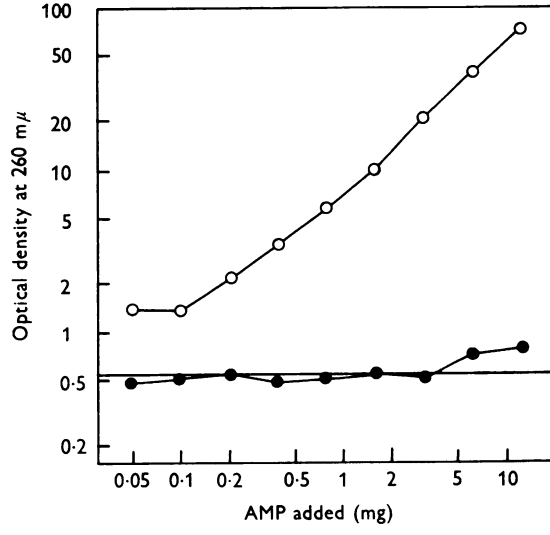

Fig. 1. The subcellular fraction P_2 from 1 g of brain was passed through anion-exchange columns. AMP (0·05–12·8 mg) was added to different samples. Estimates of optical density showed that the added AMP was practically all adsorbed. It was then eluted from the column by N-HCl and estimates of the optical density showed that the columns were not saturated by 10 mg of AMP. ●, effluent before elution; ○, eluate.

Quantities of AMP varying from 0·05 to 12·8 mg were added to P_2 fractions from 1 g of brain and run through columns. When 3·2 mg or less of AMP had been added, the optical density (measured at 260 mμ) of the

effluents was the same as that of the control tube, so that adsorption appeared to be complete. There was a small increase in the last two tubes, suggesting that less than 1 % of the AMP had passed through the columns.

The material on the columns was eluted with N-HCl; optical density measurements showed that the column adsorbed over 10 mg (or 30 μ-mole) of AMP. This is probably about equal to the total of adenine nucleotides in 10 g of brain extracted with precautions to avoid loss, and more than 10 times greater than the maximum amount present in the experiments described here.

TABLE 6. Potencies of guinea-pig P_2 fractions expressed as a ratio of the estimates given by the fowl rectal caecum to that given by the guinea-pig ileum, with crude or pure standard SP

	Crude standard		PP_1	
	Crude extract	After column	Crude extract	After column
	2·9	1·0	4·5	2·1
	2·3	0·77	3·8	4
	3·5	1·5	1·6	3·1
	1·9	1·4	5·4	3·1
	1·1	1·8	—	—
	2·2	—	—	—
	1·2	—	—	—
	2·7	—	—	—
Mean	2·25	1·29	3·82	3·08

The fowl rectal caecum was desensitized to adenosine compounds and the crude standards used were LS or IS. Estimates were made before and after passing through a column of Dowex 1 resin.

A series of P_2 fractions of guinea-pig brain were prepared and, in each case, half the fraction was reserved and half passed through an anion-exchange column. The SP equivalent of each half was estimated by various methods.

The estimates confirmed the conclusion that the fowl rectal caecum gives higher results than the guinea-pig ileum. Table 6 shows the ratio of these quantities, which was almost invariably greater than 1. The first three figures in the first column are calculated from the results in Table 4. The crude standard used for the other figures under this heading was IS, which is crude compared with PP_1. Calculations of t showed that the mean of the first column was significantly greater than the mean of the second column, which was not significantly different from 1. The effect of adsorption was to make these parallel tests agree and this seemed at first to be a satisfactory result, possibly due to the removal of AMP. This nucleotide diminishes the action of SP in both tests. Estimates with fowl rectal caecum were shown to be increased when the preparation was desensitized to adenosine compounds. Guinea-pig ileum cannot be desensitized in this way and was, therefore, expected to give low estimates.

On the other hand, Table 3 shows that IS contains an impurity which had, in this experiment at least, a comparatively large effect on the fowl rectal caecum, compared with the guinea-pig ileum. The agreement between the two tests, shown in the second column of Table 6 is, therefore, probably fortuitous. This is confirmed by the high figures in the third and fourth columns, where the extracts were compared with PP_1. The mean of the figures in the last column is significantly larger than the mean of the figures in the second column. This suggests that the brain extracts contained some other substance with a comparatively large effect on the fowl rectal caecum, resembling IS in this respect. These results suggest that the comparatively high potency of the brain extract on fowl rectal caecum is due to the presence of a substance with a comparatively large stimulating effect on this tissue. The results in Table 4 suggest that this substance is distributed in centrifuge fractions in the same way as SP.

According to Gaddum & Smith (1963) the goldfish intestine is particularly sensitive to uridine diphosphate (UDP) and may give estimates as high as 6000 u./g for the SP equivalent of extracts of guinea-pig brain. In the present experiments the mean of 3 estimates for the P_2 fraction was only 212 u./g brain and this was reduced to 103 by passage through the column. One reason for this low figure appears to be that UDP is not concentrated in the P_2 fraction, and another reason is that it is unstable. Attempts to study its distribution were, in fact, frustrated when it was found that the UDP equivalent of brain fell within 30 min of death even when the tissue remained *in situ*.

On the other hand the estimate with goldfish intestine was about 5 times the estimate with guinea-pig ileum, even after the extract had passed through an anion-exchange column. The column reduced the estimate from 212 to 103 and this reduction may have been due to the removal of UDP, but the action that was left was presumably due to something else besides SP.

In view of these facts it might be expected that estimates of the recovery of SP from extracts which had passed through the column would be erratic, and this was, in fact, the case; but the mean recovery in 9 experiments was estimated as 82% with fowl rectal caecum and 96% with guinea-pig ileum. The second figure might well have been over 100, since the column would remove AMP, but presumably there was not sufficient AMP in these fractions to affect the response of guinea-pig ileum.

DISCUSSION

The main object of this work was to decide whether assays of SP in tissue extracts by means of guinea-pig ileum did, in fact, give a true result. This method has been generally used without question, and it was pro-

posed to confirm the results by parallel assays on other tissues. This has not been achieved, since the three other tissues chosen all gave results complicated by other substances, and it has become apparent that the guinea-pig ileum does, in fact, provide the most specific test available.

The evidence shows that crude preparations of SP made from horse intestine by the usual method (including IS) contain a second active substance and that this is mainly responsible for the effects of such preparations on rat uterus, which is relatively insensitive to SP itself. Goldfish intestine and fowl rectal caecum are also sensitive to a second substance in IS, but it is not known how many such substances there are. The same may be said of brain extracts, which have more action on goldfish intestine and fowl rectal caecum than can be accounted for by their content of SP, as estimated by guinea-pig ileum.

The substances which interfere with these tests are assumed to have no action on guinea-pig ileum. It is a fortunate circumstance that the tissue which has been most widely used to estimate SP is found to give the most reliable estimate. The estimates with guinea-pig ileum in Table 4 provide the best estimate of SP possible at present. They were probably not affected by the small amounts of AMP present in these centrifuge fractions; this conclusion is confirmed by the fact that the mean recovery after passage through the columns was 96 %. The results in Table 4 confirm that the distribution of SP resembles that of acetylcholine. Activity was found in P_1, P_2 and P_3, but little or none was found in the supernatant (S_3), which was difficult to test because it contained much sucrose. The amount in each of these fractions was calculated as a percentage of the total amount recovered, and it was found that the mean percentages in P_1, P_2 and P_3 were 9, 81 and 10, respectively. The amount in each of the subfractions of P_2 was also calculated as a percentage of the total recovery in these 3 fractions, and it was found that the mean percentages in fractions *A*, *B* and *C* were 35, 58 and 7, respectively. These figures are similar to those given by Ryall (1962*a*, *b*) and by Kataoka (1962).

SUMMARY

1. Parallel quantitative assays show that most preparations of substance P (SP) are contaminated with other pharmacologically active substances.

2. The most specific of the four methods of assay used was the conventional method with guinea-pig ileum in the presence of antagonists for acetylcholine, histamine and 5-hydroxytryptamine. The activity is expressed in terms of the unofficial Standard of 1959 (IS), which has been widely accepted.

3. Acid contaminants, such as adenosine monophosphate, can be

removed from extracts by passing them through a small anion-exchange column, without appreciable loss of SP.

4. Purified SP has practically no action on rat uterus or colon, but IS has a marked action which is attributed to an impurity. This substance is present in crude extracts of intestine, but can be separated from SP.

5. There are 9 classes of pharmacologically active substances which may be present in extracts of brain, but whose effects can be excluded in tests for SP. These tests may, however, still be complicated by substances to which fowl rectal caecum and goldfish intestine are especially sensitive.

6. When subcellular particles are separated by centrifugation, SP (like acetylcholine, noradrenaline and 5-hydroxytryptamine) is concentrated in the fraction containing nerve endings.

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