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# ESTIMATION OF SUBSTANCE P IN MOUSE BRAIN. THE IDENTIFICATION OF INTERFERING NUCLEOTIDES IN THE EXTRACT

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Substance P has been extracted from brain by previously described methods and assayed on the guinea-pig isolated ileum. The concentrations showed a wide scatter which was not due to the bioassay or to variations in the extraction procedure. Adenosine-5′-monophosphate and adenosine-5′-triphosphate have been identified in the extracts by paper chromatography and by chemical reactions. The concentrations of these substances were sufficient to interfere with the assay of substance P, adenosine-5′-monophosphate diminishing and adenosine-5′-triphosphate increasing the contractions of the ileum due to substance P. Adenosine-5′-diphosphate was also detected in the extracts but its biological activity was insufficient to influence the estimations. The concentrations of adrenaline, noradrenaline, potassium ions, sodium chloride, 5-hydroxytryptamine, and γ-aminobutyric acid were below the threshold concentrations for interference with the assay of substance P.

The present work was started with the intention of determining the amount of substance P in mouse brain under various conditions. However, it soon became apparent that previously described methods of estimating substance P in brain were subject to large errors due to the presence of interfering substances of unknown composition. This paper describes the identification of these substances.

## METHODS

*Isolated smooth muscle preparations*

*Guinea-pig ileum.* This was used for the estimation of substance P by superfusion with Tyrode solution containing NaCl 8.00, KCl 0.20, $CaCl_2$ 0.20, $MgCl_2$ 0.01, $NaH_2PO_4$ 0.05, $NaHCO_3$ 1.00 and glucose 1.00 g/l.; atropine sulphate ($10^{-7}$) and mepyramine maleate ($10^{-6}$) were added to inhibit the actions of acetylcholine and histamine respectively. The length of ileum was taken from about 4 cm from the ileo-caecal junction. Animals of either sex were used, usually weighing between 200 and 300 g.

*Fowl rectal caecum.* Adenosine-5′-monophosphate was assayed in brain extract on fowl rectal caecum (Barsoum & Gaddum, 1935) in Tyrode solution containing atropine sulphate ($10^{-7}$) and mepyramine maleate ($10^{-6}$), in an organ-bath at 37° C.

*Rabbit jejunum.* Rabbit jejunum was suspended in an organ-bath at 37° C in modified Locke solution (NaCl 9.00, KCl 0.42, $CaCl_2$ 0.24, $NaHCO_3$ 0.50 and glucose 1.00 g/l. of distilled water) containing atropine sulphate ($10^{-7}$).

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*Rat uterus.* The animals were prepared by a subcutaneous injection of oestradiol monobenzoate (100 $\mu$g) 17 to 24 hr before being killed. A segment of uterine horn was either superfused or suspended in an organ-bath at 28° C. Locke solution (NaCl 9.00, KCl 0.42, $CaCl_2$ 0.24, $NaHCO_3$ 0.15 and glucose 1.00 g/l.) containing atropine sulphate ($10^{-7}$) was used in each method.

*Guinea-pig uterus.* This organ was superfused with Tyrode solution at 37° C.

*Standard substance P preparations*

Substance P standards containing 1.9 to 6.6 units/mg were prepared from cow, horse and pony intestines by precipitation with ammonium sulphate as described by Amin, Crawford & Gaddum (1954). They were standardized against preparations of substance P containing 13.8 and 75 units/mg.

*Extraction of substance P from mouse brain*

Male albino mice weighing 22 to 30 g were used. The animals were killed by a blow on the head and decapitated. The brains, after removal of the cerebellum, were homogenized in acetone and the substance P was extracted from the acetone-insoluble residue with hydrochloric acid as described by Amin *et al.* (1954).

In one experiment substance P was extracted from pooled mouse brain by the method described by Lembeck (1953), by Zetler & Ohnesorge (1957) and by Leach (1959). Substance P in one experiment was extracted from mouse jejunum by the method of Amin *et al.* (1954).

*Paper chromatography*

Salts and proteins were precipitated from the substance P preparations by the addition of four volumes of alcohol at *p*H 8 as described by Amin *et al.* (1954). For the standard preparation it was necessary to introduce a slight modification. After removal of ammonia *in vacuo* at 35° C the *p*H was readjusted to 8.0 because otherwise substance P did not move from the original point of the chromatogram.

In some experiments, in order to avoid degradation of labile nucleotides during concentration of the brain extract, nucleotides were precipitated from the extract with barium acetate, dissolved in suitable volumes of 0.1 N-formic acid, and the solutions pipetted on to the filter paper (Kunz, Schmid & Siess, 1958).

Acetone fractions of mouse brain extract were combined, evaporated and dried *in vacuo* in a desiccator. The residue was dissolved in water and then extracted four times with light petroleum (boiling point 40 to 60° C) to remove lipids as described by Amin *et al.* (1954). The water phase was centrifuged and the supernatant fluid ultrafiltered through cellophane membrane. The filtrate was concentrated *in vacuo* at 35° C and suitable volumes were pipetted on to filter paper. The solvents used for paper chromatography are shown in Table 1.

TABLE 1

CHROMATOGRAPHIC SOLVENTS

* The mixture was centrifuged; the bottom layer was kept in a beaker in the tank and the upper layer was poured into the trough. a=ascending; d=descending

| Solvent | Method | Reference |
|---|---|---|
| Acetone, trichloracetic acid (15%, w/v); 65 : 35 | a | Burrows, Grylls & Harrison (1952) |
| Acetone, trichloracetic acid (25%, w/v); 75 : 25 | a | |
| Ammonium bicarbonate (16%) | d | Hems (1959) |
| Ammonium sulphate (saturated solution), water, *i*-propylalcohol; 79 : 19 : 2 | d | Deutsch & Nilsson (1953) |
| *n*-Butanol, ethanol, water; 4 : 1 : 1 | d | Eliasson, Lie & Pernow (1956) |
| Pyridine, water; 65 : 35 | a | |
| *n*-Butanol, pyridine, water; 1 : 1 : 1 | a | Wood (1958) |
| Ether, acetic acid, water; 13 : 3 : 1 | a | |
| Methanol, formic acid, water; 16 : 3 : 1 | a | |
| *n*-Butanol, water, acetic acid; 4 : 5 : 1* | a | Partridge (1948) |
| Ethanol, hydrochloric acid (0·2 N); 1 : 1 | a | Mathia & Schachter (1958) |

*Identification of substances on chromatograms*

Substances were identified (a) by the action of eluates on guinea-pig ileum and rabbit jejunum; (b) in ultraviolet light; and (c) by chemical reactions on the paper.

(a) The chromatograms were kept in a vacuum desiccator for 2 to 3 hr to remove residual solvents. Even so, traces of butanol and acetic acid sometimes reduced the amount of substance P found by about 25 to 40%. To avoid this, these traces were removed by washing the chromatograms in benzene, the latter being evaporated from the paper *in vacuo*. The chromatograms were cut into eight pieces and eluted by shaking with 1 ml. of 0.01 N-HCl for 30 min. The elution was repeated and the combined eluates from each piece were evaporated, taken up in 1 ml. of Tyrode solution and neutralized with 0.1 N-NaOH. In other experiments elutions were performed with 2 ml. of 0.9% saline or of modified Locke solution. The biological activity of each eluate sample was estimated on the guinea-pig isolated ileum and rabbit jejunum.

(b) Chromatograms were examined under ultraviolet light at 250 and 350 mμ. Absorption was found at 250 mμ, and therefore this wavelength was used routinely.

(c) Chemical reactions were used for the detection of nucleotides as follows: phosphate reaction (Wood, 1958); uranyl acetate reaction (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950); silver nitrate-bromophenol blue reaction (Wood, 1958).

*Enzymic methods*

*Inactivation of substance P by chymotrypsin.* In order to test whether the contractions of the guinea-pig ileum elicited by both standard preparations and brain extract were due to substance P, the preparations were incubated with chymotrypsin (Amin *et al.*, 1954) in Tyrode solution at 37° C at *p*H 7.8 for 2.5 hr. After the incubation the solutions were boiled to stop the activity of the enzyme. The concentration of chymotrypsin in these experiments during the assay was between 0.12 and 200 μg/ml., and did not influence the results of the experiments.

*The effect of polyphenol oxydase on the inhibitory substance in the brain extract.* Polyphenol oxydase was extracted from mushrooms as described by Garven (1956). The extract was diluted with distilled water (1:20). Then 1 ml. of enzyme extract was added to each of the following: 1 ml. of adrenaline (1.6 μg/ml.); 1 ml. of noradrenaline (1.6 μg/ml.); and 1 ml. of brain extract containing 25 mg of dried extract (equivalent to 2.3 g of mouse brain per ml.). The solutions were incubated at *p*H 6 at room temperature for 40 min.

*Chemical estimations*

Adenosine triphosphate was estimated by the fire-fly method (Strehler & Totter, 1957).

Potassium was estimated with a flame photometer using lithium sulphate as an internal standard.

*Materials*

γ-Aminobutyric acid was presented by Dr F. Hobbiger (Department of Pharmacology, Middlesex Hospital); 5-hydroxytryptamine creatinine sulphate (Abbott); (−)-noradrenaline (Levophed, Bayer); adenosine monophosphate-$Na_2H_2O$ (Boehringer & Light); adenosine diphosphate-Na and adenosine triphosphate $Na_2.3H_2O$ (Sigma); crystallized chymotrypsin was presented by Armour Laboratories. Ultrafiltration was performed with Visking tubing (8/32 in. diameter) (Hudes Merchandizing Corporation). Other drugs and chemicals were commercial preparations.

## RESULTS

### *Assay of substance P in mouse brain extracts*

Substance P was estimated in extracts made from mouse brains by the method of Amin *et al.* (1954), which is designed to eliminate the interfering effect of 5-hydroxytryptamine by extraction with acetone. In preliminary experiments the scatter of the estimates of substance P content of individual brains was larger than would have been expected from the bioassay. The reason for this was therefore investigated. Substance P was estimated in an extract prepared from twenty pooled

TABLE 2

REPEATED ASSAYS OF SUBSTANCE P IN A POOLED MOUSE BRAIN EXTRACT

Extract was prepared from twenty mouse brains (8·22 g wet weight). Estimations were performed on four pieces of guinea-pig ileum from three guinea-pigs (first column, I to III). The number of the contractions of ileum, from which the substance P content of the preparation was calculated, is in the second column. * Weighted mean

| Guinea-pig | Number of observations | Substance P estimated (units/g brain) Mean | Range |
|---|---|---|---|
| I | 12 | 23·8 | (20·2–33·6) |
| II | 13 | 18·3 | (16·6–26·4) |
| IIIa | 25 | 15·0 | (9·8–16·9) |
| IIIb | 14 | 19·2 | (17·9–21·2) |
| | | 18·2* | (9·8–33·6) |

brains. The extract was made by the method of Amin *et al.* (1954), concentrated at 35° C *in vacuo*, dried in a vacuum desiccator, and the substance P content estimated on pieces of guinea-pig ileum taken from different animals. The estimates of the substance P content of the pooled extract showed a wide scatter (Table 2). A similar large scatter was found in the estimates for two other extracts prepared from pooled brains (twelve and twenty brains, respectively). It can be concluded from these results that the scatter derives from the method of estimation. These results provide no information about variation due to the extraction procedure, which was also investigated and the results are given later in this paper.

Discrepancies in the estimations might occur if the substance in brain extract causing contraction of the guinea-pig ileum was not identical with that in the standard preparation. This possibility was investigated by paper chromatography and by inactivation with the enzyme chymotrypsin.

Using three different solvent systems the $R_F$ values of the active substances in the standard preparation and in the brain extract were very similar (Table 3). These results incidentally confirm those of Eliasson, Lie & Pernow (1956), who showed that substance P from brain and substance P from intestine are indistinguishable.

The brain extract and standard substance P preparation were incubated at their optimal substrate concentration, which was found to be above 4 units/ml., with chymotrypsin (1 mg/ml.). There was over 90% inactivation of both the brain extract and the standard preparation, when assayed on the guinea-pig ileum. These experiments support the view that the contractions of the ileum are due to substance P.

TABLE 3

PAPER CHROMATOGRAPHY OF SUBSTANCE P IN STANDARD AND BRAIN EXTRACT

$R_F$ values were calculated from assays on guinea-pig ileum and rabbit jejunum

| No. | Solvent | Standard preparation $R_F$ Mean | Range | Brain extract $R_F$ Mean | Range |
|---|---|---|---|---|---|
| 1 | Butanol, water, acetic acid; 4 : 5 : 1 | 43 | (31–56) | 37 | (31–43) |
| 2 | Ethanol, 0·2 N-HCl; 1 : 1 | 87 | (81–93) | 87 | (81–93) |
| 3 | Pyridine, water; 65 : 35 | 87 | (75–100) | 87 | (75–100) |

*Effect of substance P preparations on rabbit jejunum*

The standard preparation of substance P contracted the rabbit isolated jejunum; however, when brain extracts, made by the method of Amin *et al.* (1954), were similarly tested, there was at first a relaxation and then, on washing out, a contraction. This clearly indicated the presence of an inhibitory substance in the brain extracts made by the acetone-hydrochloric acid method. Brain extracts made by the methods of Zetler & Ohnesorge (1957) and of Leach (1959) also contained an inhibitory substance, as did substance P extracts prepared from mouse intestine by the acetone-hydrochloric acid method. On the other hand when substance P was extracted from a standard preparation by the acetone-hydrochloric acid method, this extract did not contain the inhibitory substance (Fig. 1). Hence the presence

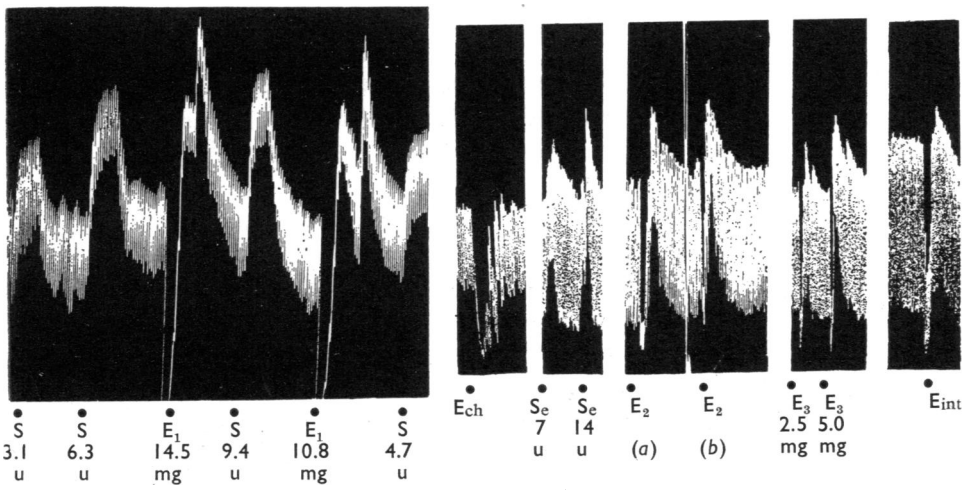


Fig. 1. Effect of substance P preparations on rabbit jejunum. S=standard preparation. $E_1$= brain extract made by the method of Amin *et al.* (1954); $E_2$=by the method of Zetler *et al.* (1957), (*a*)=fraction−a, (*b*)=fraction−b; $E_3$=by the method of Leach (1959). $E_{int}$=extract made from mouse intestine by the method of Amin *et al.* (1954). $E_{ch}$=brain extract, prepared by the method of Amin *et al.* (1954), after incubation with chymotrypsin. $S_e$=extract prepared from standard preparation by acetone-hydrochloric acid extraction method (Amin *et al.*, 1954). The figures give the amounts of substances added to a 10 ml. organ-bath (1 mg of dried preparation is equivalent to 32 mg of fresh brain).

of the inhibitory substance in extracts made from brain and from intestine by the acetone-hydrochloric acid method on one hand, and its absence from the standard preparation made by ammonium sulphate precipitation on the other, is due to the different extraction procedures.

In order to identify the interfering substance, some of its properties were investigated. Unlike substance P, it was not inactivated by chymotrypsin (Fig. 1). However, like substance P, the inhibitory substance could pass through a cellophane ultrafilter membrane and was not affected by electrolytic desalting.

*Identification of the inhibitory substance in brain extracts*

Various substances which might occur in the brain extract were tested on the rabbit jejunum and on contractions of the guinea-pig ileum induced by substance P and were compared with the inhibitory substance.

*Adrenaline and noradrenaline.* Adrenaline and noradrenaline inhibit contractions of the rabbit jejunum. When tested on contractions of the guinea-pig isolated ileum induced by substance P, adrenaline (0.75 μg/ml.) increased, and noradrenaline (5 ng/ml.) diminished, the responses. (Figures in brackets indicate threshold concentrations.)

The possibility that the inhibitory substance in the brain extract was adrenaline or noradrenaline has been investigated. The inhibitory effect of adrenaline and noradrenaline on the rabbit jejunum was abolished by boiling for 10 min at *p*H 9, while that of the brain extract was not affected. Similarly the inhibitory effect of adrenaline and noradrenaline was abolished by incubation with polyphenol oxydase extract (Garven, 1956), while that of the brain extract was not affected. The enzyme extract itself did not show any action on rabbit jejunum.

As a result of these experiments, the possibility that adrenaline and noradrenaline caused the inhibitions can be excluded.

*Adenine nucleotides and related compounds.* On the rabbit isolated jejunum adenosine triphosphate (0.12 μg/ml.) caused relaxation followed by contraction, whereas adenosine diphosphate (0.16 μg/ml.), adenosine monophosphate (0.62 μg/ml.) and adenosine (0.60 μg/ml.) caused only relaxation, and adenine was inactive (12.50 μg/ml.). (Figures in brackets indicate the concentrations in the organ-bath.)

It was possible to separate the inhibitory substance from substance P and to identify it by paper chromatography (Fig. 2). The parts of the chromatograms which contained inhibitory substances showed absorption in ultraviolet light at 250 mμ wavelength; compounds which contain adenine absorb light of this wavelength. The silver nitrate bromophenol blue reaction was positive, as occurs with compounds containing an imidazol ring (Wood, 1958). Since adenine does not inhibit contractions of rabbit jejunum, this compound was not responsible for the inhibition. Those parts of the chromatogram which absorbed ultraviolet light always reacted with the phosphate reagent, giving a blue spot after irradiation with ultraviolet light, and therefore adenosine was also excluded. Hence, the inhibitory substance was an adenine nucleotide. This was supported by the positive uranyl acetate reaction, which gave a brownish-red colour on the chromatogram. This reaction is given by nucleotides (Magasanik *et al.*, 1950).

The inhibitory substance was finally identified by paper chromatography using a solvent mixture of acetone and trichloracetic acid which separates adenosine mono- and diphosphates. In this solvent the $R_F$ value of the inhibitory substance in dried brain extract corresponded to adenosine monophosphate.

The adenosine triphosphate content of dried brain extract was estimated by the fire-fly method (Strehler & Totter, 1957) and found to be 1 μg per mouse brain (calculated as adenosine triphosphate-$Na_2.3H_2O$). This value would lead to a concentration of 0.1 μg of adenosine triphosphate per ml. in 10 ml. of brain extract, which is used for the assay of substance P. In this concentration adenosine triphos-

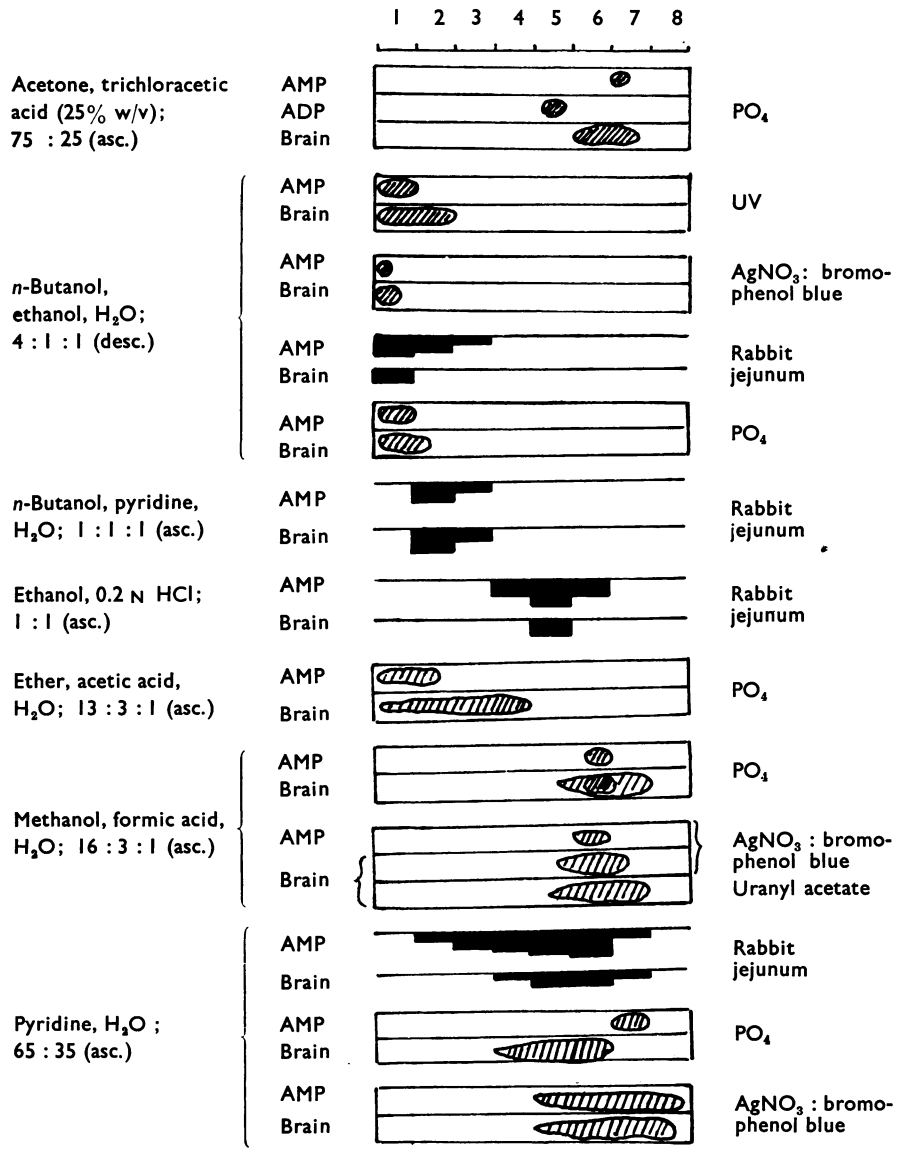


Fig. 2. Identification of the inhibitory substance by paper chromatography. The figures above indicate the different sections of the chromatograms. On the left: solvents. Asc.=ascending; desc.=descending. AMP=adenosine monophosphate; ADP=adenosine diphosphate. On the right: the test applied. ($PO_4$=phosphate reaction after irradiation with ultraviolet light; UV=absorption in ultraviolet light.) Black areas indicate sites which gave an inhibitory effect of the eluates on contractions of the rabbit jejunum.

phate does not affect the assay of substance P (see later). On the other hand when adenosine triphosphate was assayed in brain extracts immediately after extraction without evaporation of the acetone-hydrochloric acid extract, its concentration was 44.0 to 141.0 μg per brain (4.4 to 14.1 μg of adenosine triphosphate per ml. of brain extract). In this concentration adenosine triphosphate influenced the estimations of substance P.

Adenosine mono-, di- and triphosphates occur in nearly the same concentration in brain (Koransky, 1958 ; Coper, Herken & Koransky, 1958). For this reason the presence of adenosine diphosphate was reinvestigated in some experiments. The nucleotides were precipitated by barium acetate and dissolved in small volumes before chromatography, following the method of Kunz *et al.* (1958). However, owing to the incomplete separation of the nucleotides in the solvents used, the presence of adenosine diphosphate in the brain extracts remained uncertain. A further difficulty arose from the presence of inorganic phosphate, which reacted as a yellow spot on the chromatograms, and made difficult the evaluation of these chromatograms.

*Effect of nucleotides on the contractions of guinea-pig ileum induced by substance P*

The effect of those nucleotides which had previously been identified in the brain extract was investigated on the contractions of guinea-pig ileum induced by substance P, using the superfusion method. Substance P solution added to the guinea-pig ileum contained the nucleotides under investigation in different concentrations, which are given as their sodium salts.

*Adenosine monophosphate.* A concentration of 5 μg/ml. of this compound diminished the contractions induced by substance P. Higher concentrations had a greater effect, but even at 500 μg/ml. it did not abolish the contractions.

*Adenosine diphosphate.* A concentration of 10 μg/ml. made the induced contractions due to substance P more rapid, while 50 to 250 μg/ml. increased the height of contractions.

*Adenosine triphosphate.* A concentration of 10 μg/ml. usually increased the size of the contractions, but this effect was not always immediate, but only appeared after repeated doses.

*Estimation of adenosine monophosphate in the brain extract*

After the interfering action of nucleotides on the substance P estimation had been demonstrated, it remained to prove that their concentration in the brain extracts was sufficient to cause this effect. The estimation of adenosine monophosphate in brain extract was attempted on rat and on guinea-pig uteri (Bennet & Drury, 1931 ; Barsoum & Gaddum, 1935) but these test organs were found to be insensitive.

Adenosine monophosphate concentration in dried, pooled, acetone-hydrochloric acid extract prepared from ten mouse brains was estimated on rabbit jejunum and on hen rectal caecum. Before the assay, the extract was incubated with 10 μg of chymotrypsin per ml. to eliminate the effect of substance P. The amount of adenosine monophosphate per brain in the extract was 140 μg (rabbit jejunum) and

100 μg (hen rectal caecum), or 378 and 270 μg/g of brain, respectively. Chymotrypsin in the concentration used did not show any effect on these test organs.

Depending on the sensitivity of guinea-pig ileum to substance P (0.1 to 0.6 units/ml.) and on the substance P content of the brain extracts (4.0 to 13.8 units/mouse brain), the possible dilution of the extracts during the assay may vary from 6.6- to 138-fold, which leads to adenosine monophosphate concentrations of 1.0 to 21.2 μg/ml. This range overlaps the threshold concentration (5 μg of adenosine monophosphate per ml.) at which interference occurs.

Similarly, the concentration of adenosine triphosphate in the brain extract (see above) during the assay also can be sufficient to interfere with assay of substance P. The concentration of adenosine triphosphate can be as high as 21.3 μg/ml. (the ratio of 141 μg, which is the upper limit of the adenosine triphosphate content in one mouse brain, to 6.6, which is the weakest dilution of the extract; a similar calculation was used in the preceding paragraph) and thus higher than the threshold concentration (10 μg/ml.) in which adenosine triphosphate interferes with the assay of substance P.

*The distribution of nucleotides in various fractions of the brain extract*

The presence of nucleotides in the combined acetone fractions (used for the removal of 5-hydroxytryptamine) and in the protein precipitate formed in the preparation of brain extracts was also investigated. Paper chromatograms of the acetone fraction showed an ultraviolet absorbing spot and gave a positive phosphate-reaction on the day following irradiation with ultraviolet light. This reaction is characteristic of adenosine diphosphate (Wood, 1958). The protein precipitate was also tested for the inhibitory substance. After the extraction of substance P, the protein precipitate was washed with water and centrifuged. The supernatant fluid did not inhibit contractions of the rabbit jejunum. These experiments show that a certain amount of nucleotide was removed by the acetone extraction.

*Other substances*

The concentration of potassium chloride in pooled extracts varied from 1.49 to 2.02 mg/brain. Taking the highest concentration and the lowest dilution of the extract, the concentration of potassium chloride during the assay could be as high as 306 μg/ml., while the threshold concentration in which it increased the contractions induced by substance P was 400 μg/ml. Similarly, the concentration of γ-aminobutyric acid, calculated from the data of McIlwain (1959) and of Elliot & Jasper (1959), could be 18 μg/ml., but the compound did not influence the assay of substance P in concentrations up to 1 mg/ml. The concentration of 5-hydroxytryptamine (base) could be $<0.060$ μg/ml. (calculated from the data of Dr T. J. Sullivan; personal communication), but the threshold concentration at which 5-hydroxytryptamine increased the contractions induced by substance P was 0.21 μg/ml. Sodium chloride concentration in the brain extract during the assay was always below that in which it influences the contractions induced by substance P. It can be seen that none of these substances was in sufficiently high concentration to influence the assays of substance P.

*Investigations on the extraction of substance P*

The extraction of substance P from brain has been studied with a view to finding whether variations in the extraction of substance P might have contributed to the wide scatter of the estimations. This might have occurred either (1) by loss of substance P in the discarded acetone supernatant, or (2) by the incomplete extraction of substance P from the precipitate. The acetone fraction contained no detectable substance P, which agrees with the results of Amin *et al.* (1954). Substance P could be completely extracted from the acetone precipitate by two extractions with 5 ml. of Tyrode solution, and most of the substance P could be extracted by a single extraction with 10 ml. (Table 4). It would seem advisable to use 5 ml. twice for the extraction, instead of the smaller volumes suggested by Amin *et al.* (1954). It is concluded that there is no loss of substance P during the extraction with acetone and that extraction of substance P from the acetone precipitate is complete.

TABLE 4

EXTRACTION OF SUBSTANCE P FROM THE ACETONE-PRECIPITATE MADE FROM POOLED MOUSE BRAINS

The figures give the amount of substance P (in units) extracted from one brain. (First extraction: acetone precipitate was extracted with hydrochloric acid, and the volume was made up with Tyrode solution to 5 ml. or 10 ml. Second and third extractions: precipitate was extracted with the same volume of Tyrode solution)

| | Volume for the extraction | | | | | |
|---|---|---|---|---|---|---|
| | 5 ml. No. of the acetone-precipitate | | | 10 ml. No. of the acetone-precipitate | | |
| No. of the extraction | I | II | III | IV | V | VI |
| 1st | 2·32 | 2·92 | 2·81 | 3·58 | 3·67 | 4·35 |
| 2nd | 1·82 | 0·85 | 0·86 | <1·33 | <1·37 | 0·0 |
| 3rd | 0·0 | <0·76 | 0·0 | 0·0 | 0·0 | 0·0 |

## DISCUSSION

The large scatter of the results of assay of substance P in mouse brain extracts suggested the presence of a second substance influencing the assay. Similar variations in the amount of substance P in the central nervous system of different species can be found in the literature (Kopera & Lazarini, 1953 ; Amin *et al.*, 1954 ; Zetler & Schlosser, 1955 ; Pernow, 1955 ; Paasonen & Vogt, 1956), and several workers have suggested that extracts contain a substance which inhibits contractions of smooth muscle (Euler & Gaddum, 1931 ; Vogt, 1949 ; Douglas, Feldberg, Paton & Schachter, 1951 ; Florey, 1953). Euler & Gaddum (1931), Vogt (1953) and Amin *et al.* (1954) considered the possibility of it being an adenosine compound. An alternative explanation of the variation was advanced by Leach (1959), who ascribed it to the non-uniformity of the extractions of substance P from tissues.

In the investigations described here adenosine monophosphate has been identified by paper chromatography as the substance responsible for the interfering effect of dried brain extract, whereas in extracts made without drying adenosine triphosphate was found in addition to adenosine monophosphate, in concentrations sufficiently high to cause interference. Some of the adenosine monophosphate derives from adenosine tri- and diphosphates owing to some destruction of the latter nucleotides

during the extraction of substance P. The identification of these nucleotides was possible on the basis of their inhibition of contractions of the rabbit jejunum (Gillespie, 1934 ; Barsoum & Gaddum, 1935 ; Gaddum, 1936), absorption of ultraviolet light at 250 mμ and chemical reactions. Uridine and guanosine phosphates as well as diphosphopyridine nucleotide, which absorb ultraviolet light at the same wavelength (Beaven, Holiday & Johnson, 1955 ; Pabst Circular, 1956, 1961), also occur in brain, but the concentrations of the latter compounds are much lower than that of adenosine nucleotides (Coper *et al.*, 1958 ; Koransky, 1958 ; Mandel & Harth, 1961).

The presence of adenosine monophosphate in the brain extract has previously been shown by experiments in which the brain extract was incubated with adenosine monophosphate-deaminase extract ; this almost completely abolished the inhibition of contractions of rabbit jejunum (Laszlo, 1960). The end product of this reaction is 5′-inosinic acid, which has no effect on rabbit jejunum. The concentration of nucleotides estimated in the acetone-hydrochloric acid extracts of brain is comparable with those given by Heald (1960) ; the extract did not contain all the brain nucleotides because some were removed by the acetone extraction, as shown by the presence of a nucleotide, probably adenosine diphosphate, in the acetone fraction.

From the results of this investigation, it is concluded that published estimates of substance P concentrations in different parts of the nervous system and other organs have been influenced by the presence of nucleotides when their concentration relative to that of substance P exceeded a given ratio. It is calculated, from the threshold concentration of nucleotides necessary to influence the assay and from the sensitivity of the guinea-pig ileum to substance P, that these ratios are 10 μg, 10 μg and 100 μg of adenosine mono-, tri- and diphosphate respectively, per unit of substance P.

The specificity of assays of substance P have been confirmed by previous workers using various tests, such as inactivation by chymotrypsin (Pernow, 1953) and specific desensitization (Paasonen & Vogt, 1956). However, these tests would not indicate if smooth muscle contractions were influenced by an inhibitory substance or by an excitatory substance present in insufficient concentration to cause contraction by itself.

The great differences between the values of substance P published in the literature, as well as the contradictory results of assays of substance P after the treatment of animals with drugs, can be explained by the interfering action of nucleotides on the assays of substance P (Laszlo, 1962).

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