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THE ESTIMATION OF SUBSTANCE P IN TISSUE EXTRACTS

SP was first recognized in experiments in which ACh was assayed by its action on the blood pressure and the intestine of rabbits. The effect of ACh on these tissues was abolished by atropine, but certain tissue extracts were still effective after atropine and these effects were attributed to SP (Euler and Gaddum, 1931).

Our knowledge of SP still depends on bioassays, but these assays are open to criticism. ACh can be estimated by a number of different sensitive and specific methods and the results are often found to agree with one another. This agreement between parallel assays provides strong evidence that the results give the concentration of ACh itself, and do not depend on allied substances such as propionylcholine (Chang and Gaddum, 1933). Similar methods can be used to distinguish adrenaline and noradrenaline from allied substances (Gaddum, Peart and Vogt, 1949), but when SP is assayed by different methods the results do not agree with one another. This must mean that the extracts used for the assays are not pharmacologically pure; their action does not depend only on SP but also on various other interfering substances which alter the response. Many of these interfering substances are now known and their effects can be eliminated, but there is still no really specific method of assay for SP; estimates should be checked by parallel assays and it is not easy to get concordant results.

There are two ways in which methods of estimation, whether biological or biochemical, may be made more specific. The solutions may be purified, or the final test may be modified. Purified preparations of SP from intestine and brain have been shown to have the same relative potency when tested in various different ways, (Amin, Crawford and Gaddum, 1954, Eliasson, Lie and Pernow, 1956) but purification is laborious and involves loss. Much work has, therefore, been done in various laboratories with the object of making the final test more specific by using antagonists to eliminate the effects of known interfering substances.

The best known interfering substances are ACh and H, and assays of SP are generally done in the presence of antagonists for these two substances. The guinea pig's ileum provides a convenient test (Douglas, Feldberg, Paton and Schachter, 1951; Pernow, 1951) and it

has sometimes been assumed that the effects of tissue extracts on this tissue in the presence of atropine and an antihistamine are entirely due to SP. Amin et al. (1954) found that such tests were often complicated by the presence of 5-HT and precautions are, therefore, generally taken to eliminate effects due to this substance. If the tissue is extracted with 20 volumes of acetone, SP can be quantitatively recovered from the insoluble residue. 5-HT and various other interfering substances are found in the acetone, but it is difficult to extract 5-HT completely from tissues with acetone (Sharman, 1961) and reliable assays, therefore, depend on the use of an antagonist for 5-HT in the final test. The guinea pig's ileum may be densitized to 5-HT by adding tryptamine to the bath fluid (Gaddum, 1953a).

This test has provided much interesting information about SP and its distribution (Zetler and Schlosser, 1955). There is no reason to doubt the general picture given by these results, but it would be unwise to accept such estimates without independent evidence that the effect was really due to SP, and the best evidence of this depends on parallel assays, using a set of tests which have been shown to vary independently in their responses to allied drugs.

Recent work in my own laboratory has been based on 4 methods of assay, using tissues which vary widely in their responses to different drugs, but all of which are fairly sensitive to SP. Various preparations of SP and extracts of tissues have been compared with a standard preparation of SP. This was made by Messrs. Hoffmann La Roche, by Pernow's methods, and adopted by U. S. von Euler and J. H. Gaddum in 1959 as a provisional standard containing 75 units per ml.

The four methods depend on rat uterus, guinea pig ileum, hen rectal caecum and gold fish gut. The first three have been used for some years for detecting pharmacologically active polypeptides and for distinguishing between them (Gaddum, 1955). We have spent some time studying the best conditions for using the hen rectal caecum (Cleugh, Gaddum, Holton and Leach, 1961). This was first used by Barsoum and Gaddum (1935) and was shown by Pernow and Rocha e Silva (1955) to be sensitive to SP. It is also sensitive to ACh, H, 5-HT, and adrenaline, but the effects of these can be diminished by suitable antagonists. Tissue extracts normally contain sufficient ATP and other adenosine derivatives to interfere with the assay by inhibiting the rectal caecum.

Laszlo (1960) has described a method in which adenosine monophosphate is inactivated by an enzyme, but this enzyme does not destroy ATP which may also be present. We have found that these effects can be eliminated by specific tachyphylaxis. If a high concentration of two of these compounds is maintained by adding adenosine (or adenosine monophosphate) and ATP (100 µg of each) to a 4 ml bath whenever it is refilled, the interfering effects of these two substances and also of adenosine diphosphate are usually eliminated.

These adenosine compounds may also interfere with assays on guinea pig ileum, but this effect is less obvious.

The search for a sensitive test for SP has also led to the use of gold fish intestine in a microbath. For many purposes it is convenient to use the method of superfusion (Gaddum, 1953b) in which a piece of plain muscle is suspended in air and suitable salt solution allowed to run over its surface. At intervals the flow is stopped and a drug solution is applied to the surface of the muscle in a volume of 0.25 ml or more. For some purposes it is better to use a microbath (Gaddum and Stephenson, 1958), in which a small piece of muscle is bathed in 0.05 ml of salt solution or less. In the apparatus now used (Gaddum and Szerb, 1961) the bath is a horizontal circular hole (diam. 2 mm.) in a block of perspex. Salt solution runs into a vertical well (diam. 5 mm.), through a small hole into one end of the bath, and then through the bath and down a vertical face at the other end. A length of about 8 mm. of gold fish intestine is suspended between threads in the bath and its movements are amplified about 100—500 times and recorded with a pen writer. This preparation contracts in the presence of SP in a low concentration and can be used to detect quantities of the order of 1—5 milliunits.

It is also sensitive to various other substances present in tissue extracts, but the effects of many of these can be eliminated with antagonists. ACh is antagonized by hyoscine; 5-HT is antagonized by various derivatives of lysergic acid of which methylsergide (UML 491, deseryl, 1-methyl lysergic acid butanolamide) has been found particularly satisfactory, and the inhibitory effects of adrenaline and noradrenaline are antagonized by D. C. I. (dichloroisoproterenol, 3,4-dichlorophenyl-isopropylaminoethanol). ATP causes a contraction, but interference from this source can be avoided by maintaining a constant concentration of ATP in the bath fluid. The preparation is remarkably insensitive to H even without antihistamines.

By such methods all these different kinds of plain muscle — the rat uterus, the guinea pig ileum, the hen rectal caecum and the gold fish intestine can be made insensitive to most of the known pharmacologically active substances in tissues, but there are no known antagonists for the polypeptides and several of these may be present in biological fluids, including not only SP, but also bradykinin, angiotensin, oxytocin and vasopressin. All four muscle preparations are about equally sensitive to SP. The rat uterus is unspecific, being also very sensitive to bradykinin, angiotensin, and oxytocin. The guinea pig's ileum is quite sensitive to bradykinin and angiotensin, though less so than the rat uterus. It is insensitive to oxytocin and vasopressin. The fowl rectal caecum and the gold fish gut are both comparatively insensitive to these other polypeptides and should, therefore, provide specific tests for SP, provided that the effects of ACh, H, 5-HT, catecholamines and adenosine compounds are suppressed by suitable antagonists and provided that they are not affected by other unknown sub-

stances present in the solutions being tested. Experience has shown, however, that the situation is not so simple as was first supposed. When these four tests were applied in parallel to tissue extracts, the results showed discrepancies large enough to suggest the presence of unknown active substances in the extracts.

TABLE I

ASSAYS OF FIVE PREPARATIONS OF SP UNITS PER MG

	Rat Uterus	Guinea pig Ileum	Hen Rect. Caecum	Gold Fish Gut
Standard	75	75	75	75
B	110	37	28	—
D	180	340	375	—
P_3B	31	42	37	30
P_4B	11	35	57	75

Table I shows the results of assays in which four different preparations of SP were compared with the standard. Preparations B and D were obtained by Messrs. Hoffmann La Roche from the standard itself by the method described by Pernow (1953) in which SP is eluted from a column of alumina with methanol diluted with increasing quantities of water. The results show that this standard preparation contains more than one pharmacologically active substance since both of the fractions obtained from it were qualitatively different from it. If this standard preparation was pharmacologically pure the results of the parallel assays of the activity of both fractions would have agreed within the error of the tests. The tests were repeated and it was clear that this was not so. For example, the estimates of the activity of preparation D on rat uterus were 188, 188 and 163, and the corresponding results on guinea pig ileum were 288, 349, 352 and 375. Fraction D was much the most active of these fractions and the simplest assumption is that it contained most of the SP, while fraction B contained some other active substance with relatively more action on the rat uterus. This might be one of the other active polypeptides such as bradykinin, but there is, at present, no evidence of this. The preparation P_3B was made at Babraham by similar methods. There was no very definite difference between the results of the different assays, so that it would be reasonable to conclude that this preparation was similar to the standard. In the case of P_4B, however, the assay on hen rectal caecum gave much higher results than the assay on rat uterus. If the explanation given above to account for the results with preparations B and D is correct, then P_4B is particularly pure from the pharmacological point of view. It contains even less of the substance acting on the rat uterus than preparation D.

The interesting thing about these results is that they were all obtained with active preparations of SP. The preparations used in experiments with this substance have often contained between 5 and 15 units per mg and there is no reason to suppose that they were more nearly pure pharmacologically than the much more active preparations used here. The preparation made by Franz, Boissonnas and Stürmer (1961) contained 30,000—35,000 units per mg. This was presumably pure SP. Most other preparations obtained so far contain pharmacologically active impurities. On the other hand, it is important to remember that the results of experiments of this kind depend on the method of assay used. Most people use guinea pig ileum; if they had used the rat uterus exclusively they might have isolated a different polypeptide and claimed that this was pure SP.

These results have led to the conclusion that the effect of these preparations on the rat's uterus are partly due to an impurity, but we have made no serious effort to find out what this impurity is. We abandoned the use of the rat's uterus and used the other three tests in the hope that they would give concordant results. The estimates given in Table I by these three tests are reasonably concordant; the discrepancies may be due to experimental error. It is possible that any of these methods might be used to estimate the amount of SP in intestine, but there is evidence of some other substance in extracts of guinea pig's brain which complicates the results. J. Cleugh and V. P. Whittaker have been studying the distribution of SP in homogenates of guinea pig's brain after centrifuging.. The brains were homogenized in isotonic (0.32 M) sucrose solutions and tested in guinea pig ileum and hen rectal caecum in the presence of antagonists for known substances. When these homogenates were tested directly they had little activity, but they became active when they were made acid (pH 4) and heated at 100° for 10 min. and then neutralized and tested. This treatment has been shown to liberate ACh in similar circumstances by breaking up the particles in which it is contained and the results suggested that SP was contained in similar particles. When these homogenates were centrifuged most of the activity was found in the »mitochondrial« fraction (P_2) (precipitated at 450,000 g min. but not at 10,000 g min.). This confirms the observations of Lembeck (1960). This fraction was then separated in a density gradient formed by sucrose solutions of different strengths. The activity was mostly found in layer B (between 0.8 M and 1.2 M sucrose). This fraction has been shown to contain the largest amounts of ACh and cholineacetylase (Hebb and Whittaker, 1958), noradrenaline (Chruschiel, 1960) and 5-HT (Whittaker, 1959). Electron micrographs by Gray and Whittaker (1961) indicated that it consists largely of pinched off nerve endings containing synaptic vesicles. The experiments on the distribution of SP have thus led to the conclusion that it is present in the same fraction as substances known (or suspected) to be associated with chemical transmission. This lends support to the theory that it has some func-

tion in the central nervous system, but, unfortunately, the evidence that the substance estimated in these tests was SP itself is not very satisfactory. In some experiments the estimates obtained with the hen rectal caecum agreed well with the estimates obtained with the guinea pig ileum but in other experiments they were larger.

Experiments with gold fish gut revealed the presence of another substance in these extracts which was highly active on this tissue and overshadowed the effects of all the other substances. Since this substance is particularly active on gold fish gut, it was suggested, as a joke, that it should be called aureopiscin and no other name has been suggested so far. This substance is active even in doses corresponding to 10 μg of tissue. It is stable to boiling at pH 2.5 to 9.5 for 10 min. It is present in the supernatant of brain extracts after centrifuging. An extract of guinea pig liver had a similar action. There is clearly much more work to be done on this substance which is interfering with the assay of SP on gold fish gut. It may well turn out to be some substance which is already known to be present in the body, but the gold fish provides a sensitive test for it.

Summary

Four different methods for the assay of SP in tissue extracts have been compared with one another and found to give discordant results, which must be due to the presence of pharmacologically active impurities. This was true, even when the preparation tested was fairly active (75 units per mg). Some of the actions attributed to SP are probably due to these impurities. A method depending on the intestine of the gold fish (Carassius auratus) will detect small quantities of SP, because it can be used in a small bath (0.05 ml), but it is also sensitive to some unknown substance in tissue extracts. It will be necessary to remove this substance from extracts before this method can be used for the assay of SP.

ODREĐIVANJE SP U EKSTRAKTIMA TKIVA

Četiri različite metode za određivanje SP u ekstraktima tkiva uspoređene su međusobno. Rezultati se ne slažu, što mora da je posljedica prisutnosti farmakološki aktivnih onečišćenja. Ovo je zapaženo i prilikom ispitivanja prilično aktivnih preparata (75 jed./mg). Spomenuta onečišćenja, vjerojatno, izazivaju neke od efekata koji se pripisuju djelovanju SP. Pomoću metode s crijevom zlatne ribice (Carassius auratus) mogu se otkriti vrlo male količine SP, jer se određivanje može izvesti u malom kupatilu (0.05 ml), no ona je, istovremeno, osjetljiva na neku nepoznatu supstanciju u ekstraktima tkiva. Prije nego što ova metoda bude upotrebljiva za određivanje SP, treba ukloniti tu supstanciju iz ekstrakata.

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DISCUSSION

EULER: Did you extract the layers after gradient centrifugation or remove the sucrose before assay of the SP effect?

GADDUM: In the experiments I have described, the extracts were made acid and boiled to liberate the SP, but they were not extracted. The dilution was such that the sucrose did not affect the result.

LEMBECK: Do you have to dilute your tissue extracts to the same salt concentration as the Tyrode solution (1:2) when you test them on the gold fish gut?

GADDUM: Yes, the gold fish gut is sensitive to changes of tonicity. The salt concentration of the extracts was adjusted by calculation, so that it should have been the same as that of the bath fluid.

ZETLER: Are these two new biologically active substances contaminating SP destroyed by trypsin or chymotrypsin? Were there also active substances migrating to the anodic side during paper electrophoresis?

GADDUM: I have no evidence of the effects of these enzymes on the substance which is particularly active on rat uterus. They had no effect at all on the substance which is particularly active on gold-fish gut. There was some evidence suggesting that this substance migrates towards the anode at pH 3.4, but further experiments are needed.

HAEFELY: I was very interested to hear from professor Gaddum that he also failed to find a liberation of SP in the *nucleus gracilis*. We did the same experiments using the cats and the dogs as experimental animals and the tissue perfusion technique, I had the chance to see in the institute of professor Gaddum. The needle was put in the *nucleus gracilis* and the

tractus gracilis and was stimulated electrically. Before stimulation there was a weak activity measured on the guinea pig ileum, possibly due to a substance other than SP. After stimulation we failed to demonstrate the change in activity of the fluid coming out the nucleus.

PERNOW: In order to see whether the biological effects obtained by the different extracts are really due to SP we use the following tests.

(1) The specific tachyphylaxis as described by Gaddum and which works very well.

(2) Adsorbtion on aluminium oxide columns and elution with methanol and water.

(3) Inactivation by chymotrypsin is even more active in this respect. but trypsin is preferable since Rocha e Silva and I could show that trypsin inactivates SP but not bradykinin.

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