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THE RELEASE OF ACETYLCHOLINE FROM THE ISOLATED ILEUM OF THE GUINEA-PIG INDUCED BY 5-HYDROXYTRYPTAMINE AND DIMETHYLPHENYLPIPERAZINIUM

BY

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Transmural electrical stimulation of the isolated ileum of the guinea-pig (Paton, 1955) induces a measurable increase in the output of acetylcholine above that released at rest (Paton, 1957; Harry, 1962), and this stimulated release has its origin in the nervous structures of the wall. Some drugs, like nicotine, 5-hydroxytryptamine and dimethylphenylpiperazinium, contract the guinea-pig ileum by acting indirectly through the postganglionic parasympathetic nerve pathways. Their actions are inhibited by local anaesthetics in concentrations which selectively block nerves, or by atropine, and are potentiated by anticholinesterases, and so are presumably mediated by acetylcholine.

This paper reports the results of an investigation made to measure the small amounts of acetylcholine released from the isolated ileum by dimethylphenylpiperazinium, which acts on the acetylcholine receptors on the intramural ganglion cells, and by 5-hydroxytryptamine, a drug which acts specifically on a different ganglionic receptor from that on which dimethylphenylpiperazinium acts (Brownlee & Johnson, 1963).

METHODS

Adult guinea-pigs were killed and the ileum was excised. Segments of approximately 3 cm cut from the middle region of the ileum were incubated for 75 min in 100 ml. of Krebs solution containing the organophosphorus anticholinesterase *N,N*-diisopropylphosphorodiamidic fluoride (mipafox) in a concentration of 10 μg/ml. The solution was bubbled with a mixture of 95% oxygen and 5% carbon dioxide.

A segment of the ileum so treated was suspended in a 3-ml. organ-bath of bubbled Krebs solution and arranged to record longitudinal contractions isotonically. The lever had a tenfold magnification and was weighted with 0.5 g.

Drugs. These were acetylcholine chloride, 1,1-dimethyl-4-phenylpiperazinium iodide, hexamethonium bromide, 5-hydroxytryptamine creatinine sulphate, hyoscine hydrobromide, pentolinium hydrogen tartrate, physostigmine sulphate and mipafox. All drug concentrations are given as base except mipafox which is given as salt.

Experiments. A dose/response line was constructed with dimethylphenylpiperazinium or 5-hydroxytryptamine in doses which were increased logarithmically every 4 min. Two or three doses were selected which gave contractions of different magnitude and the ileum was then challenged ten times with each of these doses. The drug was kept in contact with the ileum for 45 sec after which time the bath fluid was removed and, after its volume had been noted, it was frozen at $-20°$ C. Each contraction was separated

from the next by an equivalent period of rest, so that there were two cycles each of 4 min between each drug challenge. The bath fluid was removed after each period of rest and it was treated in the same way as that removed after each drug challenge.

The bath fluid was exchanged five times after each contraction and after each rest period; the fifth exchange of bath fluid always coincided with the 3rd min of the dose cycle; this occurred at the 3rd min after the injection of the drug or at the 3rd min after the beginning of the rest period.

It has been shown previously (Brownlee & Johnson, 1963) that repeated washing of the guinea-pig isolated ileum is necessary to eliminate the possibility of tachyphylaxis to 5-hydroxytryptamine. Twenty contractions of the guinea-pig ileum, each produced in the way described to 50-ng doses of 5-hydroxytryptamine, are shown in Fig. 1; no tachyphylaxis is seen.

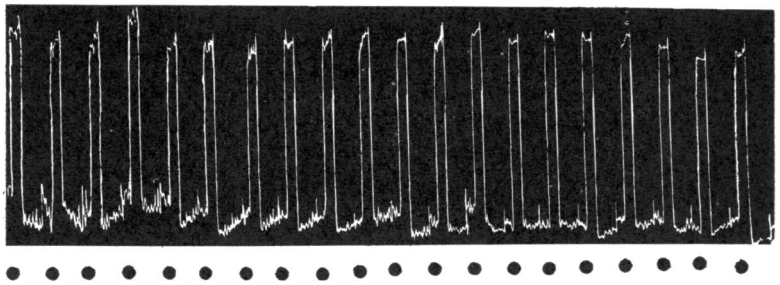


Fig. 1. The kymograph tracing shows twenty contractions of the guinea-pig isolated ileum to 50-ng doses of 5-hydroxytryptamine added (at the black dots) to a 3-ml. organ-bath of Krebs solution. The 5-hydroxytryptamine was in contact with the ileum for 45 sec and each contraction was separated from the next by a period of rest which was equal in duration to the drug cycle (4 min), making a period of 8 min between each drug challenge. When allowance is made for spontaneous fluctuations in the base-line, it will be seen that no tachyphylaxis occurred to successive doses of 5-hydroxytryptamine.

Preparation of the samples for assay. The ten samples collected for any one dose of 5-hydroxytryptamine or dimethylphenylpiperazinium were pooled in a 250-ml. flask; at the same time the ten samples obtained from the equivalent rest periods were also pooled in a separate flask. After first cooling the frozen pooled samples to the temperature of an acetone and solid carbon dioxide mixture they were freeze-dried. A 30-ml. sample was dried in this way in just over 2 hr. The dried sample was then extracted with 10 ml. of absolute ethanol, to dissolve acetylcholine and leave behind most of the Krebs salts, protein-like material and 5-hydroxytryptamine. Dimethylphenylpiperazinium is appreciably soluble in absolute ethanol.

8-ml. aliquots of this ethanolic extract were transferred to 30-ml.-capacity drug vials and the ethanol was removed under reduced pressure.

The assay. The residual smears on the bottom of the drug vials were each dissolved in 2 ml. of Krebs solution and assayed for acetylcholine on the guinea-pig ileum preparation treated with mipafox as described by Birmingham (1961), except that morphine was found unnecessary for a successful assay and was omitted (Johnson, 1963a). To eliminate the possibility that traces of 5-hydroxytryptamine carried over during the ethanol extraction might contribute to the response height of the sample during the assay, the assay preparation was treated with an excess of 5-hydroxytryptamine (5 μg/ml.) which prevented the stimulant action of further doses of 5-hydroxytryptamine (Johnson, 1963b). Treated in this way, the assay ileum would not respond to ten-times the maximum amount of 5-hydroxytryptamine which would be present had this drug been extracted completely by the ethanol. Dimethylphenylpiperazinium was always present in the samples prepared for assay but its interference was prevented by the use of ganglion-blocking concentrations of pentolinium or hexamethonium.

In each experiment in which the amount of acetylcholine released by 5-hydroxytryptamine or dimethylphenylpiperazinium is calculated four groups of assays have been made. One of these estimates the total amount of acetylcholine released during ten challenges, each of 45 sec, in the presence of the drug. A

second assay estimates the amount of acetylcholine released at rest for an equivalent period of time, but during which no drug was added. A third assay measures the fate of added acetylcholine during the equivalent period of the experiment; a fourth assay is of spasmogenic material from Krebs blanks (originally 30 ml. of Krebs solution) treated identically with the experiments above.

When all the samples had been collected, the volume of the ileal segment was measured by fluid displacement and its wet weight was noted.

Each experiment took approximately 40 hr (3 to 4 days).

Identification. The spasmogenic substance released was inhibited by hyoscine but not by a high concentration of 5-hydroxytryptamine or ganglion-blocking concentrations of pentolinium or hexamethonium. The activity was destroyed by boiling with N-sodium hydroxide but was unaffected by N-hydrochloric acid. In a single experiment a preparation of the dorsal muscle of the leech would not contract to the active principle unless it had first been treated with physostigmine. These tests eliminated histamine, 5-hydroxytryptamine and adrenaline (the latter relaxes the middle ileum preparation) as possible agents and it was concluded that the substance was an ester of choline with an order of potency identical to acetylcholine.

In the results which follow, the amount of acetylcholine has been calculated in terms of picomoles (pmoles or 10^{-12} moles) or millipicomoles (mpmoles or 10^{-15} moles) per mg of ileum per challenge; this amount of acetylcholine released per unit weight of tissue had been related to the amount of added releasing drug present in the extracellular space of unit weight of tissue. For this purpose the extracellular space has been assumed to be equivalent to one-third of the volume of the ileum, a value calculated for other intestinal preparations (Goodford & Hermansen, 1961; Burnstock, Holman & Prosser, 1963); it has also been assumed that the concentration of releasing drug is the same in the extracellular fluid as in the organ-bath fluid. Thus the units of the amounts of releasing drug will be expressed as pmoles of drug present in the extracellular volume of 1 mg of ileum (pmoles per mg-vol).

While the higher doses of 5-hydroxytryptamine or dimethylphenylpiperazinium always released measurable amounts of acetylcholine in some preparations, lower doses did not release sufficient acetylcholine to fall within the limits of the assay.

RESULTS

Acetylcholine recovery. Whether the acetylcholine set free survived the lengthy yet carefully controlled procedures was checked by simultaneous recovery experiments made on each occasion. Each segment of ileum was exposed for 45 sec to 5-ng amounts of acetylcholine and ten such samples were bulked and processed. The experimental recovery of acetylcholine in sixteen experiments, after subtracting the value for the Krebs blank, was 51.6 ± 5.5 ng (mean and standard error) compared with a theoretical yield of ten times doses of 5 ng, indicating an absence of both hydrolysis and release of the ester.

The release of acetylcholine by 5-hydroxytryptamine. The values for the amounts of acetylcholine released by different doses of 5-hydroxytryptamine for ten experiments are plotted in the two graphs of Fig. 2. It can be seen that increasing the concentration of 5-hydroxytryptamine leads to an increase in the amount of recoverable acetylcholine.

The number of molecules of 5-hydroxytryptamine required to release one molecule of acetylcholine from 1 mg of tissue was calculated for each point plotted in Fig. 2 (right-hand graph) by dividing the values for each dose of drug (pmoles/mg-vol) by the amount of acetylcholine set free (pmoles/mg/challenge), and the mean and standard error of these values were also calculated. It was found that 10 ± 2 molecules of 5-hydroxytryptamine were required to release one molecule of acetylcholine from 1 mg of guinea-pig isolated ileum during a 45-sec drug-contact period.

Acetylcholine release and contractions due to 5-hydroxytryptamine. The average equilibrium contraction height for each set of ten contractions was calculated as the percentage

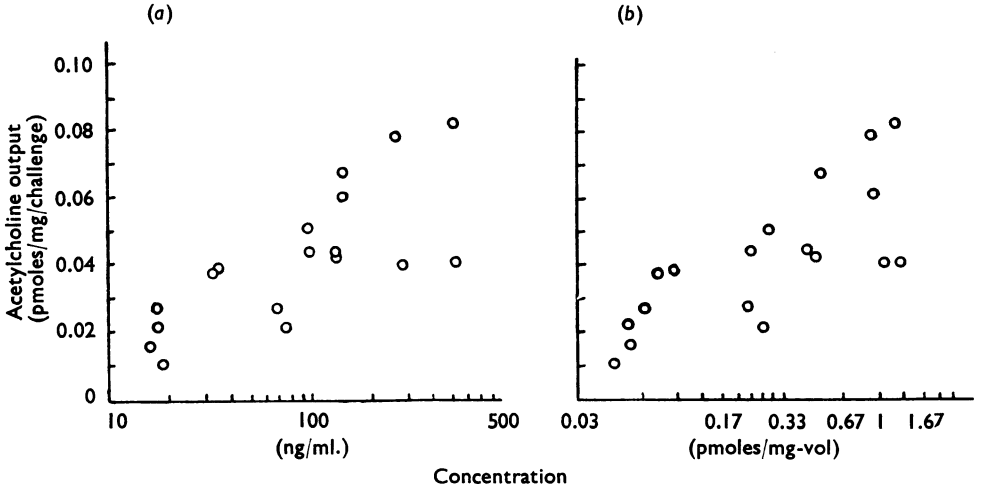
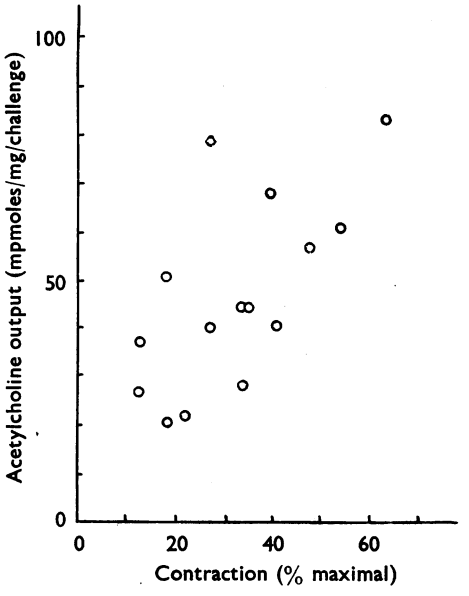


Fig. 2. (*a*) The output of acetylcholine induced by 5-hydroxytryptamine (pmoles/mg of wet ileum/challenge, ordinate) plotted against the concentration of 5-hydroxytryptamine (ng/ml. of bath fluid, abscissa). The points are taken from ten experiments. (*b*) The acetylcholine output (units as above) is plotted against the concentration of 5-hydroxytryptamine expressed as pmoles present in the volume of 1 mg of wet ileum (pmoles/mg-vol, abscissa). Both the abscissae are plotted on log scales. Increase in the concentration of 5-hydroxytryptamine leads to an increase in the amount of recoverable acetylcholine. The derived units for the 5-hydroxytryptamine concentration (*b*) did not affect appreciably the scatter of the points, which shows that the error in calculating these units is small compared with the errors associated with the experiments themselves.

of the maximal contraction of the ileum preparation. The accepted maximal contraction was the response of the ileum to 5 μg of acetylcholine. Fig. 3 shows the relation between the 5-hydroxytryptamine-induced release of the acetylcholine and the percentage of the maximal contraction of the ileum to 5-hydroxytryptamine. It can be seen that the acetylcholine output and the contractions of the ileum are simply related. Both are related to the same concentrations of 5-hydroxytryptamine.

The release of acetylcholine by dimethylphenylpiperazinium. The values for the acetylcholine outputs induced by different doses of dimethylphenylpiperazinium were derived in the same way as those for 5-hydroxytryptamine. The acetylcholine output is plotted against the concentration of dimethylphenylpiperazinium (units as described before) in Fig. 4; the values are taken from six experiments. The most prominent feature of this plot is the wide scatter of the points; the best straight line fitted to them (not shown) has a gradual negative slope. In spite of this scatter it was possible to calculate for each point the number of molecules of dimethylphenylpiperazinium that would release one molecule of acetylcholine from 1 mg of ileum by dividing the values for each dose of the drug (pmoles/mg-vol) by the amount of acetylcholine set free (pmoles/mg/challenge). The mean and standard error of these values were then calculated. From the total observations made, rejecting none, it was found that 84 ± 25 molecules of dimethylphenylpiperazinium were required to release one molecule of acetylcholine from 1 mg of ileum during a 45-sec contact period.



Fg. 3. The relation between the 5-hydroxytryptamine-induced release of acetylcholine (mpmoles/mg/challenge, ordinate) and the percentage of the maximal contraction of the ileum to 5-hydroxytryptamine (abscissa). The average equilibrium contraction height for each group of ten contractions was calculated as the percentage of the maximal contraction of the isolated ileum preparation. When allowance is made for the error associated with these experiments it seems that the acetylcholine output and contractions of the ileum are simply related.

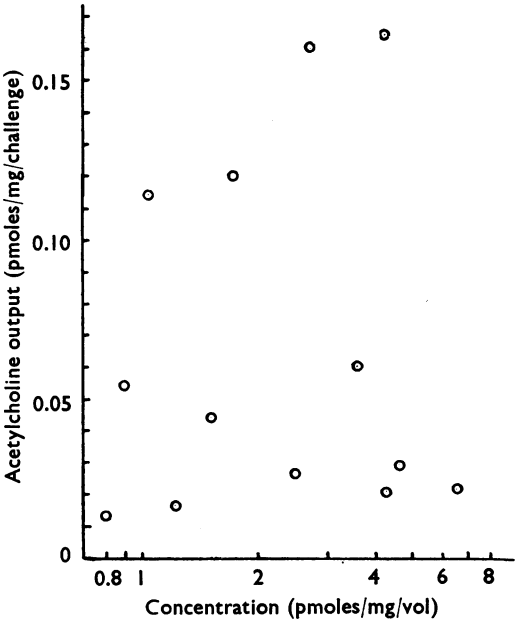


Fig. 4. The acetylcholine output from the guinea-pig isolated ileum induced by dimethylphenylpiperazinium (six experiments). The acetylcholine output (ordinate as in Fig. 2) is plotted against the concentration of dimethylphenylpiperazinium expressed as pmoles present in the volume of 1 mg of wet ileum (pmoles/mg-vol, abscissa). The abscissa is plotted on a log scale. There appears to be no obvious relation between the acetylcholine output and the concentration of dimethylphenylpiperazinium.

When the outputs of acetylcholine caused by doses of dimethylphenylpiperazinium which gave responses of the original test preparation between 25 and 50% of the maximal contraction were considered separately from the outputs corresponding to doses giving responses above 50% of the maximal, then it was found that 32 ± 9 molecules and 138 ± 69 molecules of dimethylphenylpiperazinium were respectively required to release one molecule of acetylcholine from 1 mg of tissue.

Acetylcholine release and contractions due to dimethylphenylpiperazinium. The contraction of the ileum to 5-hydroxytryptamine followed a delay period of approximately 10 sec after injection and the peak height of contraction was about the same height as the equilibrium position at the end of the 45-sec contact time (Fig. 1). The peak height of contraction to dimethylphenylpiperazinium was reached after about 3 to 5 sec but the responses to the higher doses decreased soon afterwards and reached their equilibrium position at a level considerably lower than the peak height. Sometimes the equilibrium

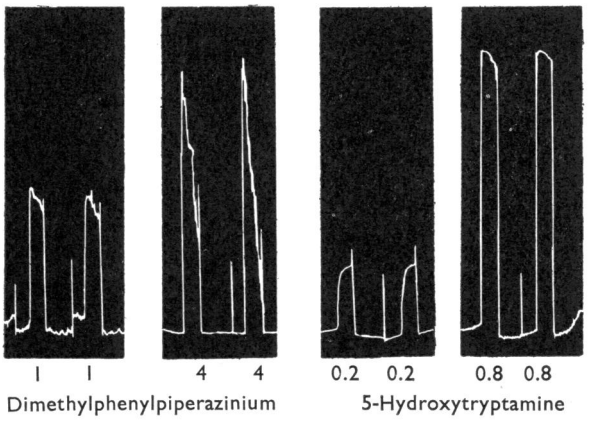


Fig. 5. Contractions of the isolated ileum preparation to two doses of 5-hydroxytryptamine are compared with contractions to two corresponding doses of dimethylphenylpiperazinium. After the 45-sec contact time the equilibrium contraction height to 5-hydroxytryptamine is not significantly different from the peak height of contraction (see also Fig. 1), but with higher doses of dimethylphenylpiperazinium (usually above ED50) the equilibrium contraction height is considerably less than the peak height of contraction. The doses given are in μg added to the 3-ml. organ-bath.

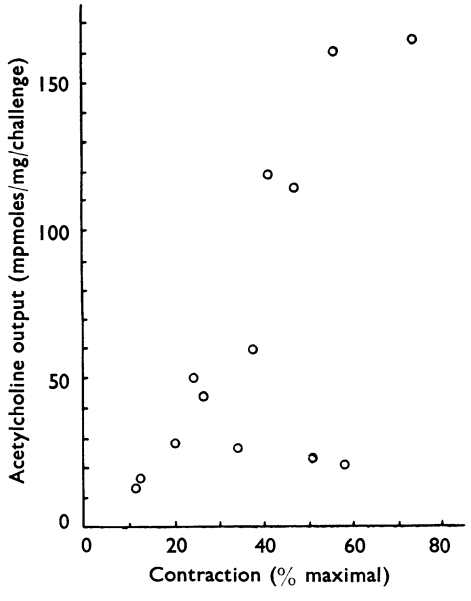


Fig. 6. The relation between the dimethylphenylpiperazinium-induced release of acetylcholine (ordinate as in Fig. 3) and the percentage of the maximal contraction of the ileum to dimethylphenylpiperazinium (abscissa). The average equilibrium contraction height for each group of ten contractions was calculated as the percentage of the maximal contraction of the isolated ileum preparation. When allowance is made for the error associated with these experiments it appears that the acetylcholine output and contractions of the ileum to dimethylpiperazinium are simply related.

level was not reached after the 45-sec contact period and in these cases the equilibrium position has been measured where the response level intersects the 45-sec position. Contractions to two doses of dimethylphenylpiperazinium are shown in Fig. 5 and are compared with contractions to corresponding doses of 5-hydroxytryptamine. The average equilibrium contraction height for each set of ten responses to dimethylphenylpiperazinium was calculated as the percentage of the maximal contraction of the ileum preparation, as were those for the 5-hydroxytryptamine responses. The relation between the dimethylphenylpiperazinium-induced release of acetylcholine and the percentage of the maximal contraction of the ileum to dimethylphenylpiperazinium is shown in Fig. 6. The acetylcholine output and contractions of the ileum seem to be simply related.

DISCUSSION

Two difficulties are met in any attempt to measure the amount of transmitter released from the intestine by drugs; the first arises because the amounts set free are small in any single drug challenge, and the second because of difficulties in assaying samples contaminated by the drug used to release the transmitter. The first problem was solved by combining the bath fluids from ten challenges at each concentration of agonist, freeze drying, extracting with ethanol and reconstituting into 2 ml. of Krebs solution. In this way concentrations of acetylcholine were achieved which fell well within the sensitivity range of the assay preparation. The possibility of interference from 5-hydroxytryptamine or dimethylphenylpiperazinium carried over into the test assays was countered by the use of specific blocking agents.

Of the four assays comprising one experiment, one estimated the amount of acetylcholine released during the 45-sec period in which the ileum was exposed to the drug; the second estimated the amount of acetylcholine released from the ileum at rest for an equivalent period of time during which no drug was added; a third assay was of spasmogenic material from the Krebs blanks; and the fourth measured the fate of added acetylcholine during the equivalent period of the experiment. That there was no loss of acetylcholine during the extraction procedure was shown by carefully controlled simultaneous experiments which gave a mean recovery and standard error of $103 \pm 11\%$ of added acetylcholine and provided no evidence of loss by hydrolysis. These experiments served to show also that the presence of acetylcholine itself did not release more acetylcholine from the intestine. An unlikely alternative must be acknowledged, that the amount of hydrolysis was exactly matched by the amount of acetylcholine simultaneously released.

There was no indication that the small concentrations of 5-hydroxytryptamine and dimethylphenylpiperazinium used in these experiments were releasing acetylcholine from the nerve endings in the circular muscle layer in any significant amounts: the acetylcholine recovered seemed to be related to the equilibrium contraction height of the longitudinal muscle. The highest concentration of 5-hydroxytryptamine or dimethylphenylpiperazinium used in the present experiments was only a fraction of the threshold dose for circular muscle contraction (Brownlee & Harry, 1963) but it must be admitted that there has been no direct evidence that these drugs did not release acetylcholine from nerve endings in the circular muscle in amounts which, although insufficient to contract the circular muscle, may have diffused across and summated with the acetylcholine released from the nerve endings in the longitudinal muscle.

Turning now to a consideration of the relation between the amount of acetylcholine recovered and the observed effect on the ileum, it may be extrapolated from Figs. 2 and 3 that about 450 ng of 5-hydroxytryptamine added to the 3-ml. organ-bath produced 50% of the maximal contraction from the segment of ileum and this corresponded to 11 pg of acetylcholine released and finally recovered from each mg of ileum. Similarly, it can be calculated from Figs. 4 and 6 that about 4 μg of dimethylphenylpiperazinium produced 50% of the maximal contraction and released 11 pg of acetylcholine from each mg of ileum. The average segment of ileum weighed 148 mg, and this when multiplied by 11 pg gave a final amount of 1.6 ng of acetylcholine released by the drugs into the organ-bath fluid. It was found from experiments made at the same time that about 5 ng of acetylcholine had to be added to the 3-ml. organ-bath to match the contractions observed when 11 pg/mg of acetylcholine was released. Thus, the sizes of the contractions of the ileum of the guinea-pig that follow the addition of amounts of 5-hydroxytryptamine or dimethylphenylpiperazinium to the bath are consistent with the activity of acetylcholine in concentrations recovered from the organ-bath fluid.

The comparison of the amount of acetylcholine released by 5-hydroxytryptamine or dimethylphenylpiperazinium can be carried a stage further by relating it to the unit weight of tissue which released it. For this purpose the assumption was made that during the period of 45-sec exposure to 5-hydroxytryptamine or dimethylphenylpiperazinium the isolated ileum concentrated neither drug within its tissues, but that these remained uniformly distributed between the extracellular tissue fluid and the bath fluid. The extracellular space of visceral smooth muscle has been shown (by the inulin method) to vary between 30 and 35% of the volume (Burnstock *et al.*, 1963); for the taenia coli of the guinea-pig it was found to be 33% (Goodford & Hermansen, 1961). If these figures are applicable to the ileum of the guinea-pig it means that one-third of the volume of unit weight of ileum contained a concentration of 5-hydroxytryptamine or dimethylphenylpiperazinium identical to that of the bath fluid. Thus the amount of drug in each mg of ileum was calculated by multiplying together the bath concentration of the drug in ng/ml. and the volume of the ileum multiplied by one-third, and dividing this total by the weight of the ileum in mg multiplied by the molecular weight of the drug.

When the figure obtained is multiplied by 1,000 the answer is given in pmoles of challenging drug per mg-volume of ileum.

Thus pmoles of challenging drug contained in the extracellular volume corresponding to 1 mg of ileum=

$$\frac{\text{drug concentration (ng/ml.)} \times \text{vol of ileum (ml.)} \times 1{,}000}{\text{weight of ileum (mg)} \times \text{molecular weight of drug} \times 3}$$

This challenging dose of drug was responsible for the increased acetylcholine output from each mg of ileum (mpmoles ; 10^{-15} moles of released acetylcholine per mg of ileum per challenge).

When an attempt was made to relate the amount of acetylcholine released by 5-hydroxytryptamine to that set free by dimethylphenylpiperazinium, a difficulty was immediately evident. Whereas increases in doses of 5-hydroxytryptamine resulted in increases in the amount of acetylcholine recovered (Fig. 2), this was not true for dimethylphenylpiperazinium and, indeed, inspection of Fig. 4 shows that this relation is a complex one.

An inspection of the responses of the ileum to doses of dimethylphenylpiperazinium used in the experiments (Fig. 5) made it at once apparent that inclusion of the larger doses of dimethylphenylpiperazinium was invalid. Whereas the peak contractions seen with low or high doses of 5-hydroxytryptamine were sustained during the period of drug contact, or in other words the peak and the equilibrium contraction height were similar, this was true only of low doses of dimethylphenylpiperazinium. With higher doses, and these were doses usually above the ED50 responses, the contraction was not sustained and no equilibrium level was seen. The observed effect of 5-hydroxytryptamine over the entire range of doses used was consistent with the view that the total activity was accounted for by released acetylcholine which was subsequently measured and found to be related to the equilibrium contraction height. The observed effect of dimethylphenylpiperazinium was consistent with the two known phases of action of the drug. The first is a stimulant action (Chen, Portman & Wickel, 1951) seen with low doses of the drug up to those producing about ED50 responses and corresponded to equilibrium contractions and which it seemed logical to relate to the output of acetylcholine. The second phase of action of the drug is seen with higher doses and is first stimulant and then blocking (Leach, 1957; Ling, 1959) at the ganglion. It may be objected that blockade of ganglia would not have been seen with such small amounts of dimethylphenylpiperazinium, but it must be borne in mind that this ileum preparation differed from those previously described in being first treated with an effective, persistent anticholinesterase drug.

For these reasons it appeared legitimate to compare directly the stimulatory phase of the dual action of dimethylphenylpiperazinium with the stimulatory action of 5-hydroxytryptamine. Thus, when the acetylcholine outputs caused by doses of dimethylphenylpiperazinium, which gave responses between 25 and 50% of the maximal contraction on the original test preparation, were considered separately from the outputs corresponding to the doses giving responses greater than 50% of the maximal, then it was found, on dividing the values for each dose of drug (pmoles/mg-vol) by the amount of acetylcholine set free (mpmoles/mg/challenge), that 32 ± 9 and 138 ± 69 molecules respectively of dimethylphenylpiperazinium were required to release one molecule of acetylcholine from 1 mg of tissue, and for 5-hydroxytryptamine 10 ± 2 molecules for both response levels. The greater variability of the effect of large doses of dimethylphenylpiperazinium is reflected in the very high standard error for values above ED50.

These results show that, irrespective of the part of the dose/response curve considered, 5-hydroxytryptamine was more potent than dimethylphenylpiperazinium in its capacity to release acetylcholine from the ileum treated with mipafox.

When the amounts of acetylcholine released by concentrations of 5-hydroxytryptamine or dimethylphenylpiperazinium which gave ED25 and ED50 contractions were compared, they were found to be the same. It appears legitimate to assume that other drugs which contract the guinea-pig ileum to the same extent by the mediation of acetylcholine at the muscarinic site will also release the same amounts of acetylcholine.

In the present instance it was calculated from the points reproduced in Fig. 3 that the doses of 5-hydroxytryptamine which caused 25 and 50% of the maximal contractions (ED25 and ED50) released respectively 41 and 61 mpmoles of acetylcholine from 1 mg of guinea-pig ileum; similarly, the doses of dimethylphenylpiperazinium which caused 25 and 50% of the maximal contractions released respectively 37 and 86 mpmoles of acetyl-

choline from 1 mg of ileum (Fig. 6). Since the errors associated with the slopes of the best straight lines calculated by the method of least squares for the points plotted in Figs. 3 and 6 overlap, this means that within the error of these estimates of slope the ED25 responses to 5-hydroxytryptamine could have been caused by the same amount of acetylcholine released as were the ED25 responses to dimethylphenylpiperazinium, and the ED50 responses to 5-hydroxytryptamine could likewise have been caused by the same amount of acetylcholine released as were the ED50 responses to dimethylphenylpiperazinium. If these results are extrapolated to include any drug which acts indirectly then it can be concluded that such a drug, when allowed to act on the guinea-pig ileum treated with an anticholinesterase and which causes it to contract to 25 or 50% of its maximal response will release respectively 0.039 and 0.074 pmoles of acetylcholine from each mg of tissue.

Why did 5-hydroxytryptamine and dimethylphenylpiperazinium differ in their capacities to release acetylcholine from the ileum? The differences in the mechanisms of action of the two drugs may first be considered under four headings: the site of action; the nature of the response; the time required by the drug to initiate its response; and the nerve pathway involved.

The site of action. A difference in the site of action of 5-hydroxytryptamine and dimethylphenylpiperazinium on the intramural nerves was reported by Day & Vane (1963), and Brownlee & Johnson (1963) presented evidence that both drugs act on parasympathetic ganglion cells, but that the 5-hydroxytryptamine receptors are different from those at which dimethylphenylpiperazinium acted; the possibilities were discussed that the two kinds of receptor could be situated on the same, or on a pharmacologically distinct cell. The demonstration of the existence of different receptors for 5-hydroxytryptamine and dimethylphenylpiperazinium did not make it necessary to consider the possibility that their different capacities to release acetylcholine arose from their differing affinities for the same receptor.

The nature of the response. This has been discussed already; in the concentrations used 5-hydroxytryptamine only stimulated, whereas dimethylphenylpiperazinium first stimulated and then blocked.

The time required by the drug to initiate its response. Yet another difference in the mode of action of 5-hydroxytryptamine and dimethylphenylpiperazinium was that between the times of onset of the contractions after the addition of the drugs to the organ-bath fluid. The response to 5-hydroxytryptamine was delayed by about 10 sec; that to dimethylphenylpiperazinium by only about 3 sec. A similar observation was made on the taenia coli preparation by Bülbring & Burnstock (1960), but using acetylcholine and 5-hydroxytryptamine; the conditions differed but the ratios of the delay times were similar to those reported here.

The nerve pathway involved. Gaddum & Hameed (1954) discussed the possibility that 5-hydroxytryptamine acts at a different kind of ganglion cell from the one at which nicotine acts, but they lacked the evidence for a ganglionic action of 5-hydroxytryptamine which was later provided by the experiments of Brownlee & Johnson (1963), who also raised the question whether the specific ganglion receptors for 5-hydroxytryptamine were located on a cholinergic nerve pathway which was separate from that stimulated by nicotine or dimethylphenylpiperazinium. Johnson (1964) has obtained evidence for this possibility from experiments made to show the effect on the responses to acetylcholine, 5-hydroxy-

tryptamine and dimethylphenylpiperazinium, of the time course of inhibition of the cholinesterases of the guinea-pig isolated ileum by mipafox. Johnson's results indicated that the acetylcholine released by 5-hydroxytryptamine is hydrolysed by a cholinesterase with different properties from that which hydrolyses the acetylcholine released by dimethylphenylpiperazinium or acetylcholine added exogenously. It is difficult to imagine how this could arise unless 5-hydroxytryptamine acts on a nerve pathway independent from that activated by dimethylphenylpiperazinium.

These discussed differences in the mechanisms of action of the two drugs provide an acceptable basis for the observation that, molecule for molecule, 5-hydroxytryptamine releases more acetylcholine from the guinea-pig ileum than does dimethylphenylpiperazinium.

SUMMARY

1. A method is described for recovering and estimating the small amounts of acetylcholine released from the isolated ileum of the guinea-pig by 5-hydroxytryptamine or dimethylphenylpiperazinium previously treated with anticholinesterase concentrations of *N,N*-diisopropylphosphorodiamidic fluoride (mipafox).

2. In each experiment the procedure involved the selection of two or three doses of 5-hydroxytryptamine or dimethylphenylpiperazinium which gave contractions of different magnitudes and with each of these doses the ileum was challenged ten times. For each dose the ten bathing fluids, each of 3 ml., were combined and frozen at $-20°$ C. The pooled samples were then freeze dried and the acetylcholine in the dried sample was extracted with ethanol. The ethanol was removed under reduced pressure and the residual smears were dissolved in 2 ml. of Krebs solution and assayed for acetylcholine on the guinea-pig ileum treated with mipafox.

3. Interference from 5-hydroxytryptamine or dimethylphenylpiperazinium carried over into the final assay was eliminated by the use of specific blocking agents.

4. Under identical conditions, sixteen recovery experiments involving ten challenges with 5 ng of acetylcholine gave $103 \pm 11\%$ (mean and standard error) recovery.

5. Irrespective of the part of the dose/response line considered, 5-hydroxytryptamine was more potent than dimethylphenylpiperazinium in its ability to release acetylcholine. Thus, for experiments in which the doses of agonists produced responses between 25 and 50% of maximal, one molecule of acetylcholine was released from 1 mg of wet ileum by 10 ± 2 molecules of 5-hydroxytryptamine or 32 ± 9 molecules of dimethylphenylpiperazinium during a 45-sec drug-contact period. Because of the blocking actions of higher doses of dimethylphenylpiperazinium, 138 ± 69 molecules were required in the dose range above ED50.

6. The size of the contraction to a dose of 5-hydroxytryptamine or dimethylphenylpiperazinium measured at the end of a 45-sec period was related to the amount of acetylcholine released.

7. The amounts of 5-hydroxytryptamine and dimethylphenylpiperazinium, and by inference of any other indirectly acting drug, that induced a 50% maximal contraction of the guinea-pig ileum treated with an anticholinesterase drug, would release the same amount of acetylcholine, corresponding to about 0.075 pmoles of acetylcholine recovered from each mg of wet ileum.

8. The discussed differences in the mechanisms of action of the two drugs appear to provide acceptable reasons for the observation that, molecule for molecule, 5-hydroxytryptamine releases more acetylcholine from the guinea-pig ileum than does dimethylphenylpiperazinium.

REFERENCES

BIRMINGHAM, A. T. (1961). Absence of spasm in a sensitive assay for acetylcholine. *J. Pharm. Pharmacol.*, **13**, 510.

BROWNLEE, G. & HARRY, J. (1963). Some pharmacological properties of the circular and longitudinal muscle strips of the guinea-pig isolated ileum. *Brit. J. Pharmacol.*, **21**, 544–554.

BROWNLEE, G. & JOHNSON, E. S. (1963). The site of the 5-hydroxytryptamine receptor on the intramural nervous plexus of the guinea-pig isolated ileum. *Brit. J. Pharmacol.*, **21**, 306–322.

BÜLBRING, E. & BURNSTOCK, G. (1960). Membrane potential changes associated with tachyphylaxis and polarization of the response to stimulating drugs in smooth muscle. *Brit. J. Pharmacol.*, **15**, 611–624.

BURNSTOCK, G., HOLMAN, M. E. & PROSSER, C. L. (1963). Electrophysiology of smooth muscle. *Physiol. Rev.*, **43**, 482–527.

CHEN, G., PORTMAN, R. & WICKEL, A. (1951). Pharmacology of 1,1-dimethyl-4-phenylpiperazinium iodide, a ganglion stimulating agent. *J. Pharmacol. exp. Ther.*, **103**, 330–336.

DAY, M. & VANE, J. R. (1963). An analysis of the direct and indirect actions of drugs on the isolated guinea-pig ileum. *Brit. J. Pharmacol.*, **20**, 150–170.

GADDUM, J. H. & HAMEED, K. A. (1954). Drugs which antagonize 5-hydroxytryptamine. *Brit. J. Pharmacol.*, **9**, 240–248.

GOODFORD, P. G. & HERMANSEN, K. (1961). Sodium and potassium movements in the unstriated muscle of the guinea-pig taenia coli. *J. Physiol. (Lond.)*, **158**, 426–448.

HARRY, J. (1962). Effect of cooling, local anaesthetic compounds and botulinum toxin on the responses of and the acetylcholine output from the electrically transmurally-stimulated isolated guinea-pig ileum. *Brit. J. Pharmacol.*, **19**, 42–55.

JOHNSON, E. S. (1963a). A note on the relation between the resting release of acetylcholine and increase in tone of the isolated guinea-pig ileum. *J. Pharm. Pharmacol.*, **15**, 69–72.

JOHNSON, E. S. (1963b). The origin of the acetylcholine released spontaneously from the guinea-pig isolated ileum. *Brit. J. Pharmacol.*, **21**, 555–568.

JOHNSON, E. S. (1964). An independent nerve-pathway for 5-hydroxytryptamine in the guinea-pig ileum. *J. Pharm. Pharmacol.*, **16**, 760–763.

LEACH, G. D. H. (1957). Ganglion blocking actions of dimethylphenylpiperazinium (DMPP). *J. Pharm. Pharmacol.*, **9**, 747–751.

LING, H. W. (1959). Actions of dimethylphenylpiperazinium. *Brit. J. Pharmacol.*, **14**, 505–511.

PATON, W. D. M. (1955). The response of the guinea-pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol. (Lond.)*, **127**, 40–41*P*.

PATON, W. D. M. (1957). The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Brit. J. Pharmacol.*, **12**, 119–127.