Brit. J. Pharmacol. (1963), **21**, 555–568.

THE ORIGIN OF THE ACETYLCHOLINE RELEASED SPONTANEOUSLY FROM THE GUINEA-PIG ISOLATED ILEUM

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(*Received September* 2, 1963)

When the guinea-pig isolated ileum had been previously treated with the anticholinesterase, *NN*-diisopropylphosphodiamidic fluoride (mipafox), and attached to an isotonic lever loaded with 0.5 g, it released acetylcholine into Krebs solution gassed with a mixture of 95% oxygen and 5% carbon dioxide. The amount of acetylcholine spontaneously released depended on the duration of the rest period. Cocaine, procaine or cooling the preparation to 25° C greatly reduced this spontaneous output of acetylcholine, thus providing evidence in support of nervous origin of the ester. Reduction of the calcium ion content of the Krebs solution to one-twentieth of its usual value or increase in the magnesium ion content four-fold, changes which inhibit the release of acetylcholine from somatic motor nerve-endings, inhibited the output of acetylcholine from the ileum. When all the calcium of Krebs solution had been replaced by strontium one-third of the control output of acetylcholine was obtained, but the smooth muscle of the guinea-pig ileum would not respond to drugs under these conditions. Strontium could thus partially substitute for calcium in nerves but not in muscle. Hemicholinium-3 (100 μg/ml.) inhibited the spontaneous release of acetylcholine and 400 μg/ml. of choline was required to reverse the inhibition. It is concluded that cocaine, procaine, cooling, reduction of calcium ion and increase of magnesium ion concentrations all reduce the spontaneous output of acetylcholine, which has its origin in the parasympathetic nerve-endings of the intramural nerve plexuses. Further, the hemicholinium experiments seem to justify the conclusion that the release of acetylcholine is reduced because synthesis is reduced.

The guinea-pig ileum, whether *in situ* or isolated, has a continuous release of acetylcholine which does not depend on the integrity of the extrinsic nerves (Feldberg & Lin, 1950).

Johnson (1963) showed that the amount of acetylcholine released per unit weight of intestine incubated in Krebs solution was proportional to the period of time the intestine was allowed to rest, and that when a segment of ileum was treated with the anticholinesterase *NN*-diisopropylphosphodiamidic fluoride (mipafox) and arranged to record longitudinal contractions the acetylcholine released induced a gradual increase in tone.

Feldberg & Lin (1950) and Chujyo (1953) thought that the acetylcholine originated in non-nervous structures but Schaumann (1957) and Paton (1957) favoured a

nervous source. The present work extends the study of the endogenous release of acetylcholine and includes an inquiry into its origin.

METHODS

Adult guinea-pigs were killed and the ileum excised. Segments, 3 cm long, from the middle and terminal regions were incubated for 75 min in 200 ml. of Krebs solution gassed with a mixture of 95% oxygen and 5% carbon dioxide and containing the anticholinesterase mipafox in a concentration of 10 μg/ml. Mipafox was chosen because of its apparent inability to release acetylcholine (Carlyle, 1963).

A segment of ileum from the middle region, so-treated, was transferred to a 3 ml. organ-bath of Krebs solution, bubbled with a mixture of 95% oxygen and 5% carbon dioxide and arranged to record longitudinal isotonic contractions. The lever had a magnification of ten-times with a load of 0.5 g.

Experiments

Dose/response lines were constructed for histamine, 5-hydroxytryptamine and nicotine, after which the ileum was washed for 15 min by repeated exchange of the bath fluid. The preparation was then allowed to rest for periods of 5, 10, 20 and 40 min. Samples for assay were obtained by removing the entire bath fluid in a 5 ml. syringe after each period of rest. The dose/response lines and rest periods were repeated in Krebs solution modified in the ways described below and, after washing for 45 min, were repeated again in normal Krebs solution.

Identification and estimation of the spasmogenic substance as acetylcholine

Samples were assayed for acetylcholine on the ileum of the guinea-pig treated with mipafox in a concentration of 10 μg/ml. (Birmingham, 1961). The identification of the active agent as acetylcholine was based on the following criteria. The spasmogenic activity was destroyed by boiling with N-sodium hydroxide solution but was not affected by boiling with N-hydrochloric acid. Hyoscine (0.1 μg/ml. in Krebs solution) antagonized acetylcholine, the sample and 5-hydroxytryptamine, but left the response to histamine unchanged. The sample often caused a small residual contraction in the presence of hyoscine and, when this occurred, its value was subtracted from the assay value of the sample.

In one experiment the sample depressed the blood pressure of a cat previously treated with mipafox (1 mg/kg) and increased the hind-limb blood flow, and both these effects were blocked by hyoscine, observations which eliminated adrenaline and 5-hydroxytryptamine as possible agents. In addition a much greater dose of 5-hydroxytryptamine than of acetylcholine was required to produce equivalent responses of the ileum. 5-Hydroxytryptamine can be eliminated by specifically desensitizing the preparation used for assay with 5 μg/ml. of 5-hydroxytryptamine (Brownlee & Johnson, 1963). The substance released had the same potency as acetylcholine in parallel assays on the guinea-pig ileum and the cat blood pressure.

Whenever a modified Krebs solution was used the standard acetylcholine was dissolved in this modified solution. Calcium-deficient Krebs solution was reconstituted for the assay. In the nanogram range this assay procedure was routinely successful.

Drugs

Acetylcholine chloride, choline chloride, cocaine hydrochloride, *NN*-diisopropylphosphodiamidic fluoride (mipafox), hemicholinium-3, histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate, hyoscine hydrobromide, nicotine hydrogen tartrate, procaine hydrochloride and strontium chloride ($6H_2O$) were used. All drugs are expressed as μg of base/ml. of Krebs solution, except hemicholinium and mipafox which are expressed as the salts.

The Krebs solution contained choline in a concentration of 1 μg/ml. unless otherwise stated. This concentration of choline, which does not contract the ileum, was included because it is a constituent of plasma in many species (Bligh, 1952).

RESULTS

Spontaneous release of acetylcholine

In twenty-two experiments the amounts of acetylcholine spontaneously released (pg/mg, means ± standard errors) were 31 ± 4 for 5 mins, 54 ± 11 for 10 min, 67 ± 9 for 20 min and 112 ± 16 for 40 min. Within the limits of the standard errors there appears to be a simple relationship between the spontaneous acetylcholine output and the period measured (Fig. 1). The output (pg/mg/min) of acetylcholine fell

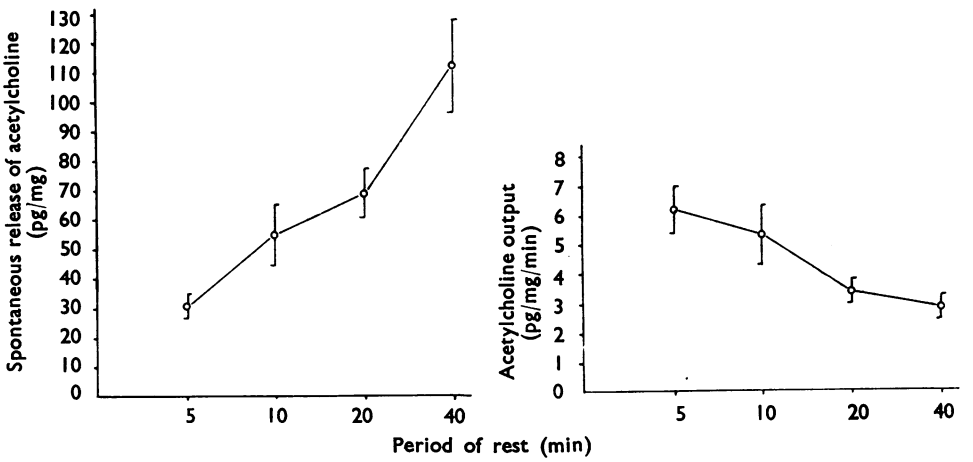

Fig. 1. Left-hand graph: the spontaneous release of acetylcholine (pg/mg of wet ileum, ordinate) for the four periods of rest (abscissa, plotted on a log scale). Each point is the mean of twenty-two experiments with the standard error (vertical line). There appears to be a simple relationship between the acetylcholine output and the duration of the rest period. Right-hand graph: the acetylcholine output is expressed as the amount released from 1 mg of wet ileum/min (ordinate) for the samples taken after rest periods of 5, 10, 20 and 40 min. The output of acetylcholine thus expressed fell progressively during the experiment.

progressively during the experiment; thus at 5 min it was 6.2 ± 0.8, at 10 min 5.3 ± 1.0, at 20 min 3.4 ± 0.4 and at 40 min 2.8 ± 0.4 pg/mg/min (Fig. 1, right-hand graph).

Local anaesthetic compounds and cooling

Cocaine. Cocaine (5 μg/ml.) inhibited the responses to 5-hydroxytryptamine and nicotine but left those to histamine unchanged. This concentration of cocaine inhibited the spontaneous release of acetylcholine during the four periods of incubation (Fig. 2).

Procaine. Procaine (10 μg/ml.) selectively inhibited the responses to 5-hydroxytryptamine and nicotine but the responses to histamine were unaffected. Procaine greatly reduced the spontaneous release of acetylcholine (Fig. 3).

Cooling. Cooling the ileum to 25° C modified the responses to the indirectly acting drugs but left the responses to histamine unaffected. There was a marked reduction of the spontaneous release of acetylcholine on cooling to 25° C (Fig. 4).

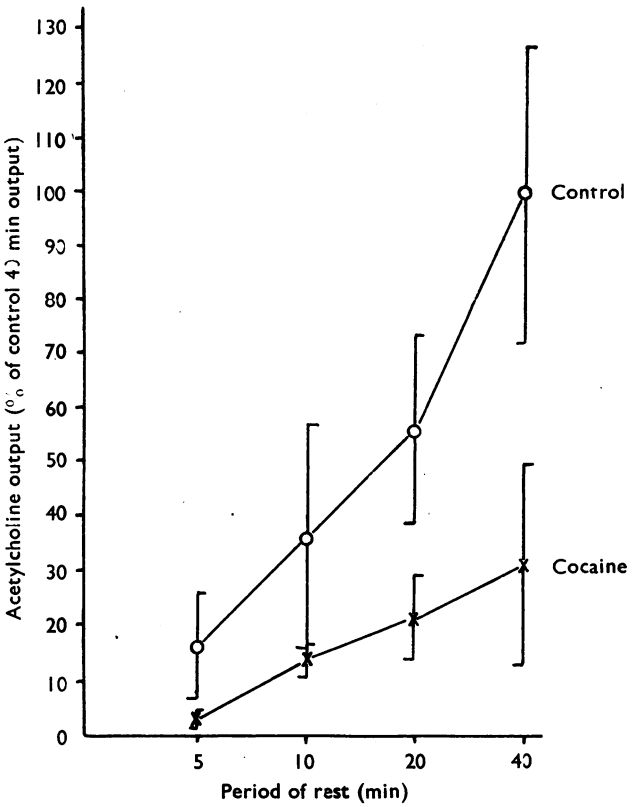

Fig. 2. The effect of cocaine (5 μg/ml.) on the spontaneous release of acetylcholine over periods of 5, 10, 20 and 40 min. The ordinate is the acetylcholine output as a percentage of the control 40 min output. The abscissa is the rest period (min) plotted on a log scale. The control values are represented by the circles and the crosses represent the release in the presence of cocaine. This low concentration of cocaine reduces the release of acetylcholine from the resting ileum. Each point is the mean output with its standard error (vertical line).

Changes in the ionic composition of Krebs solution

Calcium depletion. Reduction of the calcium to one-tenth of the usual concentration had no effect on the responses of the ileum to drugs or on the release of acetylcholine. Reduction to one-hundreth of the usual concentration abolished both the responses to drugs and the release of acetylcholine. One-twentieth of the usual calcium content modified the effects of all three agonists but inhibited the responses to 5-hydroxytryptamine and nicotine to a greater extent than those to histamine. At this reduced calcium level the spontaneous release of acetylcholine was inhibited (Fig. 5).

Substitution of strontium for calcium ions

The ileum was bathed for 15 min in calcium-free Krebs solution which was then replaced by Krebs solution containing strontium chloride with an ionic content of

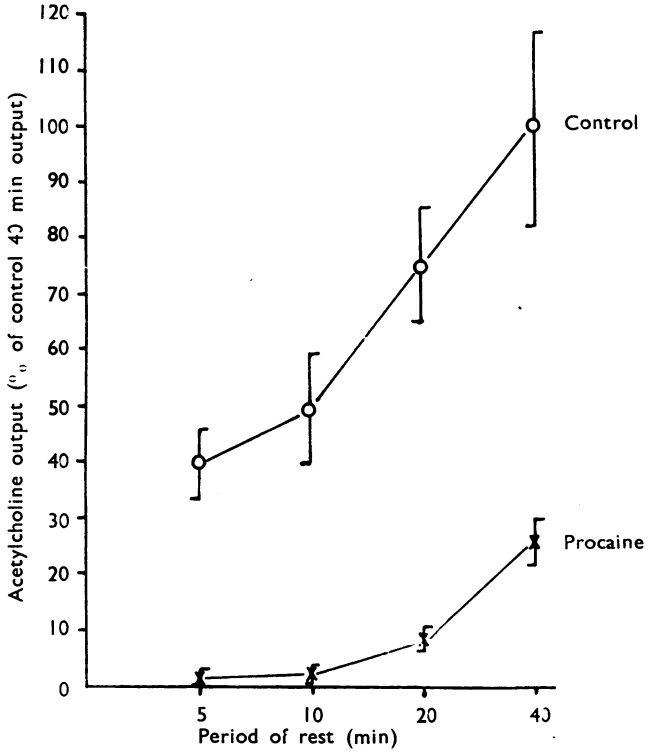

Fig. 3. The effect of procaine (10 μg/ml.) on the spontaneous release of acetylcholine over periods of 5, 10, 20 and 40 min. The ordinate, abscissa and method of plotting are as in Fig. 2. The control values are represented by the circles and the crosses represent the release in the presence of procaine, which inhibits the release of acetylcholine.

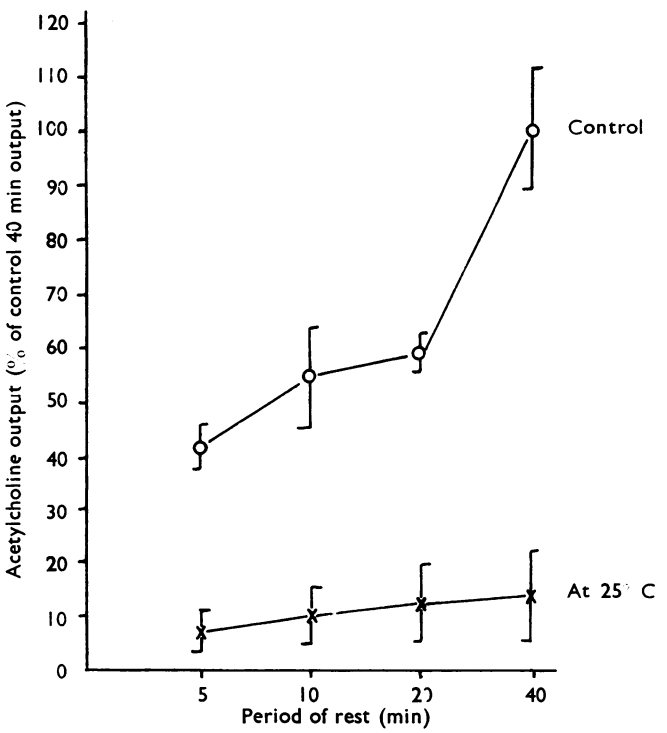

Fig. 4. The effect of cooling the fluid in the organ-bath to 25° C on the spontaneous release of acetylcholine. The ordinate, abscissa and method of plotting are as in Fig. 2. The control values are represented by the circles and the crosses represent the output under the modified conditions. Cooling to 25° C reduces the output of acetylcholine.

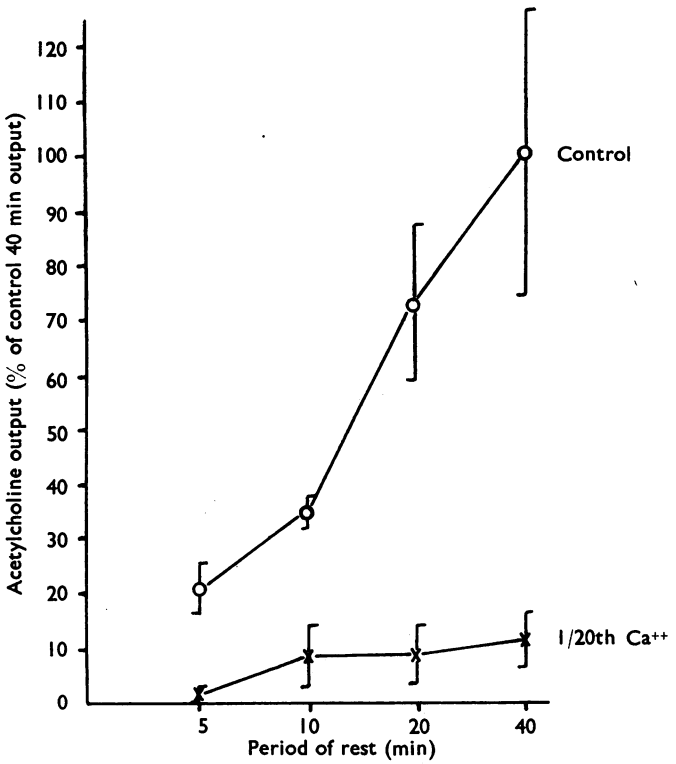

Fig. 5. The effect on the spontaneous output of acetylcholine of reducing the calcium ion content of Krebs solution to one-twentieth of its usual value. The ordinate, abscissa and method of plotting are as in Fig. 2. The control values are represented by the circles and the crosses represent the output in the calcium-depleted solution.

strontium equivalent to the calcium ion content of Krebs solution. After bathing with the strontium-Krebs for 30 min, during which time the bath fluid was exchanged several times, the ileum was found on dosing with histamine, 5-hydroxytryptamine and nicotine to be irresponsive and there was no detectable change in tone even when the preparation was allowed to rest for 40 min, indicating an absence of spontaneously released acetylcholine. But when the samples for the four periods were assayed they were found to contain appreciable amounts of acetylcholine, approximately one-third of the normal output. When the strontium-Krebs was replaced by normal Krebs solution the preparation recovered its former sensitivity to drugs and responded to its resting output of acetylcholine. The responses of the ileum to histamine, 5-hydroxytryptamine and nicotine and the increase in tone caused by the release of acetylcholine before, during and after treatment with strontium-Krebs solution are shown in Fig. 6. The corresponding acetylcholine outputs are plotted against the four periods of time in Fig. 7.

Increased magnesium. A four-fold increase in the magnesium content of Krebs solution did not alter the responses to histamine, 5-hydroxytryptamine or nicotine but greatly reduced the spontaneous output of acetylcholine (Fig. 8).

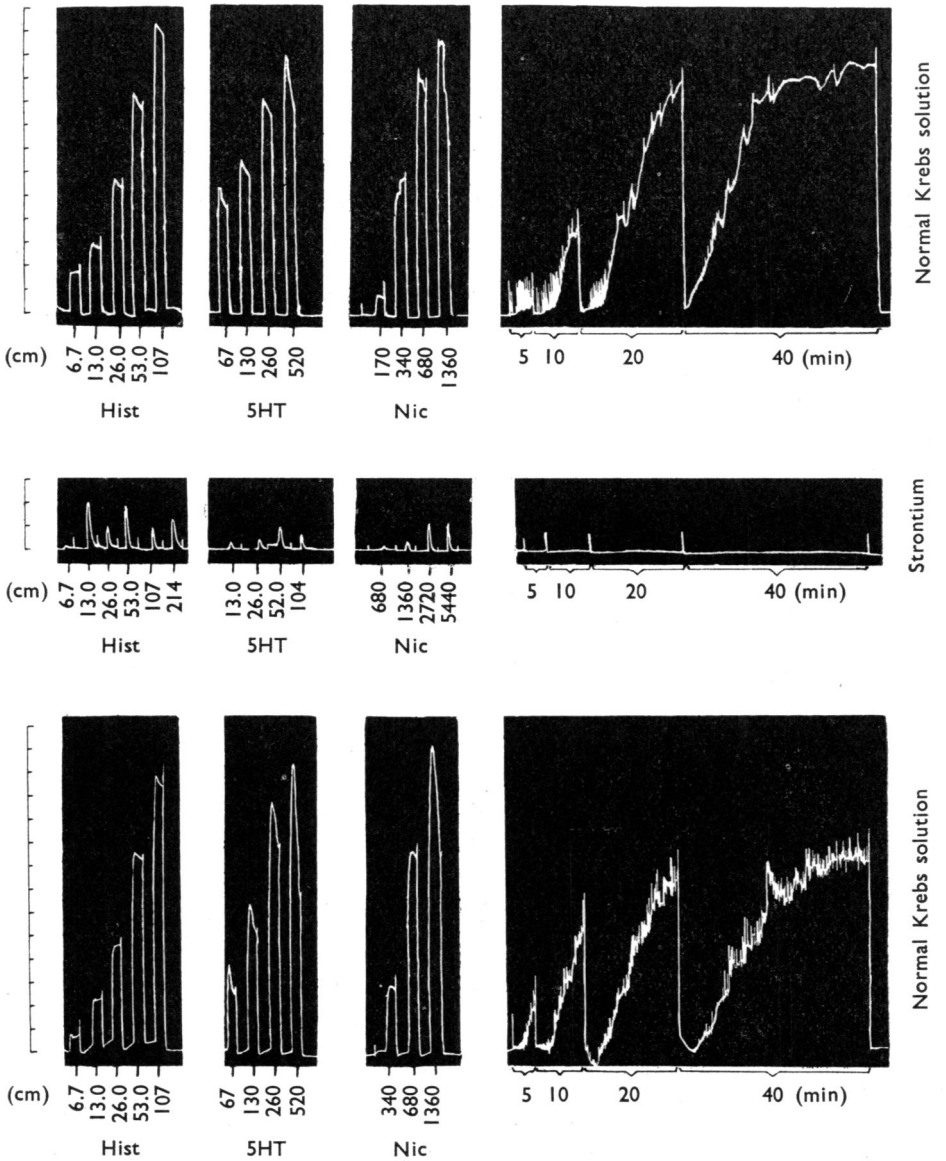

Fig. 6. The uppermost kymograph tracings are records of the responses of the guinea-pig isolated ileum to graded doses of histamine (Hist), 5-hydroxytryptamine (5HT) and nicotine (Nic). The top right-hand tracing shows the gradual increase in tone of the ileum (drum speed reduced) caused by the accumulation of acetylcholine in the bath fluid during periods of 5, 10, 20 and 40 min. The middle tracings show the vestigial responses to drugs and the abolition of the responses to released endogenous acetylcholine after treatment of the ileum for 30 min with Krebs solution in which all the calcium ions had been replaced by strontium. The lowest records demonstrate the reversal of the inhibition of the responses of the ileum, obtained when the Krebs solution containing strontium had been replaced by normal Krebs solution. Each dose of drug was in contact with the ileum for 30 sec and was separated from the next by an interval of 3 min. Doses (three left-hand columns) are in ng/ml., times (right-hand column) are in min. Contraction scales (on left) are in cm on the kymograph.

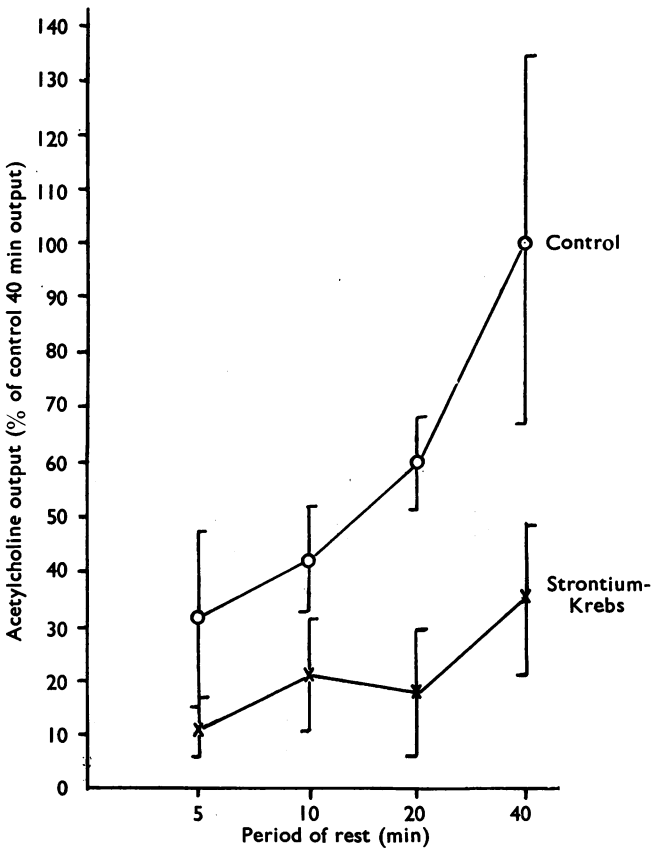

Fig. 7. The effects of substituting strontium ions for calcium in Krebs solution on the spontaneous output of acetylcholine from the guinea-pig ileum. The ordinate, abscissa and method of plotting are as in Fig. 2. The control values are represented by the circles and the crosses represent the output in the calcium-free strontium-Krebs solution.

Inhibition of acetylcholine synthesis

Hemicholinium. When the ileum was suspended in choline-free Krebs for 90 min with hemicholinium (100 μg/ml.), there was little inhibition of the contractions due to 5-hydroxytryptamine and nicotine, and those of histamine were unaffected; but the output of acetylcholine was much inhibited (Fig. 9). In three of the experiments the preparations treated with hemicholinium were treated with choline (400 μg/ml.) for 75 min, after which time the choline was removed from the bath fluid, and in each experiment this treatment resulted in a partial reversal of the block of acetylcholine output by hemicholinium (Fig. 10).

DISCUSSION

Dikshit (1938) compared the ability of minced tissue from different organs of the dog, rabbit and guinea-pig to form acetylcholine. He found that all parts of the gastrointestinal tract could synthesize acetylcholine but the small intestines formed

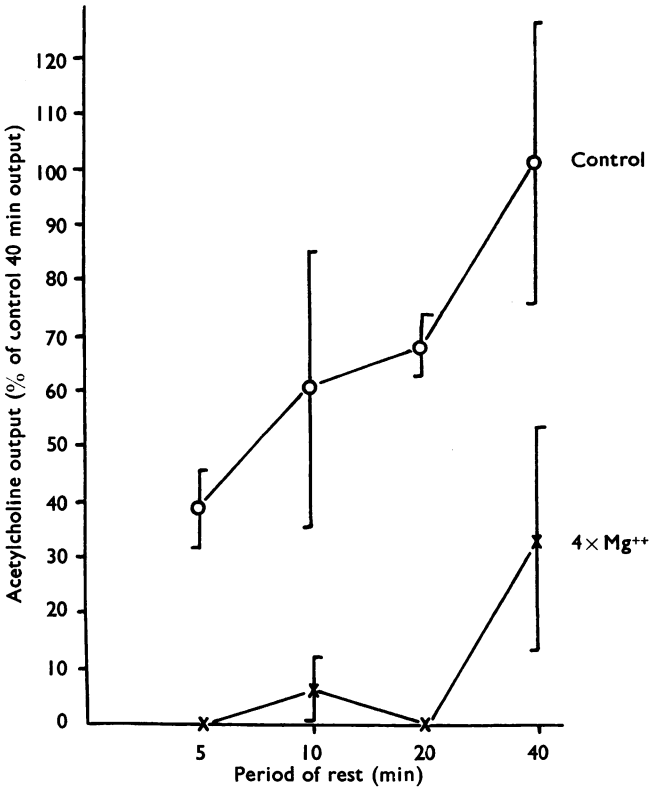

Fig. 8. The effect of a four-fold increase in the magnesium ion content of Krebs solution on the spontaneous output of acetylcholine from the guinea-pig isolated ileum. The ordinate, abscissa and method of plotting are as in Fig. 2. Control values are represented by the circles and the crosses represent the output in Krebs solution containing four-times its usual magnesium ion content. Increasing the magnesium ion content greatly reduces the output of acetylcholine.

more than did other regions; for unit weight of tissue more acetylcholine was formed by the small intestine of guinea-pigs than by those of the other species studied. He considered that the nervous plexuses were responsible for the ester formation. In agreement with this, Welsh & Hyde (1944) showed that the acetylcholine content of the guinea-pig myenteric plexus was higher than that of any other mammalian nervous tissue.

From the experiments described in this paper the spontaneous outputs of acetylcholine from ileum (pg/mg of tissue, means and standard errors) were 31 ± 4, 54 ± 11, 67 ± 9 and 112 ± 16 pg/mg for 5, 10, 20 and 40 min periods respectively. Although the total amount of released acetylcholine increased with time, the rate of release (pg/mg/min) decreased with time, the values being 6.2 ± 0.8, 5.3 ± 1.0, 3.4 ± 0.4 and 2.8 ± 0.4 pg/mg/min for the respective periods.

As the concentration of acetylcholine rises with time there is also seen a gradual increase in tone (Johnson, 1963). Thus the possibility existed that this muscular action itself activated the release of additional amounts of acetylcholine. Yet

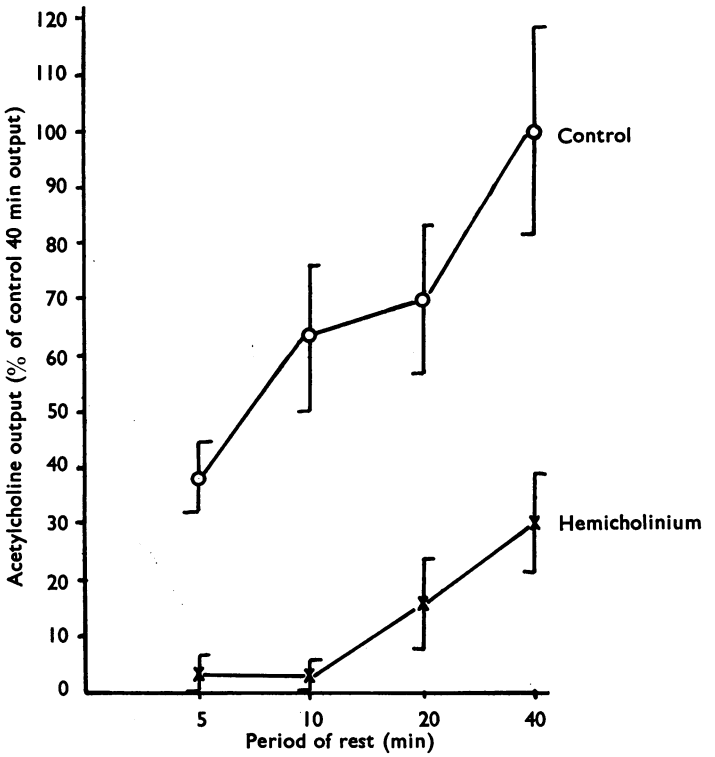

Fig. 9. The effect of treatment with hemicholinium for 1 hr on the acetylcholine output from the ileum. The ordinate, abscissa and method of plotting are as in Fig. 2. The control values are represented by the circles and the crosses represent the output in Krebs solution containing hemicholinium (100 μg/ml.). The acetylcholine output was reduced by treatment with hemicholinium.

no support for this hypothesis is provided by the evidence that the total output for a 40 min period was not greater than the sum of the outputs for eight 5 min periods. Schaumann (1957) also found a greater total output of acetylcholine from repeated fluid changes compared with a continuous incubation period. He postulated an equilibrium between bound and free acetylcholine ; as the acetylcholine concentration rose in the organ-bath further release from the guinea-pig ileum was prevented. A similar phenomenon was seen by Brodkin & Elliott (1953) with acetylcholine from brain slices, and Birks & MacIntosh (1961) made a parallel observation with the acetylcholine output from the cat superior cervical ganglion.

Feldberg & Lin (1949) showed that nicotine, tubocurarine and cocaine did not inhibit the spontaneous release of acetylcholine from the intestinal wall of the rabbit or guinea-pig and they concluded that the release was probably independent of the innervation. Chujyo (1953) also favoured a non-nervous site of acetylcholine production in the guinea-pig ileum. On the other hand, Schaumann (1957) discovered that low concentrations of morphine, which abolished both the peristaltic reflex and the contractions due to nicotine of the guinea-pig intestine, also reduced

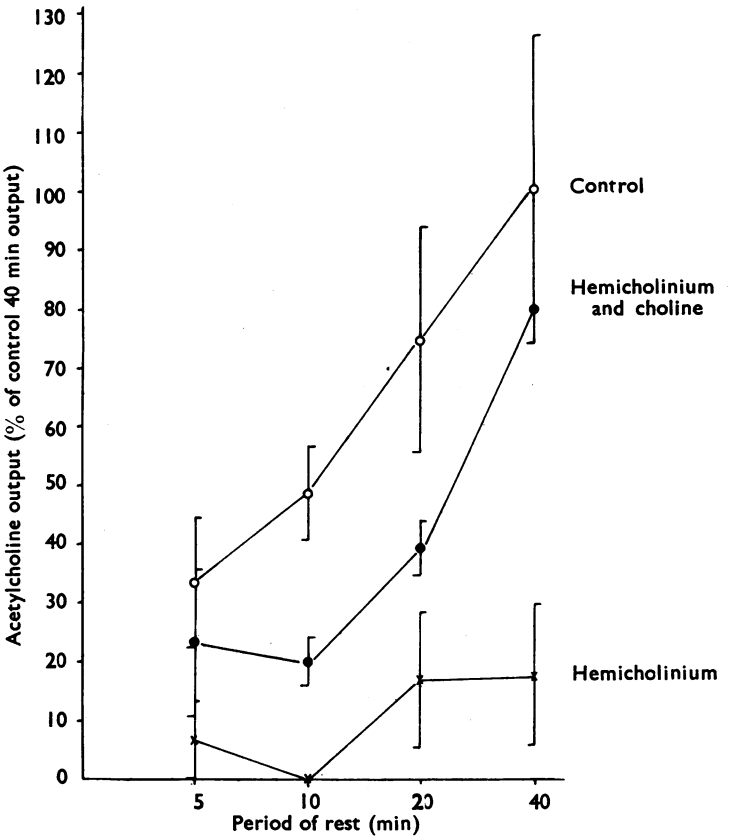

Fig. 10. The reversal by choline of inhibition by hemicholinium of the spontaneous release of acetylcholine from the guinea-pig isolated ileum. The ordinate, abscissa and method of plotting are as in Fig. 2. The empty circles represent the control values, the crosses represent the output after treatment with Krebs solution containing hemicholinium (100 ng/ml.) and the filled circles represent the output from a preparation treated with hemicholinium and then treated for 75 min with choline (400 μg/ml.). Since the choline itself contracted the preparation, it was washed out before the responses to drugs were repeated. Under these conditions a partial reversal of the block by hemicholinium was achieved.

the release of acetylcholine and he discussed additional information favouring a nervous source for acetylcholine. Paton (1957) also showed that morphine reduced the resting output of acetylcholine from the guinea-pig isolated ileum.

In the present experiments a concentration of cocaine as low as 5 μg/ml. inhibited acetylcholine output. Procaine (10 μg/ml.) reduced the release of acetylcholine below the threshold of assay during the 5 and 10 min periods, and for the 40 min period the output was about one-quarter of the control value. Similarly, the experiments made after cooling the ileum to 25° C showed a reduced acetylcholine output and provide good evidence for siting the origin of the acetylcholine in nerves. Cooling was used by Ambache (1946) and by Innes, Kosterlitz & Robinson (1957) to antagonize drugs acting indirectly on the guinea-pig ileum.

Additional evidence from experiments with ions reported above support this view. Depletion of the calcium or increase of the magnesium content of physiological solutions interferes with the release of acetylcholine from motor nerve-endings (Liley, 1956 ; Straughan, 1959). In the present experiments similar results were obtained for smooth muscle. Thus a reduction of the calcium content of Krebs solution to one-tenth of the usual value had no effect on the responses of the ileum to drugs or on the release of acetylcholine, but depletion to one-hundreth of the usual content abolished both output and responses to drugs. One-twentieth of the usual calcium content, whilst antagonizing all the agonists, antagonized the drugs acting indirectly to a greater extent, and at this reduced level of calcium the spontaneous output of acetylcholine was inhibited also. A similar result was obtained when the concentration of magnesium in Krebs was increased. Thus a four-fold increase in magnesium ion content did not affect the responses of the ileum to the agonists, but reduced the spontaneous output of acetylcholine.

These results are consistent with the view that the source of the acetylcholine is the intramural nerve-endings of the smooth muscle. Also, these nerve-endings appear to be more resistant to changes in the ionic environment than are those of skeletal muscle.

The evidence we have now discussed shows that the spontaneous output of acetylcholine is more easily reduced by the presence of local anaesthetics, by cooling, by reduction of calcium ion or increase of magnesium ion concentrations than are the responses of the ileum to drugs under the same conditions. For this reason the results of experiments in which strontium was substituted for the calcium of Krebs solution are surprising in that the spontaneous acetylcholine output was only partially inhibited when the smooth muscle no longer responded to drugs. Since the results described in this paper indicate that the acetylcholine released from a resting preparation originates in the nerve-endings in the wall of the intestine, the experiments with strontium may mean that nervous tissue is capable of utilizing strontium instead of calcium whereas the intestinal muscle is not. The results cannot be explained by the presence of calcium stores in the nerves concerned since the output of acetylcholine in the presence of calcium-free strontium Krebs solution was approximately three-times that of the ileum in Krebs solution containing one-twentieth of the usual calcium concentration, and there was no measurable output of acetylcholine when the calcium content of Krebs solution was reduced further. Frank (1962) showed that strontium could partially replace calcium ions in supporting a potassium- or caffeine-induced contracture of the toe muscle of the frog but, if the store of calcium ions in the muscle were first depleted by prolonged exposure to a calcium-free solution, strontium would not restore either contracture.

Since the output of acetylcholine in unit time was less for a 40 min period than for the sum of eight 5 min periods, it appears that the rate of synthesis is the limiting factor in the amount of acetylcholine released. Hemicholinium has proved useful in investigating the course of synthesis of acetylcholine. Schueler (1955) first suggested that hemicholinium-3 might interfere with cholinergic mechanisms, and later this compound was shown to inhibit acetylcholine synthesis in the cat superior cervical ganglion (MacIntosh, Birks & Sastry, 1956). A currently accepted hypo-

thesis postulates that hemicholinium prevents the transport of choline from the extracellular fluid to the point of acetylation (Gardiner, 1957; MacIntosh, 1961). In the present experiments a high concentration of hemicholinium inhibited the spontaneous release of acetylcholine from the guinea-pig ileum but this concentration had little effect on the contractions in response to 5-hydroxytryptamine or nicotine and had no effect on those to histamine. In the three preparations treated with hemicholinium and incubated with choline, the inhibition of the acetylcholine output was reversed.

It is concluded that cocaine, procaine, cooling, reduction of calcium ion and increase of magnesium ion concentrations all reduce the spontaneous output of acetylcholine, which compound originates in the parasympathetic nerve-endings of the intramural nerve plexuses. Further, the hemicholinium experiments seem to justify the conclusion that the release of acetylcholine is reduced because its synthesis is reduced.

I am indebted to Professor G. Brownlee for his helpful advice and criticism, and for his assistance in the preparation of this paper for publication. I am grateful to Professor F. W. Schueler (Tulane University, New Orleans, U.S.A.) for a gift of hemicholinium. This work was supported by a scholarship from the Medical Research Council.

REFERENCES

AMBACHE, N. (1946). Interaction of drugs and the effect of cooling on the isolated mammalian intestine. *J. Physiol. (Lond.)*, **104**, 266–287.

BIRKS, R. I. & MACINTOSH, F. C. (1961). Acetylcholine metabolism of a sympathetic ganglion. *Canad. J. Biochem.*, **39**, 787–827.

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

BLIGH, J. (1952). The level of free choline in plasma. *J. Physiol. (Lond.)*, **117**, 234–240.

BRODKIN, E. & ELLIOTT, K. A. C. (1953). Binding of acetylcholine. *Amer. J. Physiol.*, **173**, 437–442

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.

CARLYLE, R. F. (1963). The mode of action of neostigmine and physostigmine on the guinea-pig trachealis muscle. *Brit. J. Pharmacol.*, **21**, 137–149.

CHUJYO, N. (1953). Site of acetylcholine production in the wall of the intestine. *Amer. J. Physiol.*, **174**, 196–198.

DIKSHIT, B. B. (1938). Acetylcholine formation by tissues. *Quart. J. exp. Physiol.*, **28**, 243–251.

FELDBERG, W. & LIN, R. C. Y. (1949). The effect of cocaine on the acetylcholine output of the intestinal wall. *J. Physiol. (Lond.)*, **109**, 475–487.

FELDBERG, W. & LIN, R. C. Y. (1950). Synthesis of acetylcholine in the wall of the digestive tract. *J. Physiol. (Lond.)*, **111**, 96–118.

FRANK, G. B. (1962). Utilization of bound calcium in the action of caffeine and certain multivalent cations of skeletal muscle. *J. Physiol. (Lond.)*, **163**, 254–268.

GARDINER, J. E. (1957). Experiments on the site of action of a choline acetylase inhibitor. *J. Physiol. (Lond.)*, **138**, 13*P*.

INNES, I. R., KOSTERLITZ, H. W. & ROBINSON, J. A. (1957). The effects of lowering the bath temperature on the responses of the isolated guinea-pig ileum. *J. Physiol. (Lond.)*, **137**, 396–409.

JOHNSON, E. S. (1963). 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.

LILEY, A. W. (1956). The quantal components of the mammalian endplate potential. *J. Physiol. (Lond.)*, **133**, 571–587.

MACINTOSH, F. C. (1961). Effect of HC-3 on acetylcholine turnover. *Fed. Proc.*, **20**, 562–568.

MACINTOSH, F. C., BIRKS, R. I. & SASTRY, P. B. (1956). Pharmacological inhibition of acetylcholine synthesis. *Nature (Lond.)*, **178**, 1181.

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.

SCHAUMANN, W. (1957). Inhibition by morphine of the release of acetylcholine from the intestine of the guinea-pig. *Brit. J. Pharmacol.*, **12**, 115–118.

SCHUELER, F. W. (1955). A new group of respiratory paralysants. 1. The " hemicholiniums.' *J. Pharmacol. exp. Ther.*, **115**, 127–143.

STRAUGHAN, D. W. (1959). *The Effect of Changes in Temperature, Ionic Environment and of Drugs on the Release of Acetylcholine from Skeletal Nerve-Muscle Preparations.* Thesis for the degree of Ph.D. at the University of London.

WELSH, J. H. & HYDE, J. E. (1944). Acetylcholine content of the myenteric plexus and resistance to anoxia. *Proc. Soc. exp. Biol. (N.Y.)*, **55**, 256–257.