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THE MECHANISM OF ACETYLCHOLINE RELEASE FROM PARASYMPATHETIC NERVES

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SUMMARY

1. The output of acetylcholine from the plexus of the guinea-pig ileum longitudinal strip has been used to study the mechanism of acetylcholine release. From the effects of hexamethonium and tetrodotoxin, it was inferred that 60 % of the normal resting output is due to propagated activity in the plexus, and 40 % to spontaneous release. Tetrodotoxin virtually abolishes the increase in output in response to electrical stimulation.

2. Resting acetylcholine output is increased when the bathing medium is changed in the following ways:

(*a*) sodium replacement by sucrose, trometamol or lithium;

(*b*) addition of ouabain or *p*-hydroxymercuribenzoate (PHMB), or withdrawal of potassium;

(*c*) the combination of PHMB and partial sodium replacement;

(*d*) addition of potassium; this increase in output becomes greater in the absence of sodium.

3. The resting output is virtually abolished by calcium withdrawal, and is restored by barium substitution for calcium. It is also reduced by raising the magnesium concentration.

4. The enhanced resting output in response to sodium withdrawal also occurs in the absence of calcium.

5. Cooling to 5° C greatly reduces both the resting output and the output in response to raised potassium concentration or to electrical stimulation.

6. The increase in resting output due to potassium excess is slight up to 25 mM $[K^+]_o$, but increases thereafter with about the fourth power of the potassium concentration; it is resistant to tetrodotoxin.

7. Synthesis of acetylcholine by the longitudinal strip is increased when output is enhanced by electrical stimulation, by potassium excess or by

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addition of barium, so that the acetylcholine content of the strip is maintained approximately normal. Synthesis is reduced, in relation to output, by potassium lack or by treatment with ouabain, and is virtually abolished by sodium withdrawal.

8. The theory is discussed that acetylcholine release depends on inhibition of the activity of a $(Na^+ + K^+ + Mg^{2+})$-activated ATPase at the axonal membrane.

INTRODUCTION

The principal studies of the mechanism of acetylcholine release have been made on the neuromuscular junction and on the autonomic ganglion. To the classical evidence that established the theory of chemical transmission has been added new evidence made possible by the discovery of quantal release of acetylcholine at the motor end-plate and the development of new electrophysiological methods (*v.* Katz, 1962). But there is less evidence for parasympathetic structures. Acetylcholine release in the salivary gland was studied by Emmelin & Muren (1950); and there is a considerable amount of work on acetylcholine output from the alimentary tract, but this has been complicated by uncertainty as to the origin of the acetylcholine.

The finding that, in the longitudinal strip of guinea-pig ileum, acetylcholine content and release are restricted to Auerbach's plexus, and that the smooth muscle neither contained nor released acetylcholine (Paton & Zar, 1968) made possible an examination of the factors controlling acetylcholine release by this parasympathetic plexus. It has already been used for a study of the action of catecholamines (Paton & Vizi, 1969). The main advantage of the preparation is that it is suitable, from the thinness of the strip, for *in vitro* study, and that relatively large amounts of acetylcholine are released which can be directly assayed.

METHODS

The methods described by Paton & Zar (1968) and Paton & Vizi (1969) were used for the preparation and mounting of longitudinal muscle strips of guinea-pig ileum, and for assay of acetylcholine output and tissue content. The weight of the strips varied from 18 to 80 mg, depending on the animal and on the method of preparation. The composition of the normal Krebs solution and its modification were as follows. (*a*) Normal Krebs (mM), NaCl 113; KCl 4·7; $CaCl_2$ 2·5; KH_2PO_4 1·2; $MgSO_4$ 1·2; $NaHCO_3$ 25; and glucose 11·5. (*b*) Sodium-substituted solutions contained equimolar amounts of sucrose, lithium chloride, or trometamol (Tris) chloride; the $NaHCO_3$ was replaced with $KHCO_3$, KCl and KH_2PO_4 being omitted. (*c*) Potassium-free solutions were prepared by omitting KCl and KH_2PO_4, either without adjusting osmolarity (as in the experiment of Fig. 6) or by appropriate NaCl reduction as in the experiments of Figs. 11 and 12. (*d*) Potassium-rich solutions were made by adding KCl, up to 49 mM. (*e*) Calcium-free solutions were made by omitting $CaCl_2$. (*f*) For

barium-enriched solutions, $BaCl_2$ was added to calcium-free Krebs, to a final concentration of 1·25 mM, and magnesium chloride replaced an equimolar amount of magnesium sulphate. Before assay of such solutions for acetylcholine content, barium was precipitated by adding Na_2SO_4 to a concentration of 15 mM of Na_2SO_4 in the assay fluid; after shaking, the fluid was filtered and assayed directly. In all assays, the standard acetylcholine solutions were made up in appropriately modified Krebs solution.

In experiments at low temperature, the organ bath was arranged in a trough of ice; the temperature was reduced to 3–5° C.

Drugs. The drugs used were acetylcholine chloride or bromide (values given in the paper are cited as acetylcholine salt, mol. wt. 181·7), physostigmine sulphate (Burroughs Wellcome & Co.), (±)-adrenaline bitartrate (Burroughs Wellcome & Co.), (−)-noradrenaline bitartrate (Koch Light Laboratories Ltd.), hexamethonium bromide, morphine sulphate, cocaine hydrochloride (May & Baker Ltd.), procaine hydrochloride, sodium *p*-hydroxymercuribenzoate (Sigma Chemical Co.), tetrodotoxin (Sankyo), ouabain (Strophanthidin, B.D.H.), and trometamol (Tris; tris-(hydroxymethyl)-aminomethane, Sigma Chemical Co.) which was converted to the chloride by titration with hydrochloric acid at pH 7·3.

RESULTS

Analysis of the resting release of acetylcholine

For the analysis of release mechanisms, there is a choice between the use of resting or of evoked output. With the longitudinal strip, the resting output, while varying from one preparation to another, is reasonably constant for a given preparation for long periods (see, for example, Fig. 9). The mean value for eighty-four experiments was $51 \pm 1{\cdot}5$ ng/g.min. The evoked output, however, usually changes as stimulation is prolonged, in a manner varying with the stimulation used. Further, if changes in the ionic environment are made, difficulty arises with field stimulation in securing the same conditions of nervous excitation as are used in control periods. Accordingly, even though resting output is lower in magnitude than that obtainable by excitation, it has been used for this analysis.

The first question that arises is how far what is termed resting output originates from propagated activity in cholinergic nerves, and how far it corresponds to what will be termed 'spontaneous' release from nerve fibres free of action potentials. It is useful in considering this problem to distinguish three possible types of cholinergic fibre; 'extrinsic' fibres, i.e. those with cells of origin outside the strip; 'interneuronal' fibres, i.e. those with cell bodies located within the plexus and synapsing with other ganglion cells; and 'pre-effector' fibres, i.e. those with cell bodies located in the plexus, whose nerve endings come into relation not with nerve cells but smooth muscle. The terms pre- and post-ganglionic are confusing in this context since both terms could be applicable to an interneuronal axon. Interneuronal cholinergic cell bodies could be uninnervated (activated

perhaps by axon reflex), or innervated by non-cholinergic fibres (e.g. of local sensory origin), or innervated by cholinergic fibres (possibly as part of a re-entrant cholinergic network). With each of the types of cholinergic fibre, acetylcholine release could occur either spontaneously or due to action potentials, so that six possible fractions of acetylcholine output can be distinguished (Fig. 1).

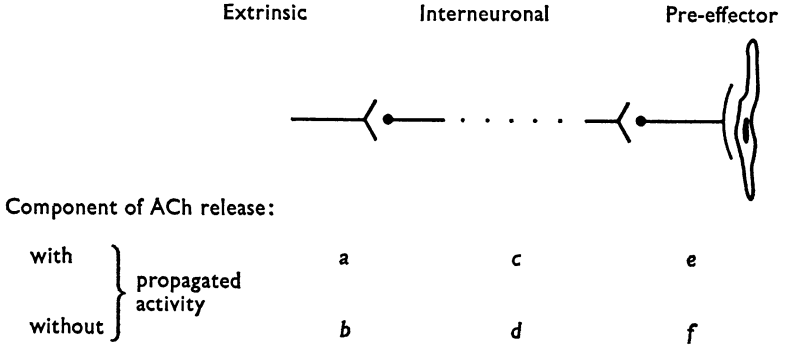

Fig. 1. Diagram of possible sources of acetylcholine (ACh) in the nerve plexus of the longitudinal muscle strip. See text.

TABLE 1. Inhibition by drugs of resting acetylcholine release from longitudinal strip: % ± S.E. Number of experiments in brackets. Exposure time, 20 min

	g/ml.	
Hexamethonium	5×10^{-5}	61·9 ± 4·8 (4)
Hexamethonium	10^{-4}	53·0 ± 6·6 (5)
Tetrodotoxin	5×10^{-7}	54·8 ± 3·2 (6)
Hexamethonium Tetrodotoxin	10^{-4} 5×10^{-7}	61·7 ± 2·4 (3)
Adrenaline*	10^{-6}	71·2 ± 5·7 (6)
Procaine	2×10^{-5}	83·3 (2)
Morphine	10^{-6}	81·5 ± 2·7 (4)
Hexamethonium Adrenaline	10^{-4} 5×10^{-7}	80·2 ± 10·1 (5)
Hexamethonium Morphine	10^{-4} 10^{-6}	81·7 ± 6·2 (8)

* Taken from Paton & Vizi (1969).

Table 1 shows the action of a number of drugs on resting output. First, hexamethonium in a concentration of 5×10^{-5} or 10^{-4} g/ml. reduced the resting output by about 57 %. The concentration used is 5–10 times greater than that required to abolish both the peristaltic reflex (Paton & Zaimis, 1949) and the spontaneous activity of the guinea-pig ileum (Feldberg, 1951), and must be supposed to abolish cholinergic ganglionic transmission

in the strip. On this basis, the residual output after hexamethonium, about 40% of normal, must at most represent (using the symbols of Fig. 1) fractions *a*, *b*, *d* and *f*.

Secondly, tetrodotoxin, 5×10^{-7} g/ml. (1·6 μM), reduced resting output in six experiments by 55%. Fig. 2 shows further experiments on the dose–response curve of the inhibition; it was readily detectable at 0·15 μM and is virtually maximal at concentrations of 1 μM or above. From what is now known of the properties of tetrodotoxin (Narahashi, Moore & Scott, 1964;

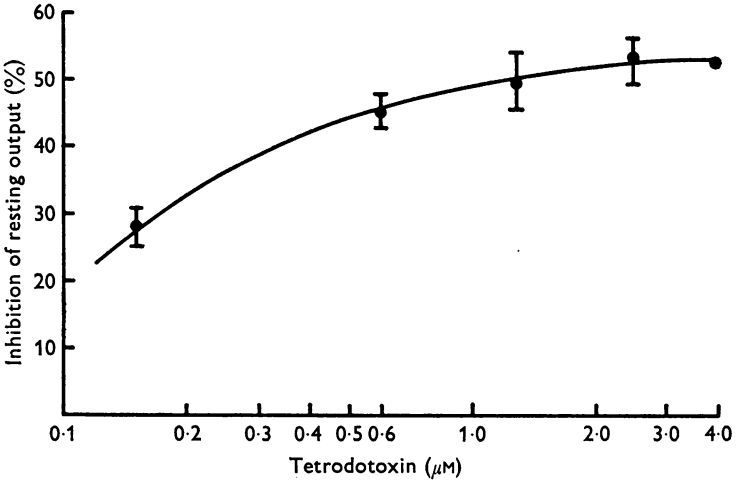

Fig. 2. Inhibition of resting acetylcholine output from the longitudinal muscle strip of guinea-pig ileum by tetrodotoxin. Abscissa: concentration of tetrodotoxin (μM). Ordinate: % inhibition of the resting acetylcholine output. Mean of six experiments; vertical bars indicate S.E. The control resting output was $44{\cdot}7 \pm 3{\cdot}2$ mg/g.min.

Katz & Miledi, 1967) it must be assumed that all propagated activity in nerve fibres is abolished, but that spontaneous quantal release of acetylcholine at nerve terminations is retained. Fig. 3 shows that tetrodotoxin abolishes the response to stimulation, at rates both of 0·3 Hz and 10 Hz, as well as reducing resting output. If it is supposed that quantal activity such as occurs at the end-plate is responsible for all spontaneous release unaccompanied by action potentials (and therefore is all tetrodotoxin-resistant), then the residual output under resting conditions after tetrodotoxin, about 45% of normal, must represent the fractions *b*, *d* and *f*. The quantitative similarity of the effect of hexamethonium to that of tetrodotoxin is interesting, and indicates that acetylcholine release of extrinsic origin (i.e. fraction *a*) is negligible. This is further supported by three experiments in which the output in the presence both of hexamethonium (10^{-4} g/ml.) and of tetrodotoxin (5×10^{-7} g/ml.) was determined; output

was reduced by 61·7%, a value not differing significantly from that obtained with either drug alone. The insignificance of an extrinsic component is in any case probable, in the light of what is known of the distribution of the vagus to the alimentary tract in the cat (Agostini, Chinnock, de Burgh Daly & Murray, 1957). The results also suggest that propagated activity

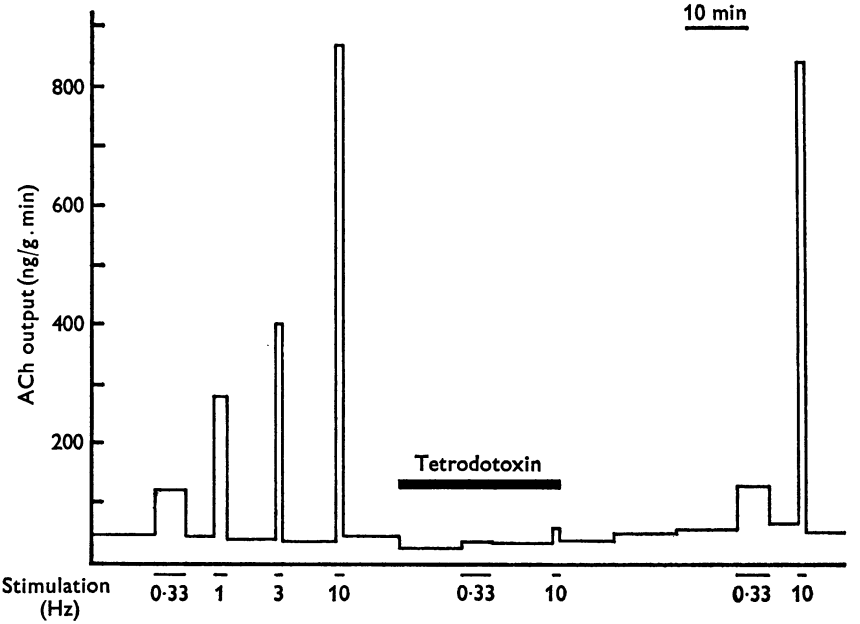

Fig. 3. Inhibition by tetrodotoxin of acetylcholine output due to electrical stimulation. Longitudinal muscle strip of guinea-pig ileum in eserinized Krebs solution. Tetrodotoxin (5×10^{-7} g/ml.) present as indicated. The values of acetylcholine output (ng/g.min) are means of two experiments. The preparation was stimulated for periods of 5 min at 0·33 Hz, 2 min at 1 Hz and 1 min at 10 Hz. Note the almost complete but reversible inhibition of acetylcholine output by tetrodotoxin.

resulting in acetylcholine release in the resting strip arises only from activity by cholinergic mechanisms; if this were not the case, then cholinergic neurones activated by some other mechanism would remain active in the presence of hexamethonium, but not of tetrodotoxin, and the latter would have a greater effect in reducing acetylcholine output. We have assumed, therefore, that 40% of the resting output is due to spontaneous activity and the remainder arises from propagated activity evoked by local cholinergic mechanisms.

The action of catecholamines, cocaine, procaine and morphine

Certain drugs are able to depress resting output still further. In a previous paper (Paton & Vizi, 1969) it was reported that adrenaline, 10^{-6} g/ml.,

reduced resting output by $71{\cdot}2 \pm 5{\cdot}7$ %. Procaine and morphine produce similar inhibitions: procaine 2×10^{-5} g/ml., 83·3 % (two experiments); morphine 10^{-6} g/ml., $81{\cdot}5 \pm 2{\cdot}7$ (four experiments). In higher concentrations, morphine does not exert any greater effect. Similar inhibitions have been found with whole intestine; thus Johnson (1963) has found that procaine and cocaine reduced the output from whole ileum by about 60–85 %, and we have confirmed that cocaine ($1{\cdot}5 \times 10^{-4}$ g/ml.) reduces output from whole ileum by $66{\cdot}7 \pm 4{\cdot}6$ %. But these data on whole gut cannot be considered quantitatively without corresponding studies of the effects on whole gut of tetrodotoxin and hexamethonium.

In the light of the previous section, the ability of these drugs to depress output from the longitudinal strip by 70–80 % implies that they must act both on release by propagated activity and on spontaneous release. They might, therefore, be annulling spontaneous release and only partially reducing propagated activity. This possibility can be excluded by the fact that if the strip is exposed simultaneously to adrenaline or morphine and to hexamethonium, the reduction in output is not significantly greater (Table 1). With adrenaline and morphine, therefore, and probably also with procaine, release due to propagated activity must be abolished, together with part of the spontaneous release. The possible mechanism involved for catecholamine action has been discussed by Paton & Vizi (1969).

The mechanism of release

Effect of sodium replacement. Evidence has been put forward (Birks, 1963) that the release of acetycholine in autonomic ganglia and at the neuromuscular junction depends on a rise of internal sodium concentration in the nerve ending, and this has led to a later suggestion (Birks & Cohen, 1968) that it acts not directly, but by increasing calcium influx. We have tested such a role of sodium on output from the longitudinal strip by exposing the strip to sodium-deficient media. Fig. 4 shows the change in output which follows treatment with media in which the sodium was wholly replaced with trometamol (Tris), with sucrose, or with lithium. In each case, a considerable rise in output developed, most rapid and largest with lithium substitution. This was an unexpected result, both in the light of Birks's findings, and because it would be expected that a fall in output would in any case result from an abolition of propagated nervous activity following sodium withdrawal. This latter effect can in fact be seen with reduced sodium concentrations. Fig. 5 shows the output with media containing no sodium, 4 % normal sodium, and 20 % normal sodium, with sucrose substitution. With 20 % sodium, a pure depression of output to about 20–30 % of normal occurs; with 4 % an initial depression passes into an increased output; with zero sodium, the enhanced output already noted

was observed. But if lithium was used in place of sodium with the 20% medium, only an increase in output was observed. Under these last conditions, it must be assumed that propagated activity will be retained, since lithium is known to substitute in this respect fully for sodium. It follows that under conditions where propagated activity can persist, and sodium cannot enter the nerve fibre, acetylcholine output is enhanced. Any simple

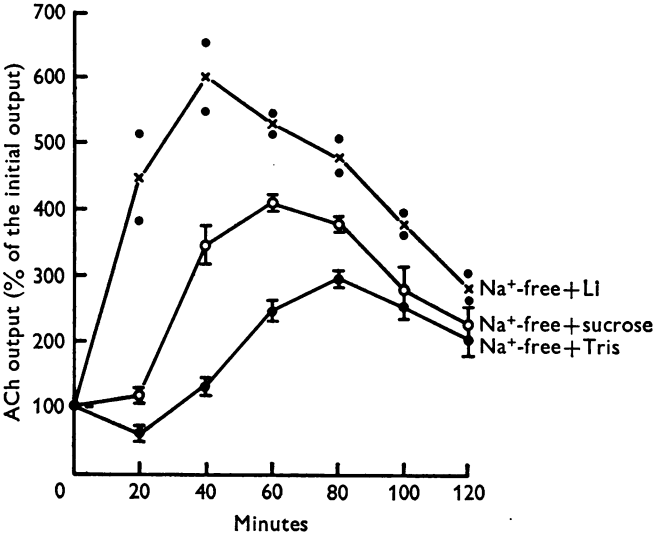

Fig. 4. Increase of resting acetylcholine output by total sodium deprivation. Eserinized longitudinal muscle strip of the guinea-pig ileum in modified Krebs solution. Sodium was replaced as indicated by lithium or sucrose or Tris (trometamol). For details see Methods. The initial rate of acetylcholine output in the presence of normal amount of sodium in Krebs solution has been taken to be 100 and the subsequent rates of acetylcholine release, on treatment with sodium-free solutions, have been expressed as % of the initial rate. Each point represents the resting acetylcholine output during the preceding 20 min. In two lower curves, bars give S.E. of mean of three experiments; upper curve drawn through means of two experiments, the individual values being shown.

correlation between output and rise in internal sodium concentration cannot therefore be accepted; and in fact the experiments quoted would support a view that a fall in axonal sodium concentration favoured release.

Another observation should be mentioned here. Fig. 6 showed the effect of sodium deprivation on the increase in output produced by raised external potassium concentration. This treatment enhances the acetylcholine release by up to twofold.

Effect of interfering with sodium extrusion. The converse experiments, with procedures calculated to raise intra-axonal sodium, by interfering with the sodium pump, were made with ouabain, reduced external potas-

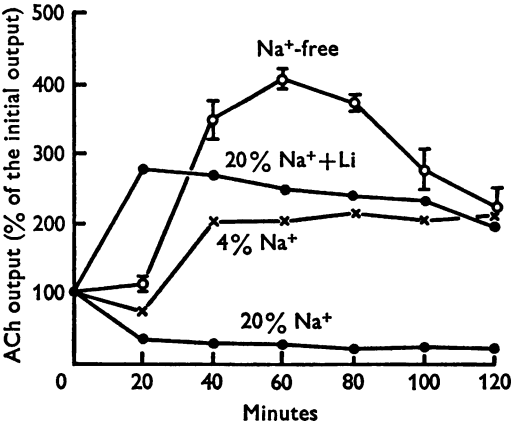

Fig. 5. Resting acetylcholine output from eserinized longitudinal muscle strip exposed to various concentrations of sodium in modified Krebs solution. Sucrose substitution except when lithium was used. Initial rate of acetylcholine output in presence of normal Krebs solution taken as 100%. ○—○ sodium-free solution, three experiments; bars give S.E. ×—× 4% normal sodium, one experiment. ●—● 20% normal sodium, one experiment, and 20% normal sodium, lithium substitution, one experiment.

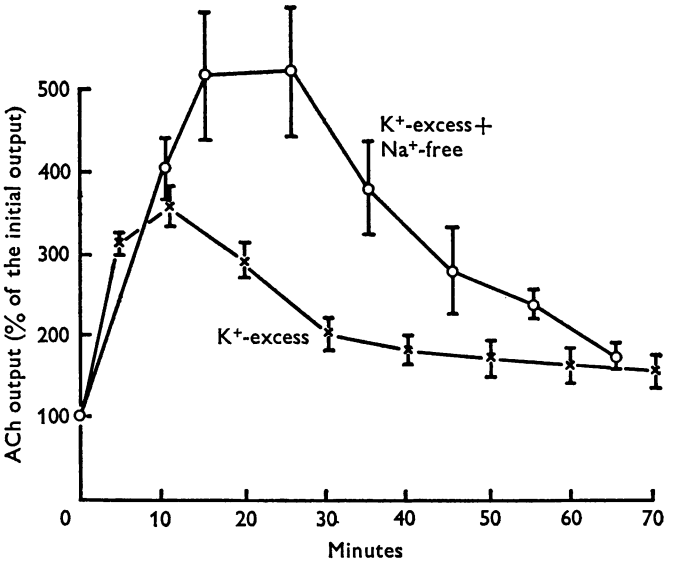

Fig. 6. Effect of complete sodium deprivation on acetylcholine output from eserinized longitudinal muscle strip of guinea-pig ileum exposed to excess potassium (40 mM). Tris was used as the substitute for the sodium. Control resting output, $103 \pm 7{\cdot}1$ ng/g.min, taken as 100%. Bars give S.E. of mean of three experiments. Each point represents the acetylcholine output during the preceding period.

sium and *p*-hydroxymercuribenzoate (PHMB). Fig. 7*a* shows the effect on acetylcholine output of treatment with ouabain, 10^{-6} ($1{\cdot}4 \times 10^{-6}$ M) and 10^{-5} ($1{\cdot}4 \times 10^{-5}$ M) g/ml. With 10^{-6} ouabain, the maximum action was reached in the first 20 min, and increased acetylcholine output by $60{\cdot}6 \pm 10{\cdot}5$ % of the initial value: this was significantly different from the control,

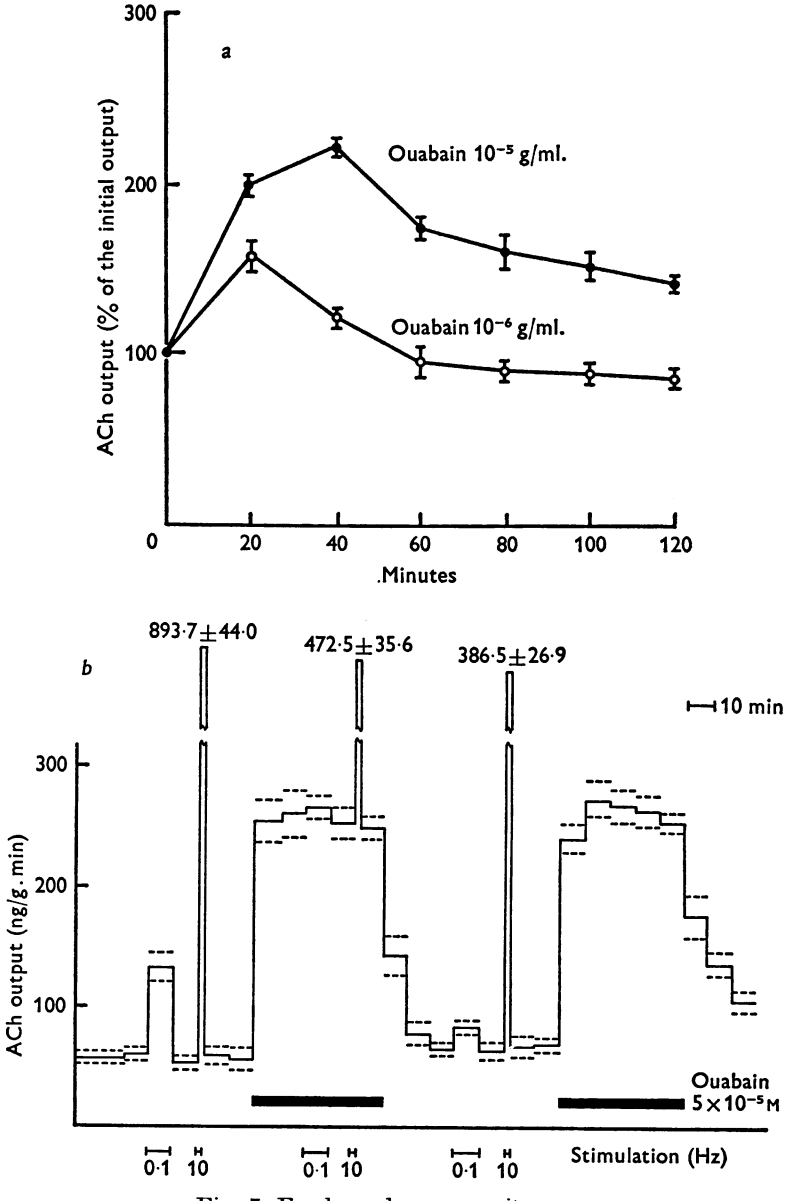

Fig. 7. For legend see opposite page.

but the increase in output in the following period, by 23 % ± 6·7, was not statistically significant.

With potassium-free solution (Fig. 8) the maximum effect was reached in the period from 90 to 120 min after removal of potassium, output being

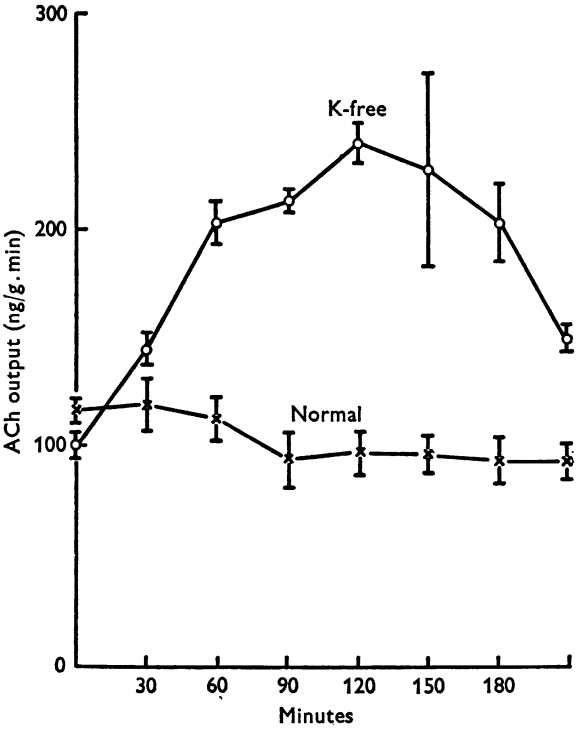

Fig. 8. Increase of acetylcholine output as a result of K_o^+ deprivation. Longitudinal muscle strip of guinea-pig ileum. The rate of acetylcholine output is plotted against time (min). Each point represents the acetylcholine output during the preceding 30 min. Potassium-free Krebs solution was prepared by omitting KCl and KH_2PO_4.

Legend to Fig. 7.

Fig. 7. Augmentation by ouabain of resting acetylcholine output from longitudinal muscle strip of guinea-pig ileum. (*a*) Time course of ouabain action. Abscissa: time in min. Ordinate: change of acetylcholine output in percent; the initial rate of acetylcholine output has been taken to be 100. Each point represents the mean value of acetylcholine output in three experiments during the preceding 20 min. Bars indicate the S.E. (*b*) The effect of ouabain (5×10^{-5} M) on the acetylcholine output under conditions of rest and of stimulation. The values of acetylcholine output (ng/g.min) are the means of four experiments with identical schedules. Dashed lines indicate the S.E. errors. Stimulation periods were 10 min at 0·1 Hz, 1 min at 10 Hz. Note the increase of acetylcholine output during rest and the decrease of stimulation output as a result of ouabain.

increased from an initial value of 103·3 ± 6·3 ng/g.min to 243·3 ± 10·0 ng/g.min. The Figure also shows the resting output under normal conditions; the output over a period of 4 hr ranged from 96·5 to 116·5 ng/g.min, a variation of less than ± 7 % from the mean value over the period.

PHMB in a concentration of 10^{-5} M caused a long-lasting and significant

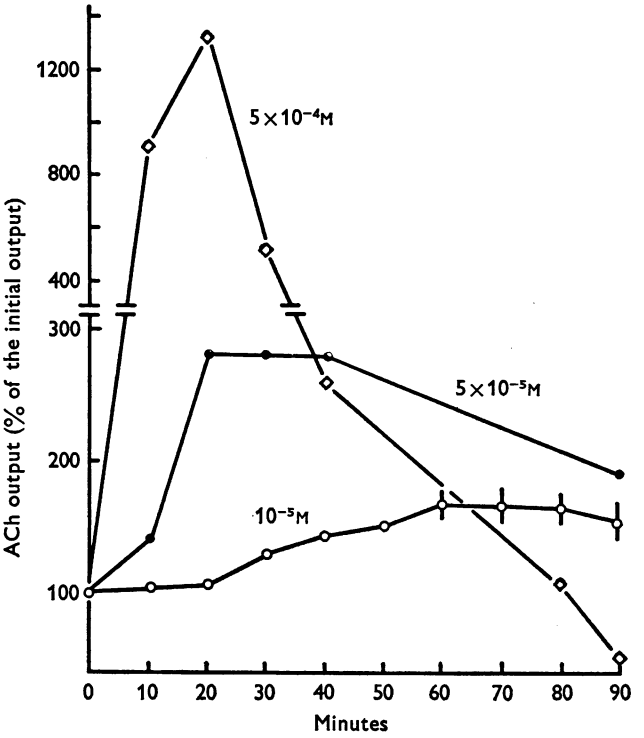

Fig. 9. Increase of acetylcholine output from the longitudinal muscle strip of guinea-pig ileum by sodium salt of *p*-hydroxymercuribenzoate (PHMB). The initial rate of acetylcholine output has been taken to be 100 and the subsequent rates of output, on treatment with PHMB, have been expressed as % of the initial rate. The following concentrations were used: ○ 10^{-5} M (three experiments, vertical bars indicate the S.E.), ● 5×10^{-5} M (one experiment). ◇ 5×10^{-4} (one experiment). Note that with 10^{-5} PHMB for the period from 20–30 min onwards the values differ significantly from the control ($P < 0{\cdot}01$). Each point represents the mean acetylcholine output during the preceding period.

increase in output, the increase reaching a maximum of 66·6 ± 9·1 % in the 50–60 min period. The increase was statistically significant from the 20 to 30 min period onwards (Fig. 9). A larger increase, by 180 % between 10 and 40 min, was produced in a single further experiment with 5×10^{-5} M-PHMB. In a final single experiment a concentration of 5×10^{-4} M caused an

increase in output of 810 % in the first 10 min, and a peak effect of 1260 %: this very greatly increased output could not be maintained, and at the end of 90 min output had fallen below normal.

A further set of experiments was made with a higher concentration of ouabain ($3{\cdot}7 \times 10^{-5}$ g/ml., 5×10^{-5} M) using identical dose and stimulation schedules for each experiment (Fig. 7*b*), in which its action on output to stimulation was also tested. This concentration of ouabain caused a fivefold increase in resting output: in the presence of this enhanced resting output, the response to stimulation at 0·1 Hz was undetectable and at 10 Hz reduced in size. After withdrawal of ouabain, responses to stimulation were present but still reduced. A subsequent exposure to ouabain again increased the resting output by about the same amount as previously. On withdrawing the drug, resting output returned to normal with a half-time of about 5–10 min, recovery becoming slower with repeated dosage.

It was also found, as described later, that calcium deprivation, in the presence of normal sodium and potassium, reduces output. This procedure, too, is likely to increase intra-axonal sodium (Frankenhaeuser & Hodgkin, 1957) and has been found to increase sodium content of smooth muscle (Paton & Rothschild, 1965).

Finally, Fig. 10 shows an experiment in which partial sodium deprivation was combined with treatment with PHMB. This was designed to test the possibility that acetylcholine release resulted merely from a disturbance (in either direction) of intra-axonal sodium. We have no direct knowledge of the actual sodium concentration; Krnjević (1955) estimated the intracellular sodium of cat sciatic nerve as 41 m-mole/kg water, and the interstitial sodium as 245 m-mole/kg water. Rang & Ritchie (1968) give the intra-axonal sodium content of rabbit desheathed vagus nerve as 76·6 m-mole/kg fibre water. For these experiments with non-myelinated nerve, if the sodium content of the external fluid were reduced to 20 % of the normal content, and simultaneously a sodium pump inhibitor applied, there should be a much smaller change in the sodium concentration of intra-terminal axoplasm, through the great reduction in electrochemical gradient for sodium between the outside and inside of the nerves. Fig. 10 shows, however, that the addition of PHMB after low sodium treatment increased acetylcholine output to a peak value after 80 min of 3·5 times the initial level and 19 times the level attained in the low sodium medium.

These experiments show that an increase in acetylcholine output results from measures which would reduce intra-axonal sodium content (sodium withdrawal, with or without lithium substitution), or increase it (treatment with ouabain or PHMB, or potassium or calcium withdrawal), or tend to minimize such changes (the combination of partial sodium withdrawal with PHMB treatment). It seems, therefore, that some factor

other than the intra-axonal sodium content is involved in the control of acetylcholine release.

Effect of excess external potassium. A much simpler view, for which there is considerable support, is that acetylcholine output is a function of the membrane potential of the nerve endings. It has long been known that raised potassium concentrations increase acetylcholine output from the

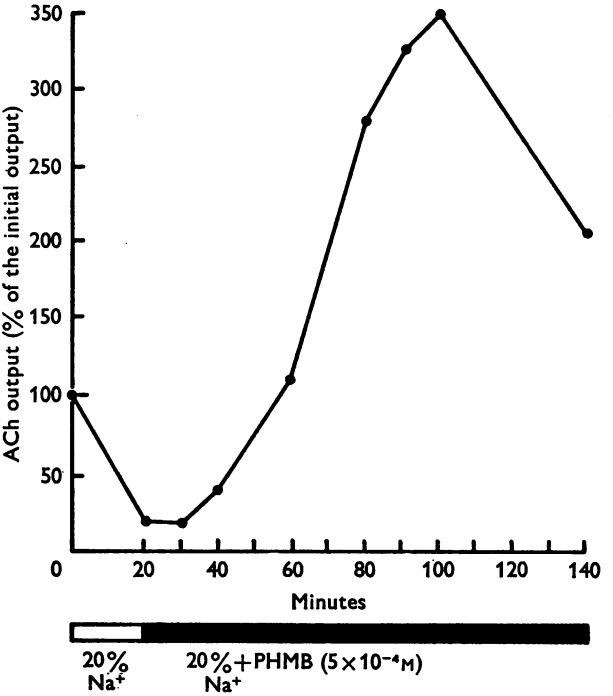

Fig. 10. Increases of acetylcholine output as a result of sodium salt of *p*-hydroxymercuribenzoate (PHMB) in a low sodium medium. 20 % sodium solution was prepared by replacing the rest of the sodium with equimolar amount of sucrose to maintain isosmolarity. The change of acetylcholine output has been expressed in percent taking the control output as 100. One experiment.

ganglion (Brown & Feldberg, 1936); and Liley (1956*a*) has shown a corresponding effect on rate of miniature discharge at the neuromuscular junction. Fig. 11*a* shows how resting output from the longitudinal strip varies with external potassium concentration (K_0). For small changes around the normal potassium content, there is little effect, but as concentration is raised, output increases dramatically. Liley (1956*a*) made similar observations and found that the rate of miniature discharge increased with the fourth power of K_0. A corresponding analysis of our results requires a preliminary correction. Evidence has been given earlier

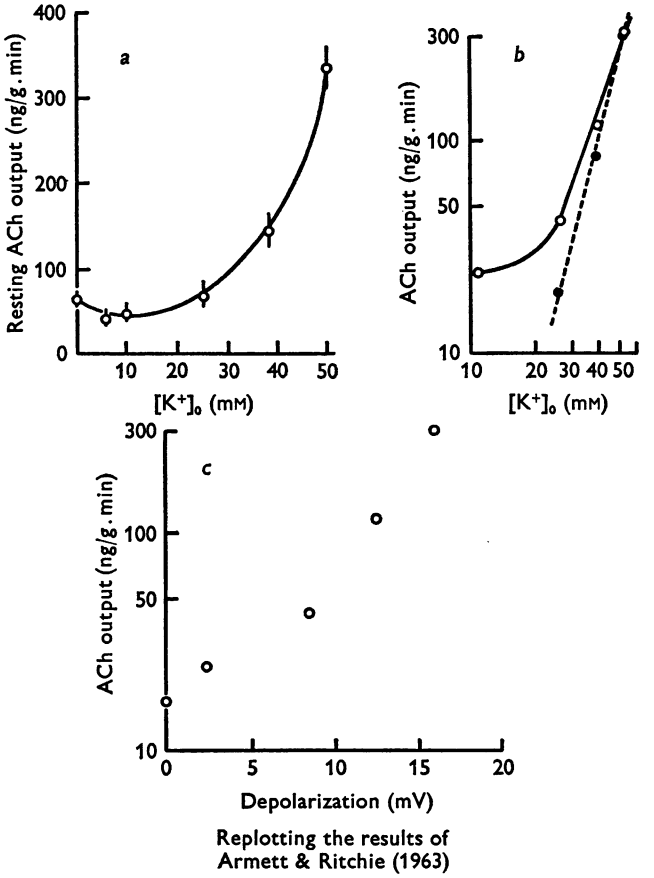

Fig. 11. Relation between acetylcholine release, external potassium concentration (K_o^+) and estimated depolarization of nerve terminals of eserinized longitudinal muscle strips of guinea-pig ileum. (*a*) Relation between acetylcholine release and K_o^+. To maintain isosmolarity, NaCl concentration of Krebs was lowered to the same extent as the KCl concentration was raised. Mean of six experiments; S.E. are indicated. (*b*) Correlation between K_o^+ and acetylcholine resting release. Data are plotted on double log scales. The tetrodotoxin-sensitive component of the control resting release of acetylcholine in 5·8 mM-K_o^+ has been subtracted from the outputs observed at the increased K_o^+ concentrations. The slope of the line from 27 mM-K_o upwards is 3·28. If the potassium-insensitive component of output (50·0 ng/g.min) is subtracted, the dashed line of potassium-sensitive release is obtained, with slope of 4·2. (*c*) Correlation between the depolarization produced by K_o^+, estimated by re-plotting the results of Armett & Ritchie (1963), and acetylcholine output induced by different concentrations of K_o^+. Abscissae: potential (x) is expressed relative to the resting potential obtaining at the normal potassium concentration (see Armett & Ritchie, 1963): the acetylcholine output (y) at different K_o^+ concentrations is plotted on a logarithmic scale. The tetrodotoxin-sensitive part of resting release has been subtracted from the values of acetylcholine output. The equation is $\log y = 0{\cdot}113x + 0{\cdot}6838$.

that the output consists of two main fractions, one associated with propagated activity and sensitive to hexamethonium and tetrodotoxin, the other not. Fig. 12 shows that the augmented output induced by potassium, unlike part of the resting output, is resistant to tetrodotoxin. Presumably nerve conduction is impaired by the exposure to high potassium concentration (cf. Lundberg, 1948). In any case, this result implies that comparison should be made only with the tetrodotoxin-resistant fraction of normal output; this could be regarded as spontaneous release associated with the normal resting membrane potential. Accordingly, 60 % of normal resting

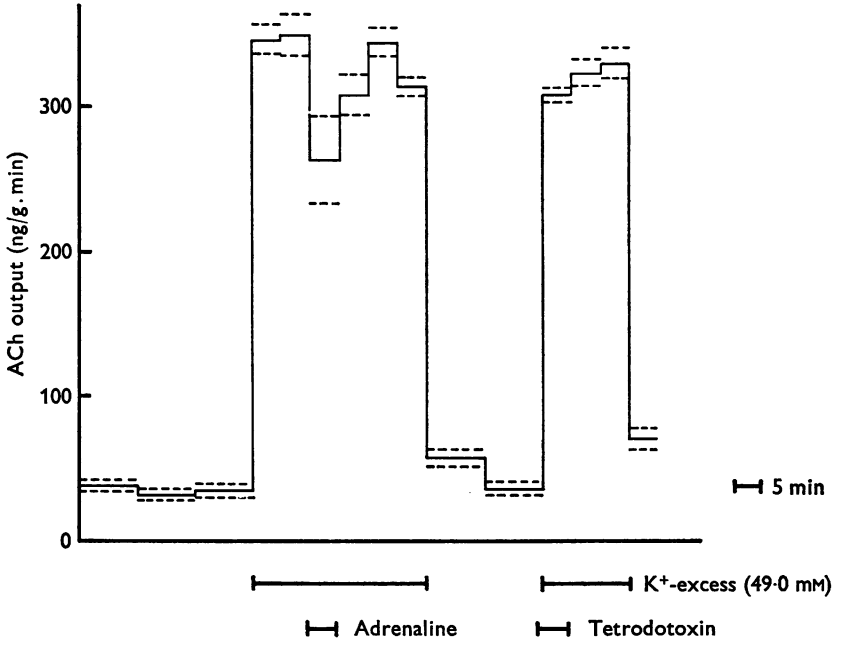

Fig. 12. The resistance to tetrodotoxin of acetylcholine output evoked by high K_o^+. Longitudinal muscle strip of guinea-pig ileum. Potassium, 49·0 mM, was present as indicated. Adrenaline, 10^{-6} g/ml., is present as indicated. Three experiments were made using identical dose and time schedules. Dashed lines represent the S.E. of the mean.

output has been subtracted from the acetylcholine outputs determined. Fig. 11*b* shows the relation of these outputs to K_o, plotted with logarithmic scales. With high K_o the output increases with a power of K_o of about 4. If assumptions are made as to the permeability of the nerve-ending membrane, estimates of the connexion between membrane potential and output could be made; but on comparing our results with those of Liley, it must be supposed that the region where the membrane potential is potassium-determined is only from 20 mM or beyond. A more direct approach is available, from the data of Armett & Ritchie (1963) on

the change of membrane potential, measured by a sucrose-gap method, with K_o, for desheathed vagus nerve C fibres. Fig. 11*c* gives the corrected acetylcholine output plotted against membrane depolarization, calculated on the assumption that the nerve fibres of the longitudinal muscle plexus correspond to vagus nerve C fibres in their response to change in K_o. The slope of the relationship varies so that for lower values of K_o, 15 mV depolarization is required for a tenfold rise in output (or a compound increase of 15 % per mV), but for high values of K_o only about 10 mV.

It is evident that the sensitivity of acetylcholine output to changes in membrane potential is adequate to account for some of the changes we have described. Thus the output in response to ouabain and PHMB could readily be attributed to depolarization resulting from sodium entry and potassium loss. The effect of potassium withdrawal is less obvious, but it is known that this can lead to spasm of smooth muscle, and to membrane depolarization (Holman, 1958; Paton, 1961), despite the expectation on the basis of the Nernst equation for a potassium electrode that hyperpolarization would occur. The depolarization could arise from a fall in potassium permeability as a result of potassium withdrawal, allowing other cation movements a more prominent role. There is no evidence, however, that sodium withdrawal should cause a depolarization of the nerve endings, and possibly the reverse might be expected. Nor can the augmentation of potassium-induced release by sodium withdrawal be plausibly explained on the basis of a further membrane depolarization.

The role of calcium and magnesium. The discrepancy mentioned could be accounted for, and a mechanism provided for the connexion between depolarization and release, if it were entry of calcium in the membrane that is critical for acetylcholine release, and if sodium competes with it in the way described for heart muscle and other tissues (Luttgau & Niedergerke, 1958; Colomo & Rahamimoff, 1968; Douglas & Rubin, 1963). It would then only be necessary to suppose that depolarization induces calcium entry and that sodium removal also increases calcium influx, as is known for squid axon (Baker & Blaustein, 1968; Hodgkin & Keynes, 1957). There is, indeed, abundant evidence that acetylcholine release in autonomic ganglia is normally calcium-dependent, since the pioneer work of Harvey & MacIntosh (1940). With the longitudinal strip, we have found that calcium is likewise normally essential for acetylcholine output (Fig. 13). In addition Ba^{2+} is found to substitute for it, giving a greatly enhanced output in the period immediately after substitution. Similar results have been obtained on the superior cervical ganglion by Douglas, Lywood & Straub (1961) and on m.e.p.p.s (Elmqvist & Feldman, 1965*a*). Further, magnesium is known to antagonize many actions of calcium (Hubbard, 1961; Skou, 1960) and would therefore be expected to interfere with acetylcholine output. A

fivefold increase of Mg^{2+} in the bath fluid from 1·2 to 6·0 mM reduced the resting acetylcholine output from 45·7 ± 2·0 to 17·4 ± 1·6 ng/g.min (n = 3). A similar finding has been made by Cowie, Kosterlitz & Watt (1968) who found that resting acetylcholine output from the longitudinal strip exposed to 0·1 mM-Mg^{2+} was roughly 10 times greater than with 5 mM-Mg^{2+}.

It would be expected on this basis, however, that all acetylcholine release should be calcium-dependent. But we have found that release of acetylcholine from the longitudinal strip caused by sodium deprivation

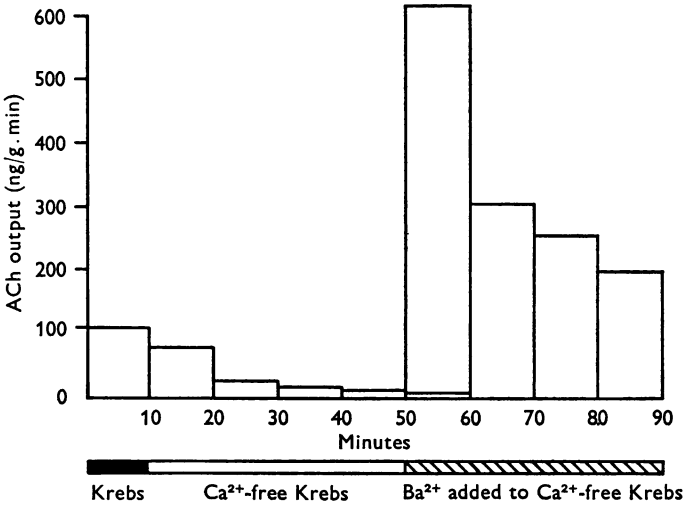

Fig. 13. Effect of Ca^{2+} lack and its substitution by Ba^{2+} on resting acetylcholine output. Mean of two experiments, which differ by less than 10 % at each value. Calcium-free solution was prepared by omitting calcium chloride from the bathing solution. Barium-Krebs was prepared by replacing $CaCl_2$ with $BaCl_2$. For method of barium extraction from the Krebs assay, see Methods.

does not depend on the presence of calcium (Fig. 14). MacIntosh (1963) has already reported that the same is true for the superior cervical ganglion.

It is thus possible to find conditions where enhanced acetylcholine release can take place without dependence on movements of sodium, nor on nerve terminal depolarization, nor on the presence of calcium in the extracellular fluid. This result, particularly the finding that deprivation of both sodium and calcium led to an augmented rate of acetylcholine release from the strip, prompted us to consider another theory (Zar, 1965): that the release of acetylcholine depends on the inhibition of ($Na^+ + K^+ + Mg^{2+}$)-activated ATPase associated with the membrane of the nerve endings. For on this hypothesis, once the ATPase has been inactivated through the absence of sodium, the presence of calcium would no longer be required for

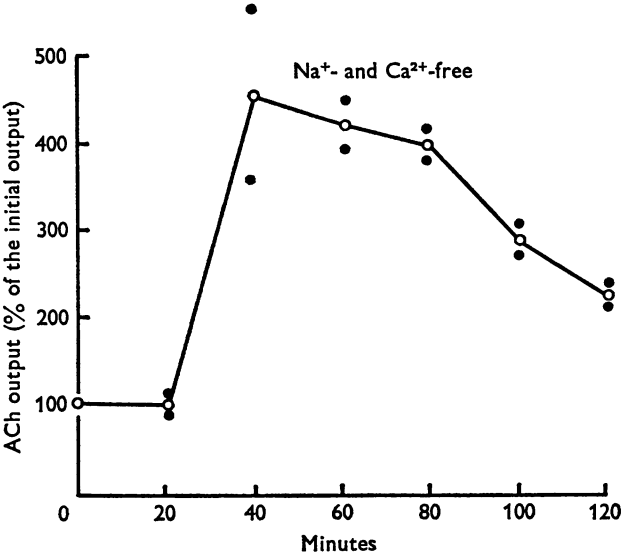

Fig. 14. Increase of acetylcholine output from the longitudinal muscle strip of guinea-pig ileum as a result of withdrawing sodium and calcium. Sucrose was used as the substitute for sodium and $CaCl_2$ was omitted. The value of acetylcholine output (○) represents the mean of two experiments. The individual values are indicated (●). The acetylcholine output in normal Krebs was taken as 100 and the subsequent values obtained have been expressed as % of the initial output.

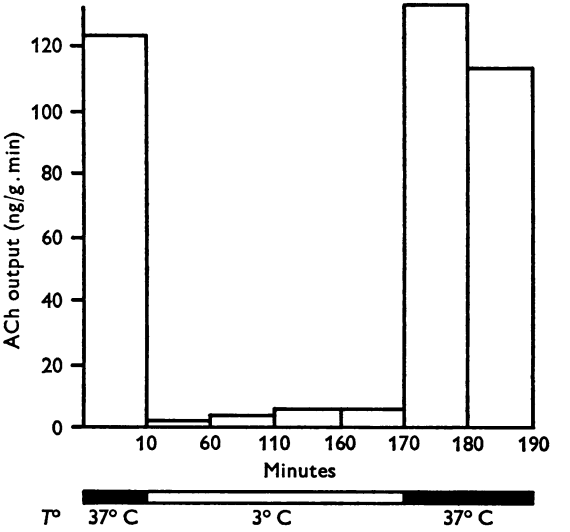

Fig. 15. The effect of lowering the temperature on the acetylcholine output from the longitudinal muscle strip of guinea-pig ileum. The acetylcholine output is expressed in ng/g.min. Mean of two experiments.

acetylcholine release. The manner in which this could account for our experimental findings is discussed later. But an attempt to test this hypothesis further was made in which the enzyme was inhibited not by added drugs, but by cooling to near 0° C without any other change in conditions. Fig. 15 shows the result, that resting output, so far from being increased, is reduced to almost undetectable levels. But it was also found that this fall of temperature had a similar effect on release evoked by stimulation or by

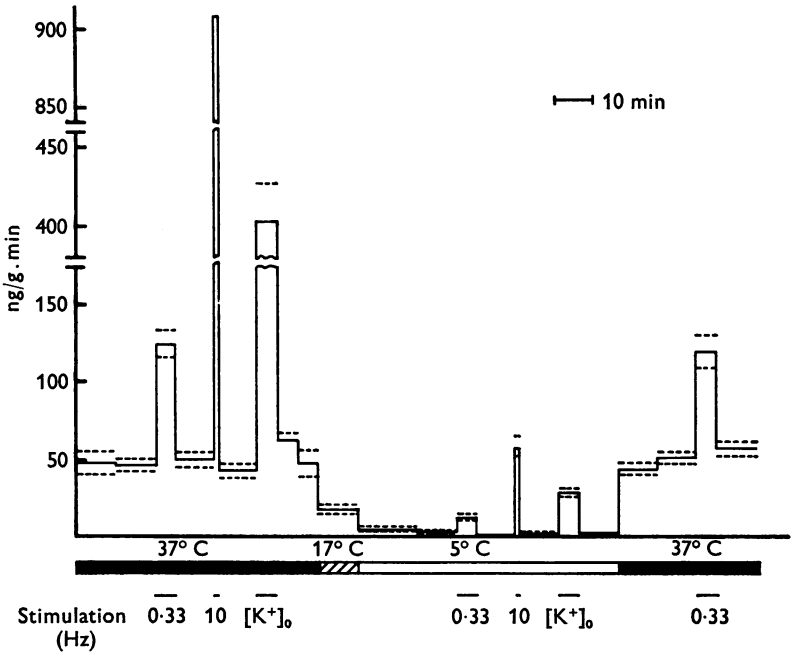

Fig. 16. Effect of low temperature on acetylcholine output by longitudinal muscle strip under resting conditions, stimulation at 0·33 or 10 Hz, or during exposure to 49·7 mM potassium in the bathing fluid, as indicated. Mean of three experiments with identical treatment schedules; dashed lines indicate S.E. Stimulation periods were 5 min at 0·33 Hz and 1 min at 10 Hz.

added potassium (Fig. 16). Cooling to 5° C was found to reduce by 90% or more the response both to stimulation at 0·33 Hz or at 10 Hz, and also that to 50 mM potassium. The figure also shows the prompt reversibility of the effect, showing that no permanent damage takes place. It was thus not possible to use cooling for a crucial experiment, since it reduced the acetylcholine output under the four widely different conditions tested. It should be noted that the reduction of output in response to cooling is probably not attributable to a failure of nerve conduction, since Greengard & Straub (1958) found that conduction in rabbit sympathetic fibres survived cooling to 7° C, the negative after-potential being greatly increased.

Acetylcholine synthesis by the longitudinal muscle strip

It is known from work with cat superior cervical ganglion that although 85% of the acetylcholine content of the ganglion is available for release, yet under normal conditions the content is maintained remarkably constant when the ganglion is stimulated even though more than the original content has been released (Birks & MacIntosh, 1961). As MacIntosh (1963) particularly has emphasized, there must be some control mechanism linking acetylcholine release and acetylcholine synthesis. Experiments of a similar type were made with the longitudinal muscle strip, in which the effect of a number of the procedures already discussed on acetylcholine was determined, thus allowing the total synthesis rate to be calculated (Table 2) The control figures quoted were all obtained after pre-incubation in eserinized Krebs solution for 60–90 min.

At rest, the tissue acetylcholine content did not change significantly, the rate of synthesis, $3{\cdot}4 \pm 0{\cdot}5$ μg/g.hr, being sufficient fully to make good the resting output. Similarly, with electrical stimulation at 10 Hz or exposure to raised external potassium concentration (49·0 mM), synthesis rose to a level, $19{\cdot}8 \pm 1{\cdot}3$ and $20{\cdot}3 \pm 0{\cdot}6$ μg/g.hr respectively, sufficient or almost sufficient to compensate for release. It is possible that during stimulation synthesis lagged a little behind release. With barium treatment, in addition, synthesis and release were closely matched. The fact that these three procedures each induced a synthesis rate of about 20 μg/g.hr, together with the fact that acetylcholine output per minute does not increase with higher rates of stimulation than 10 Hz, suggests that this represents the maximal synthesis rate. At the opposite extreme may be cited the experiments in which calcium was withdrawn. The abolition of acetylcholine release by this procedure has been already described; the acetylcholine content of the tissue fell, but the change in these experiments was of doubtful significance. Although more evidence is required, it appears that with calcium lack, also, release and synthesis are matched.

In contrast, synthesis was impaired by sodium or potassium lack or by ouabain treatment, although acetylcholine release was increased. The effect of sodium deprivation was the most striking, synthesis being reduced below the resting level, and possibly being arrested altogether. With ouabain and potassium lack, synthesis did not rise above the resting level. This impairment of synthesis probably affects the estimate of the acetylcholine-releasing capacity of these procedures, since it would be expected that the release would have been bigger had the tissue not become depleted of acetylcholine. The depletion may also contribute to the falling away of the rate of release with time; but since the release induced by raised potassium concentration also falls away with time, other factors

TABLE 2. Effect of various procedures on acetylcholine content of and acetylcholine release from nerve terminals of eserinized longitudinal muscle strips from guinea-pig ileum and on calculated rates of acetylcholine synthesis

Condition	n	ACh content (μg/g ± S.E.) before	ACh content (μg/g ± S.E.) after	Gain (+) or loss (−) (μg/g ± S.E.)	Total release (μg/g ± S.E.)	Total synthesis (μg/g ± S.E.)	Rate of synthesis (μg/g.h ± S.E.)
1. Resting (1 hr)	3	21·8 ± 1·6	22·5 ± 1·4	+ 0·7 ± 0·3	2·6 ± 0·2	3·4 ± 0·5	3·4 ± 0·5
2. K^+ excess (49 mM) (20 min)	3	23·4 ± 0·6	25·7 ± 1·3	+ 2·3 ± 0·7	6·0 ± 0·2	8·3 ± 0·6	20·3 ± 0·6*
3. Stimulation 12/min (3 hr; no eserine)		20·4 ± 3·7 (4)	17·7 ± 2·1 (5)	− 2·7	36 (estimated)	34·3	11·4***
4. Stimulation 10 Hz (1 hr)	5	22·2 ± 0·6	17·5 ± 0·7	− 4·7 ± 1·1	25·2 ± 0·7	19·8 ± 1·3	19·8 ± 1·3
5. K^+ lack (2 hr)	2	25·8 (2)	8·4 (2)	− 17·4	26·1	8·7	4·4**
6. Na^+ lack (2 hr)	2	28·2 (2)	2·5 (2)	− 25·7	26·9	1·2	0·6**
7. Ouabain (1 hr)	5	23·5 ± 0·6	17·2 ± 0·8	− 6·1 ± 0·4	11·0 ± 0·9	4·8 ± 0·7	4·8 ± 0·7
8. Ca^{2+} lack (2 hr)	2	21·1	17·5	− 3·5	2·5 } (estimated)	(− 1·0)	(− 0·5)
		34	25·3	− 8·7	2·5 }	(− 6·2)	(− 3·1)
9. Ba^{2+} treatment (69 min; includes 2 min stimulation at 10/sec)	2	25·9 (2)	26·0 (2)	+ 0·1	26·0	26·1	22·7

The solutions and conditions used were: (1) resting (Krebs solution); (2) K^+ excess (49·0 mM-KCl with NaCl lowered to maintain isosmolarity); (3) stimulation 0·2 Hz (Krebs solution, no eserine present); (4) stimulation 10 Hz for 1 hr (Krebs solution); (5) K^+ lack; (6) Na^+ lack; (7) Ouabain 5×10^{-5} M; (8) Ca^{2+}-free; (9) Ba^{2+}.

* Calculated for 1 hr from 20 min exposures; ** Calculated for 1 hr from 2 hr exposures; *** calculated for 1 hr from 3 hr exposures. Values are given of mean ± S.E. (except (5), (6), (8) and (9) when values or mean of two experiments is cited. n refers to the number of experiments.

such as depletion of the readily mobilized stores (Birks & MacIntosh, 1961; Paton & Vizi, 1969; Collier & MacIntosh, 1969) may be important. It was interesting that in the sodium withdrawal experiment, an appreciable output was still taking place when acetylcholine content must have been drastically reduced.

DISCUSSION

Most of our recent knowledge concerning acetylcholine release has been derived from experiments on nerve-striated muscle preparations (Fatt & Katz, 1952; Katz, 1962; Liley, 1956*a*, *b*; del Castillo & Katz, 1954; Katz & Miledi, 1965, 1967) or the cat superior cervical ganglion (Brown & Feldberg, 1936; Perry, 1953; Birks & MacIntosh, 1961; Birks, 1963; MacIntosh, 1963). The longitudinal muscle strip preparation of guinea-pig ileum provides information about acetylcholine release at a different type of neuroeffector junction. It possesses the advantages that relatively large amounts of acetylcholine are released; it is now established that the acetylcholine comes only from the nervous tissue (Paton & Zar, 1968); and the preeffector cholinergic axones can be assumed still to be in continuity with their cell bodies.

It would be expected that acetylcholine is released by a process corresponding to the quantal release at motor end-plate or ganglionic synapse. Although there is no direct evidence, such as miniature junction potentials, for quantal release in this preparation, vesicles with a diameter ranging from 250 to 600 Å are found in the terminal nerve bundles of the plexus (Fig. 17). Since close approximation of nerve terminal to smooth muscle cell is not common (W. D. M. Paton, unpublished), discrete junction potentials may in fact not be readily detectable. At the same time, since release from the nerve terminals of the longitudinal muscle strip is far more sensitive to drugs such as morphine or noradrenaline than is the case at motor end-plate or ganglion, the possibility of some other process of release must be kept in mind; in view of the presence of cholinesterase in the membrane of cholinergic nerves, however, it is difficult to envisage a physiologically plausible mechanism.

The principal disadvantage of the preparation is that the state of activity of the nervous tissue is less well defined than for the other synapses. It was concluded, from experiments with tetrodotoxin and hexamethonium, that in the resting state (i.e. without external electrical stimulation) about 60% of the resting acetylcholine output depended on propagated activity. A similar partition of miniature junction potential discharge into tetrodotoxin-sensitive and tetrodotoxin-resistant has been made at the neuromuscular junction, and the latter is usually held responsible for the acetylcholine output in the absence of stimulation (Katz & Miledi, 1967;

Elmquist & Feldman, 1965*b*). It is not yet clear how far the tetrodotoxin-resistant part of acetylcholine release from neuromuscular junction can properly be compared with that from longitudinal muscle strip, but at least it is now known that both can be increased by potassium.

We have described above how the theories that acetylcholine release is ultimately controlled by axonal sodium concentration, by membrane potential, or by calcium failed to account for all our results. The fact that

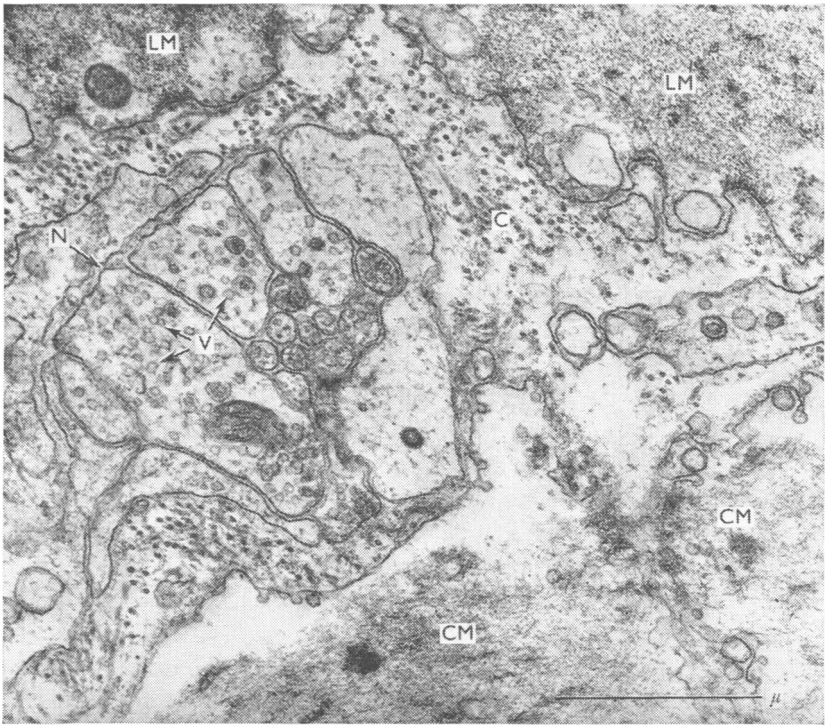

Fig. 17. Electron micrograph of section of longitudinal muscle strip showing a small nerve bundle (N) between longitudinal smooth muscle cells (LM) cut transversely and circular smooth muscle cells (CM) cut longitudinally. Osmium fixation, Araldite embedding and lead citrate after-staining. Collagen bundles (C) usually cut transversely lie between the nerve bundle and the muscle fibres. Vesicles (V) are present in the axons of the nerve bundle. Bar 1 μm.

acetylcholine release as a result of sodium deprivation did not require the presence of calcium suggested that release might depend on inhibition of the activity of a $(Na^+ + K^+ + Mg^{2+})$-activated ATPase at the axonal membrane. This enzyme has been shown to be present in the membrane of nervous tissues (Skou, 1957; Bonting, Caravaggio & Hawkins, 1962; Somogyi, 1964) as well as in the erythrocyte and cardiac muscle membrane

(Dunham & Glynn, 1961; Repke, 1965). It remains to consider how far such a mechanism could account for our results generally.

(*a*) The activity of magnesium-activated ATPase of crab nerve is almost abolished by sodium deprivation (Skou, 1957). Whittam (1962) has shown that the membrane ATPase of the erythrocyte membrane has vectorial properties, so that it is probable that the inhibition would develop only slowly in experiments on whole tissues, as the sodium was lost from the interior of the axon. Lithium has been found to be a far less effective activator of the enzyme than is sodium; it competes with sodium (Skou, 1965) and in its presence the intraneuronal sodium concentration falls (Wespi, 1969; Baker, Blaustein, Hodgkin & Steinhardt, 1969). Substitution of sodium by lithium could, therefore, lead to a more rapid functional sodium deprivation and hence ATPase inactivation. Acetylcholine release developed more rapidly when sodium replacement was with lithium rather than with Tris or sucrose (Fig. 4). Acetylcholine release by such a mechanism would not be expected to be calcium-dependent.

(*b*) The same enzyme has also been shown to be dependent on potassium, and very low levels of activity prevail in its absence (Skou, 1957).

(*c*) Ouabain was one of the first inhibitors of the enzyme to be identified (Skou, 1957; Bonting *et al.* 1962), and it is now known to be active against ATPase from brain, red cells, striated muscle, cardiac muscle and mucous membrane (Bonting *et al.* 1962; Dunham & Glynn, 1961; Repke, 1965; Smith, 1967). Maximal inhibition may require about 10^{-4} M ouabain (Repke, 1965), but half-maximal inhibition of enzyme preparations is usually obtained with about $0{\cdot}1\text{–}1 \times 10^{-6}$ M (Smith, 1967; Bonting *et al.* 1962). These results have been obtained on tissue homogenates in which the glycoside would have ready access to the enzyme. In our experiments, an effect was obvious with 10^{-6} g/ml. ($1{\cdot}4 \times 10^{-6}$ M) and considerably greater with $3{\cdot}7 \times 10^{-5}$ g/ml. (5×10^{-5} M). The concentrations of ouabain needed to produce an effect appear high compared to those required for ATPase inhibition; but this could be due partly to restraints on the access of the glycoside to the enzyme in intact unhomogenized tissue, and partly to a simultaneous release of catecholamine (Banks, 1970), which is known to reduce acetylcholine output (Paton & Vizi, 1969; Kosterlitz, Lyden & Watt, 1970; Knoll & Vizi, 1970). The experiments of Elmquist & Feldman (1965*a*) raise a question about ouabain action. They found that although ouabain continued to cause an increased discharge of m.e.p.p.s in the rat diaphragm in the absence of calcium, even if EDTA was added at the same time as the ouabain, ouabain failed to be effective if the tissue was incubated previously in calcium-free EDTA-containing medium. They suggested, therefore, that ouabain works in part by mobilizing sequestered calcium; on other grounds, they concluded that its action was also due in

part to inhibition of the sodium pump. In either case, however, ATPase inhibition would be expected.

(*d*) PHMB has been shown to inhibit the ATPase of ox brain (Skou, 1963); in a concentration of 10^{-5} M, 60 % inhibition was produced. It is, however, an inhibitor of less specificity than the cardiac glycosides, being a general sulphydryl reagent.

(*e*) It has long been known that acetylcholine release does not take place in the absence of calcium, and there is now convincing evidence that release as a result of a change in membrane potential of the nerve ending, produced by a propagated action potential, by local depolarization, or by added potassium, depend on the presence of calcium and its interaction with or entry into the membrane. Calcium is also a very effective inhibitor of ATPase; Skou (1957) found that 50 % inhibition is produced by about 0·5–1 mM, in the presence of 120 mM potassium, 80 mM sodium and 3 mM magnesium; the inhibition is sensitive to the concentration of other ions, and magnesium in particular antagonizes its action (Skou, 1957, 1960; Rendi & Uhr, 1964). Somogyi (1964) showed, with membrane ATPase from rat brain, that the concentration of Ca^{2+} to produce half-maximal inhibition was 0·5 mM, in the presence of 100 mM sodium, 20 mM potassium and 5 mM magnesium. In our experiments, therefore, the output evoked by stimulation or by K^+ excess could be due to Ca^{2+} movement with resultant ATPase inhibition. The ability of Mg^{2+} to reduce acetylcholine output could, conversely, be attributed to ATPase activation (Skou, 1957, 1960).

(*f*) Barium is known to be able to substitute for calcium in transmitter release (Douglas *et al.* 1961). In a concentration of 10^{-6} M it inhibits ATPase of calf kidney by 100 % (Rendi & Uhr, 1964). Elmqvist & Feldman (1965*a*) showed that barium increases m.e.p.p. discharge, and can do so when calcium is removed with EDTA.

Our results can, therefore, be accounted for qualitatively in an economical way on the assumption that acetylcholine release depends on inhibition of $(Na^+ + K^+ + Mg^{2+})$-activated ATPase in the nerve terminal membrane. But consideration of the data of Table 2 raises another question. A crucial experiment was the observation that release occurred in response to simultaneous sodium and calcium deprivation. In Table 2 it is shown that sodium deprivation leads to a virtual suppression of synthesis, and a similar observation has been made by Birks (1963) on the cat superior cervical ganglion. It could be suggested therefore that the increased rate of release found in the absence of sodium is in some way linked to this suppression of synthesis, perhaps through interference with acetylcholine storage sites (cf. MacIntosh, 1963), and that this entails a release process distinct from that normally involved. The output resulting from the other procedures used could in turn be plausibly linked to calcium mobilization

or influx. There is, however, no reason to suppose that interference either with storage or with synthesis of acetylcholine by sodium deprivation will lead to an *increased* rate of release. If impairment of acetylcholine storage by sodium deprivation led to the appearance of free acetylcholine within the nerve ending, the situation would be analogous to that of 'surplus' acetylcholine in the superior cervical ganglion. Surplus acetylcholine was the term applied by Birks & MacIntosh to the increase in content that follows treatment of the superior cervical ganglion with eserine, an increase sufficient to double the acetylcholine content of the ganglion. They concluded that it was probably acetylcholine surplus to that required for vesicular acetylcholine, normally destroyed by cholinesterase, but preserved in the axoplasm in the presence of eserine. They found, however, that in the presence of this surplus acetylcholine, neither resting output nor output in response to preganglionic stimulation was increased and they concluded that it was not released during normal transmission. A possibly analogous finding is the observation by Potter (1967) that although in the presence of reserpine and an amine oxidase inhibitor a sympathetic nerve may still contain noradrenaline, free in the axoplasm, it cannot be released by nerve stimulation. Nor does the mere inhibition of synthesis entail release of acetylcholine; HC3 in suitable doses virtually abolishes synthesis, but does not itself lead to acetylcholine release (MacIntosh, 1963).

The suggestion that ATPase inhibition is necessary for acetylcholine release might imply that ATPase activity generally is necessary for stability of the nerve terminal membrane; and that only regions of instability can be used for exocytosis. Alternatively, there might be a more specific link with the ATPase-dependent transport mechanisms such that only inhibited channels were available for use in the process of exocytosis.

The control of synthesis of acetylcholine by the nerve plexus remains puzzling. MacIntosh (1963) has stressed the way in which the superior cervical ganglion maintains a remarkably constant acetylcholine content under varying conditions of synaptic activity, the rate of synthesis being in some way adjusted both to the activity and to the capacity of the stores. The longitudinal muscle strip behaves in the same way, at rest, or when stimulated electrically, or by potassium, or with barium. With widely varying rates of release, acetylcholine content is held rather constant. It is difficult, however, to argue that sodium entry controls this homoeostatic process, since treatments with ouabain or with potassium-deprivation which lead to sodium entry (Hodgkin & Keynes, 1955) produce depletion of the tissue acetylcholine content, the synthesis rate being only about sufficient to compensate for normal resting output. It is equally doubtful whether calcium entry could be held to control the process, since ouabain is known to increase calcium uptake of cortex (Stahl & Swanson, 1969), yet

the synthesis when exposed to ouabain is not increased significantly above the resting level; sodium deprivation also leads to increased calcium influx (Baker & Blaustein, 1968), yet here, as already discussed, synthesis is almost arrested. It is, however, suggestive that there are three procedures, namely treatment with ouabain, withdrawal of sodium and withdrawal of potassium, which both impair synthesis and promote release.

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