J. Physiol. (1972), **221**, *pp.* 669–682
With 9 text-figures
Printed in Great Britain

AN ELECTROPHYSIOLOGICAL ANALYSIS OF THE SYNTHESIS OF ACETYLCHOLINE IN PREGANGLIONIC NERVE TERMINALS

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(*Received 3 September 1971*)

SUMMARY

1. An electrophysiological analysis has been made of the synthesis of acetylcholine (ACh) in the preganglionic nerve terminals of the isolated superior cervical ganglion of the guinea-pig. The mean amplitude of excitatory post-synaptic potentials recorded intracellularly was taken as a measure of the ACh output per impulse from the terminals of a preganglionic axon.

2. Prolonged repetitive stimulation of the cervical sympathetic trunk at 10 and 20 Hz led to a decline in ACh output over the first 5–15 min and then a maintained output for periods of up to an hour. The mean level of maintained output was 0·4 of the peak initial output.

3. The maintained level of output was shown to be equal to the rate of synthesis of new transmitter and was not dependent on the addition of choline (3×10^{-5} M) to the fluid in the organ bath.

4. The ACh output per minute was shown to be directly proportional to the frequency of stimulation.

5. A model has been proposed of the storage and synthesis of ACh in preganglionic nerve terminals during prolonged stimulation, in which choline from the hydrolysis of released ACh is the main source of substrate for synthesis of new transmitter, and the rate at which synthesis proceeds is controlled by the rate at which transmitter is released.

INTRODUCTION

During prolonged high frequency stimulation, the amount of acetylcholine (ACh) overflowing into the venous circulation of perfused sympathetic ganglia decreases over the first few minutes and is then maintained at a constant level over long periods (Brown & Feldberg, 1936; Perry, 1953; Birks & MacIntosh, 1961). This maintained output of transmitter is

presumably a consequence of the synthesis of new ACh in the preganglionic terminals, as output is not maintained in the absence of synthesis (Kahlson & MacIntosh, 1939; Birks & MacIntosh, 1961; Collier & MacIntosh, 1969; Bennett & McLachlan, 1972). It appears that synthesis replenishes, at least in part, the store from which transmitter is released.

A supply of choline at plasma levels is necessary to maintain the output of ACh during prolonged stimulation of the perfused superior cervical ganglion of the cat in the presence of physostigmine (Brown & Feldberg, 1936; Birks & MacIntosh, 1961). Perry (1953) suggested that, in the absence of anti-cholinesterase, the choline derived from the hydrolysis of released ACh by acetylcholinesterase might be an important source of choline for resynthesis during maintained activity. It has since been proposed that about half of the choline formed from released ACh during long trains of stimuli is immediately recaptured and used in the resynthesis of ACh (Collier & MacIntosh, 1969). If it is true that choline from released ACh is the main source of substrate for resynthesis, this conservation of substrate should be able to maintain constant the amount of transmitter released per impulse at different frequencies of stimulation (Perry, 1953). This implies that the rate at which synthesis proceeds will be directly proportional to the stimulation frequency.

In electrophysiological studies of the release of ACh from motor nerve terminals, Elmqvist & Quastel (1965) found that the ACh output fell rapidly to 25% of the initial output in about 2 min and then levelled out during prolonged repetitive stimulation. However, their use of a curare-blocked preparation, together with apparent conduction failure in the nerve, prevented the detection of low levels of transmitter output during long trains of impulses. Nishi, Soeda & Koketsu (1967) measured the post-ganglionic nerve action potential from the toad sympathetic ganglion during maintained stimulation at different frequencies, and showed an early decrease in amplitude over 5–16 min, followed by a negligible decline over the succeeding hour.

In the preceding paper (Bennett & McLachlan, 1972), we have shown that, during prolonged stimulation, ACh is released from a single store in preganglionic terminals, and that this store is depleted if synthesis is blocked. The present paper examines the way in which newly synthesized transmitter replenishes this store so as to maintain the transmitter output during prolonged periods of stimulation. The ganglion *in vitro* does not seem to be dependent on supplementary choline to maintain the output of ACh over periods of stimulation of up to an hour, and the implication of this finding will be discussed.

METHODS

Isolated superior cervical ganglia from guinea-pigs (150–250 g) were used in all experiments. The experimental arrangement and techniques were the same as those described in the preceding paper (Bennett & McLachlan, 1972). The cervical sympathetic trunk was stimulated repetitively with or without the addition of choline at plasma levels (3×10^{-5} M) to the organ bath. Physostigmine (8×10^{-6} M) was applied in some experiments. Samples of excitatory post-synaptic potentials (EPSPs) from ganglion cells were recorded at intervals of $\frac{1}{2}$ or 1 min for the first 10 min after the beginning of stimulation and then at 2 or 5 min intervals after the amplitude of the epsps had become relatively constant. The amplitude of the action potential generated occasionally by a depolarizing current pulse was used as the criterion of maintained impalement.

The data were analysed in the same way as described in the preceding paper (Bennett & McLachlan, 1972). The mean amplitude of 20 EPSPs was measured at intervals during stimulation and corrected for non-linear summation (Martin, 1955), assuming an equilibrium potential for ACh of -10 mV (Nishi, Soeda & Koketsu, 1965). The values were normalized to the peak value for each terminal, and have been taken to be proportional to the ACh output per impulse. The pooled data for a number of terminals is representative of the pattern of release from the whole population (Bennett & McLachlan, 1972).

RESULTS

The release of ACh during maintained stimulation

Stimulation of the cervical sympathetic trunk at 10 Hz gave rise to EPSPs in the ganglion cells which facilitated to a maximum in $\frac{1}{2}$–1 min and then decreased in amplitude. This decrease continued for 5–15 min and thereafter the EPSPs remained relatively constant as long as the impalement was maintained (30 min to over an hour) (Fig. 1).

In two of fifteen cells, the EPSP amplitude was maintained at almost the peak value for the first few minutes. Thereafter the amplitude declined to a new steady level by 15 min after the beginning of stimulation. This release pattern was seen in a similar proportion of terminals stimulated in the absence of synthesis (Bennett & McLachlan, 1972).

The maintained level of ACh output from different terminals ranged from 0·2 to 0·7 of the peak initial output level. However, the pooled data from a number of terminals stimulated at 10 Hz gave an over-all maintained level of 0·4 (Fig. 2). This pattern of an early decline over 5–15 min followed by a levelling off of the ACh output is very similar to that reported from experiments in which the ACh overflowing into the veins from perfused ganglia was measured (Perry, 1953; Birks & MacIntosh, 1961).

Some terminals (20 %) showed periods of asynchronous release of ACh after 30 min or more of high frequency stimulation (Fig. 3). This phenomenon was observed more often during stimulation at 20 Hz than at 10 Hz. The pattern of release sometimes consisted of complete failures followed by bursts of large EPSPs. It is unlikely that

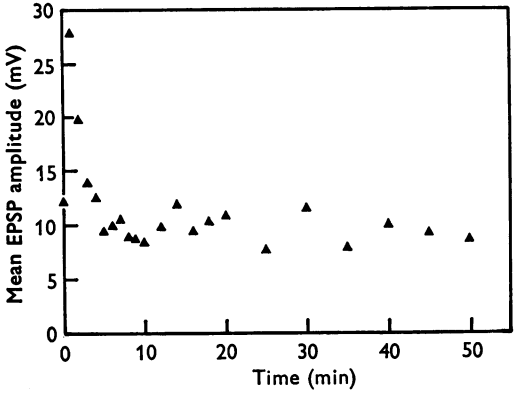

Fig. 1. The changes in amplitude of the excitatory post-synaptic potential (EPSP) recorded in a ganglion cell during continuous stimulation of the cervical sympathetic trunk. Each filled triangle represents the mean amplitudes of twenty EPSPs (corrected for non-linear summation) sampled at intervals after the beginning of stimulation. Frequency of stimulation, 10 Hz. Choline-free medium. Dihydro-β-erythroidine (5×10^{-7} g/ml.). The EPSP amplitude declined over the first 5 min until a steady amplitude was reached which then remained approximately constant for over 40 min of continuous stimulation.

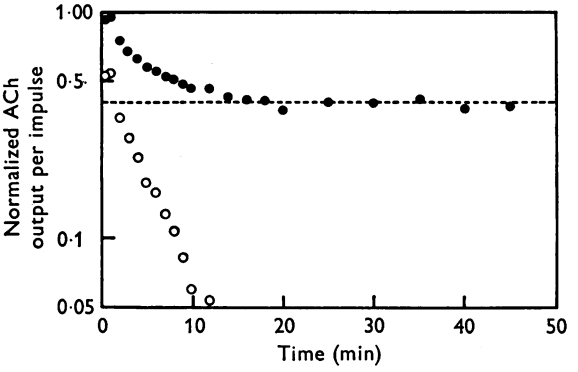

Fig. 2. The changes in ACh output per impulse from preganglionic terminals during continuous stimulation. The filled circles represent the mean ACh output from fifteen individual terminals determined at intervals after the beginning of stimulation. S.E. of the means were always less than $\pm 0{\cdot}10$. The dashed line gives the maintained level of ACh output, which is 0·4 of the initial maximum output. The open circles, derived by subtracting 0·4 from the values given by the filled circles, describe the depletion of the stores of transmitter formed before the beginning of stimulation. Frequency of stimulation, 10 Hz. Logarithmic ordinate. Choline (3×10^{-5} M) was present in the perfusion fluid of about half of the preparations.

the failures were due to presynaptic failure of the nerve impulse (Krnjević & Miledi, 1959), as the mean epsp amplitude was about the same during the periods of asynchrony as it was before and after such periods when the pattern of release was regular.

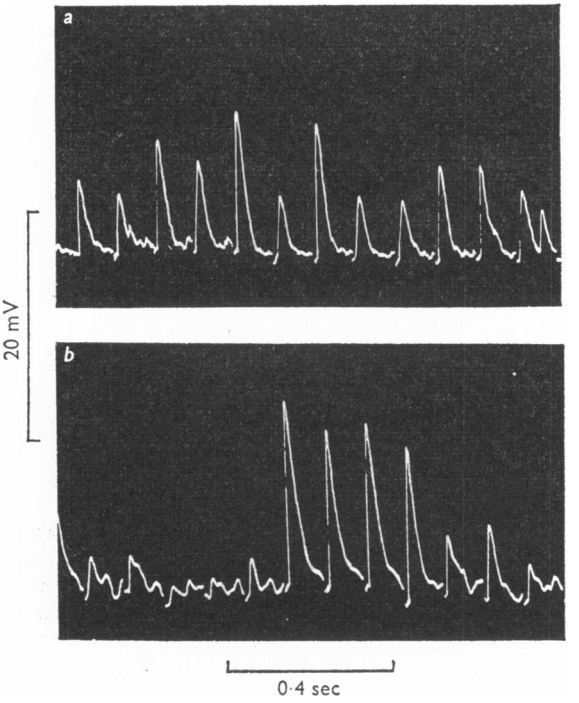

Fig. 3. EPSPs in a ganglion cell during continuous stimulation of the cervical sympathetic trunk recorded at 15 min (*a*) and 40 min (*b*) after the beginning of stimulation. Frequency of stimulation, 10 Hz. Choline (3×10^{-5} M) added to the perfusion fluid. The amplitude of individual EPSPs recorded at the earlier time fluctuated, but after prolonged stimulation bursts of large EPSPs occurred followed by a number of small EPSPs. However, the mean EPSP amplitude of each sample was approximately the same.

The release of newly synthesized ACh

As the output of ACh from preganglionic terminals declines exponentially to zero during inhibition of synthesis (Bennett & McLachlan, 1972), the maintained output during continuous stimulation in the untreated preparation must be a consequence of the synthesis of new ACh. The subtraction of the maintained level of output from the overall output curve was originally performed by Perry (1953) to describe the depletion of the stores present in the terminals before the beginning of stimulation. If the maintained level of 0·4 is subtracted from the pooled results for all terminals stimulated at 10 Hz (Fig. 2), an approximately exponential

curve is obtained. This curve follows closely the time course of depletion of the store in the terminals determined in the absence of synthesis (Fig. 4). If this interpretation is correct, then it seems likely that the transmitter available for release during prolonged stimulation is maintained by synthesis, and furthermore that synthesis continues at the same rate throughout the period of stimulation.

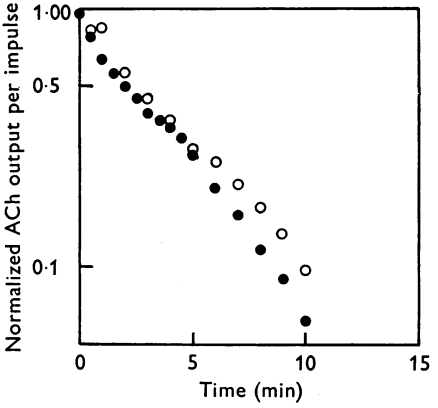

Fig. 4. Comparison between the changes in ACh output from preganglionic terminals during continuous stimulation in the presence of hemicholinium (HC-3), and that predicted on the basis that the steady level of release in the absence of HC-3 is solely maintained by the synthesis of new transmitter. The filled circles represent the mean ACh output per impulse for terminals stimulated in the presence of HC-3 (data from Bennett & McLachlan, 1972). The open circles are the values derived by subtraction of the maintained output level of 0·4 from the mean ACh output per impulse determined when normal synthesis was occurring (data from Fig. 2). Frequency of stimulation, 10 Hz. Logarithmic ordinate.

The origin of choline required to maintain synthesis

Unlike the case in perfused ganglia in the presence of physostigmine, a maintained output of ACh from preganglionic terminals *in vitro* exists in the absence of a supply of choline at plasma levels. The pooled results of ACh output for terminals stimulated in the presence of added choline are not significantly different from those obtained in normal Krebs solution (Fig. 5). The mean EPSP amplitudes at the peak output period were not different for the two groups. The slightly lower mean values after 10 min of stimulation in choline-free perfusion medium are not unexpected as a result of some leaching out of choline from the ganglion. This finding implies that the ganglion contains and/or synthesizes adequate choline for normal ACh synthesis during periods of stimulation of up to 50 min.

The use of an anti-cholinesterase in experiments on perfused ganglia would prevent the hydrolysis of released ACh at the synapse and so remove

this source of choline for uptake during stimulation. To test if the choline derived from the break-down of released ACh was the main source of substrate for resynthesis, the output of ACh was studied during stimulation in the presence of physostigmine and the absence of added choline. Repetitive stimulation in the presence of physostigmine leads to gradual depolarization of the ganglion cells as the concentration of ACh in the ganglion rises. It therefore becomes impossible to determine the ACh output from a single terminal by measuring the EPSP amplitude. Furthermore,

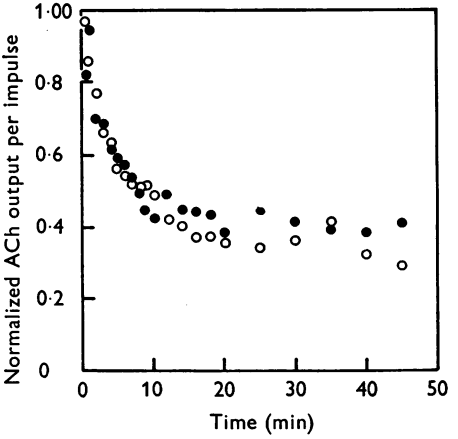

Fig. 5. Comparison between the ACh output from preganglionic terminals during continuous stimulation in the presence (●) or absence (○) of added choline. The filled circles represent the mean ACh output per impulse for terminals (n = 7) measured at intervals after the beginning of stimulation when the fluid bathing the ganglion contained choline (3×10^{-5} M). The open circles give the mean data for terminals (n = 8) stimulated in choline-free media. S.E. of the means were always less than $\pm 0{\cdot}12$ and showed considerable overlap. Frequency of stimulation, 10 Hz. The maintained release of transmitter did not appear to be dependent on a supply of choline at plasma levels.

hyperexcitability and repetitive firing of ganglion cells in the presence of physostigmine, as described by Eccles (1935, 1944) and Emmelin & MacIntosh (1956), made it especially difficult to obtain clear records. Several attempts were made to activate a single low threshold fibre to a ganglion cell by stimulating only a part of the preganglionic trunk using low stimulus strengths, thus avoiding the depolarization due to excessive accumulation of ACh in the ganglion.

In a cell in which a single EPSP was obtained in this way, it was clear that the output of ACh from one terminal decreased markedly and was not maintained at a steady level (Fig. 6). In fact, the pattern of output is almost identical to that seen during inhibition of synthesis. It thus appears

that the choline derived from the break-down of newly released ACh is the main source of substrate for the synthesis of releasable ACh during repetitive stimulation.

A similar conclusion was reached by Perry (1953), on the basis that physostigmine prevented the replenishment of transmitter stores during a period of rest after a long train of high frequency stimulation, suggesting that the use of the drug removed the source of substrate for synthesis.

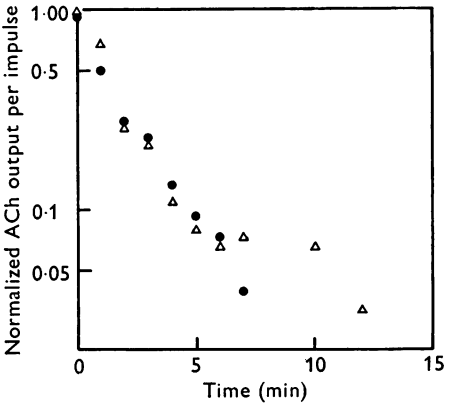

Fig. 6. Comparison of the effect of an anti-cholinesterase on the ACh output from a preganglionic terminal during continuous stimulation with the output from another terminal in which synthesis was inhibited. The filled circles represent the ACh output per impulse from a terminal stimulated in the presence of HC-3 (2×10^{-5} M) (data from Bennett & McLachlan, 1972). The open triangles give the ACh output per impulse from a terminal stimulated in the presence of physostigmine (8×10^{-6} M). Frequency of stimulation, 10 Hz. Choline-free media. Logarithmic ordinate. The decline in ACh output in the presence of anti-cholinesterase has a very similar time course to that seen in the absence of synthesis.

The addition of choline to the perfusion fluid in experiments on perfused ganglia overcomes this effect of anti-cholinesterase (Matthews, 1963), thus implying dependence of the maintained output of ACh on added choline.

The dependence of the rate of transmitter synthesis on stimulation frequency. In order to investigate the manner in which the rate of synthesis is controlled, it is necessary to evaluate the actual quantities of transmitter released during the period of steady output during stimulation at different frequencies. As it is extremely difficult to hold an impalement for long enough to examine these events in one terminal, the peak initial output of ACh per impulse was determined at different frequencies in the same terminal, and the relative levels of maintained output measured in a number of other terminals at the same frequencies.

The mean EPSP amplitude during continuous stimulation reached a maximum value between ½ and 1 min after the beginning of stimulation. This peak initial output of ACh was measured in several cells during stimulation at different frequencies, allowing 10 min of rest between each stimulation period for the restoration of control conditions. The responses recorded at 5, 10 and 20 Hz in a number of cells measured at this time had approximately the same mean amplitude and therefore the same ACh

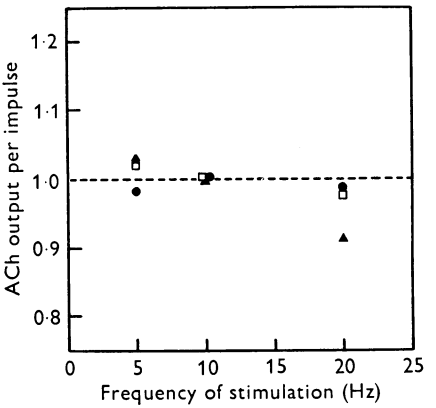

Fig. 7. The effect of frequency of stimulation on the ACh output per impulse at 30 sec after the beginning of continuous stimulation. The filled circles, open squares, and filled triangles represent the data from three different terminals. The cervical sympathetic trunk was stimulated for 30 sec at each frequency. The mean amplitude of twenty EPSPs recorded in each ganglion cell was measured at the end of each period of stimulation. The values have been normalized to the output per impulse at 10 Hz for each terminal. Ten minutes of rest between periods of stimulation. The ACh output per impulse is independent of the frequency of stimulation.

output per impulse (Fig. 7). This agrees with the finding of Birks & MacIntosh (1961), that, over short trains of impulses, the ACh output per volley at frequencies from 4 to 64 Hz was constant in experiments on the perfused ganglion of the cat. The results are also consistent with the conclusion in the preceding paper (Bennett & McLachlan, 1972) that each impulse releases a constant fraction of the amount of transmitter in the store.

The output of ACh was measured during prolonged stimulation at 20 Hz (n = 4) and compared with the results obtained at 10 Hz (n = 15). The pooled results for each frequency showed that there is no difference in the level (0·4) of the maintained output of transmitter relative to the initial maximum output per impulse (Fig. 8). These results also agree with observations of Birks & MacIntosh (1961) for the output of ACh from ganglia

after 20 min of stimulation relative to the initial output at different frequencies.

These results have shown that there is a constant output per impulse, and a constant maintained level proportional to the initial output, at different frequencies of stimulation. It follows that the mean EPSP amplitude must be the same during the steady level of output after prolonged stimulation

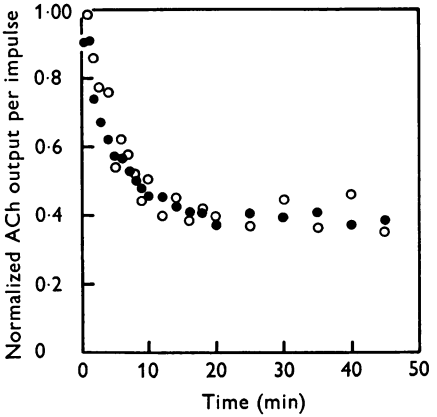

Fig. 8. The effect of frequency of stimulation on the ACh output from preganglionic terminals. The filled circles represent the mean ACh output per impulse for terminals stimulated at 10 Hz (n = 15) determined at intervals after the beginning of stimulation. The open circles give the mean data for terminals stimulated at 20 Hz (n = 4). Standard errors of the means were always less than $\pm$ 0·10 and showed considerable overlap. The maintained level of ACh output during prolonged stimulation is the same proportion (0·4) of the initial maximum output for each frequency.

at different frequencies. The actual quantity of ACh being released per minute at 20 Hz must therefore be twice the amount released at 10 Hz. This implies that synthesis is accelerated proportional to frequency, so as to maintain a constant output per impulse, and that the release of transmitter probably controls the rate at which synthesis proceeds.

DISCUSSION

The output of transmitter substances during long trains of high frequency stimulation has been studied in a number of different nerve terminals, and the pattern of release appears to be the same in all of them. The output declines from an initial high rate over 5–15 min, and then a steady level of output is reached which is maintained (Brown & Feldberg, 1936; Perry, 1953; Straughan, 1960; Birks & MacIntosh, 1961; Paton, 1963; Kopin,

Breese, Krauss & Weise, 1968). The use of radioactive labelling techniques for the marking of stores of transmitter formed prior to stimulation suggests that there is a preferential release of newly synthesized transmitter from nerve terminals (Kopin *et al.* 1968; Collier, 1969; Potter, 1970; Stjärne & Wennmann, 1970). It seems likely then that the phenomena involved in the turnover of transmitter substances during activity are the same in different nerve terminals.

The steady level of maintained output appears to be due to the synthesis of new transmitter as output is not maintained in the absence of synthesis (Birks & MacIntosh, 1961; Bennett & McLachlan, 1972). The rate of synthesis during the stimulation period seems likely to be equal to the rate of maintained output for two reasons. First, the subtraction of the maintained level of output from the over-all curve of released transmitter results in a curve identical to that seen for the depletion of stores determined in the absence of synthesis. This has been shown both electrophysiologically (Fig. 4) and for transmitter overflowing from the perfused ganglion (Birks & MacIntosh, 1961). Secondly, the mean rate of synthesis over 1 hr of stimulation has been calculated from measurements of the total amount of synthesized ACh released by the ganglion during this period (Birks & MacIntosh, 1961). The results gave a value which was the same as the value of the maintained rate of ACh output.

The results discussed above imply that the rate of synthesis is constant throughout the period of stimulation. It has been suggested that the acceleration of synthesis during activity is delayed in onset (Collier, 1969), on the basis that there is an increased proportion of preformed (labelled) ACh released from the perfused ganglion during the first five minutes of stimulation. As a large proportion of the preformed store which synthesis does not replenish is released during this period, this result is not incompatible with a constant rate of synthesis throughout.

The rate of synthesis of new transmitter during activity appears to be dependent on the rate of release. We have shown that the ACh output per impulse is approximately the same during stimulation at 5, 10 and 20 Hz, and this implies that the output per minute is directly proportional to the frequency. As this output per minute represents the rate of synthesis of transmitter at the time when the steady level of release is reached, the rate of synthesis must also be proportional to the frequency of stimulation. The rates of ACh synthesis observed in intact nervous tissue (Banister & Scrase, 1950; Hebb, Krnjević & Silver, 1964) are well below the rates dictated by the activity of the enzyme or the concentration of the substrate (Potter, Glover & Saelens 1968). There is only limited inhibition of choline acetyltransferase by ACh at concentrations approaching those which probably occur in the nerve terminals (Kaita & Goldberg, 1969; Glover &

Potter, 1971). The rate of synthesis of ACh is more likely to be limited by mass action. The release of ACh from the terminal by nerve impulses would result in a decrease in the concentration of ACh in the neuroplasm, and so accelerate synthesis. It seems probable then that this is the mechanism whereby the release of ACh during stimulation governs the rate at which synthesis proceeds.

A model of the storage and synthesis of ACh in preganglionic terminals, derived from the results of the electrophysiological experiments reported in this and the preceding paper (Bennett & McLachlan, 1972), is summarized

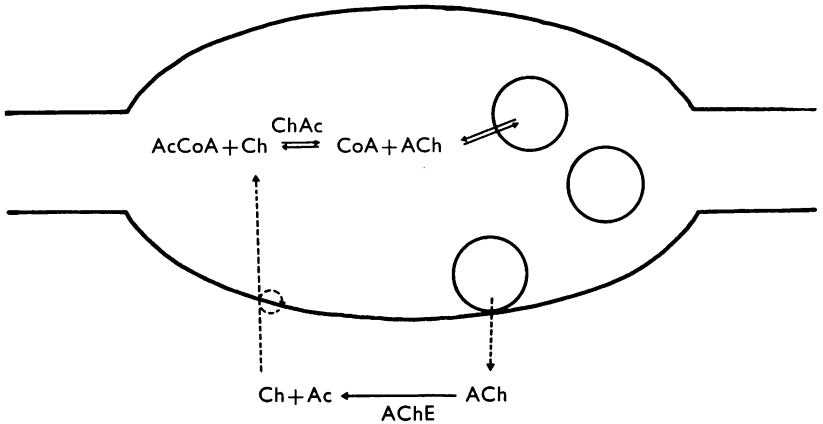

Fig. 9. Diagram of a varicosity of a preganglionic nerve terminal showing the principal features of the storage and synthesis of ACh. Ac, acetyl group; CoA, coenzyme-A; Ch, choline; ChAc, choline acetyltransferase; ACh, acetylcholine; AChE, acetylcholinesterase. ACh is synthesized in the cytoplasm and stored in the synaptic vesicles. The population of vesicles represents the single store from which transmitter is released by nerve impulses. Released ACh is hydrolysed by acetylcholinesterase and the choline from this reaction is taken up into the terminals and used in the resynthesis of ACh. The rate of synthesis is accelerated by the decrease in the concentration of transmitter in the terminal which is a consequence of its release by nerve impulses.

in Fig. 9. During prolonged stimulation, ACh is released from a single store in the terminal, which may be represented by the population of vesicles, and the amount released is probably a constant fraction of the quantity of transmitter in the store at any time. The rate of depletion of this store is dependent on the frequency of stimulation, as the amount of transmitter released by each impulse is constant. The hydrolysis of the transmitter by acetylcholinesterase at the synapse produces free choline which is taken up into the nerve terminal, thus providing an efficient homoeostatic mechanism for the supply of substrate to be used in the synthesis of new ACh. Resyn-

thesis of transmitter occurs at a rate which is dependent on the frequency of stimulation and therefore is probably dependent on the concentration of ACh in the terminal. This synthesis maintains the store at about 40 % of the maximum content during continuous stimulation, which is sufficient to allow a steady release of transmitter over long periods.

We are indebted to Professor W. Burke for helpful comments on this manuscript. We wish to thank Merck, Sharp and Dohme Research Laboratories for supplying us with dihydro-β-erythroidine.

This work was supported by the Australian Research Grants Committee.

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