Brit. J. Pharmacol. (1960), **15**, 417.

THE RELEASE OF ACETYLCHOLINE FROM MAMMALIAN MOTOR NERVE ENDINGS

BY

D. W. STRAUGHAN

From the Department of Pharmacology, King's College, London

(RECEIVED MARCH 16, 1960)

The release of acetylcholine from rat and guinea-pig isolated diaphragm preparations stimulated through the phrenic nerve was optimal at 37° in Krebs solution with 5×10^{-6} neostigmine methylsulphate. The amount of acetylcholine released by a 20 min. tetanus was reduced by cooling. At frequencies of stimulation above 6/sec. the release was less than that predicted. This " failure " was unaffected by the addition of 1×10^{-6} choline. The acetylcholine release declined with continued stimulation at 25/sec. In the absence of nerve stimulation, there was a small continuous resting release of acetylcholine which seemed to originate in the muscle fibres. These results are discussed in the light of current electrophysiological knowledge of the quantal release of acetylcholine.

Nearly a quarter of a century has passed since Dale and his colleagues first showed that acetylcholine was released at the neuromuscular junction by motor nerve stimulation, and could be measured directly in the extracellular fluid if steps were taken to prevent its destruction by cholinesterase (Dale and Feldberg, 1934; Dale, Feldberg, and Vogt, 1936). Since that time it has been generally accepted that acetylcholine is the chemical mediator of nerve impulses at all neuromuscular junctions. Recently, Dale's observation has been confirmed and extended by others, in a perfused muscle preparation (Emmelin and MacIntosh, 1956) and in isolated diaphragm preparations (Burgen, Dickens and Zatman, 1949; Barnes and Duff, 1954; Brownlee, 1957; Brooks, 1954).

The present experiments are concerned with the factors which modify the amounts of the transmitter released from motor nerve endings; and in particular with the effect of changes in the frequency and duration of stimulation, changes in temperature and the presence of choline on acetylcholine release.

METHOD

The Collection of Acetylcholine

The method was based on that of Burgen, Dickens, and Zatman (1949), and has been described in detail by Straughan (1959).

Female albino rats between 200 and 300 g. and female albino guinea-pigs of 200 g. were used. After decapitation, a half diaphragm with its attached phrenic nerve was put in a flat glass diaphragm bath containing between 2.5 and 3.0 ml. Krebs solution. The small volume has the advantage of increasing the acetylcholine concentration in the bath fluid. The upper intercostal margin of the diaphragm was connected by fine stainless wire to a semi-isometric spring lever.

To ensure complete inhibition of cholinesterase, the preparation was allowed to stand for 30 min. in 5×10^{-6} neostigmine methylsulphate before an experiment was begun. The temperature of the muscle was kept constant at $37° \pm 0.25°$ and the phrenic nerve was stimulated for 20 min. periods at a rate of 25/sec. with supramaximal rectangular pulses 0.03 millisecs. in duration, except where indicated.

Less acetylcholine was found to be released by the first than by subsequent periods of stimulation; to offset this a " conditioning " 3 min. period of stimulation was applied to the preparation before each experiment. An intervening rest period of 10 min. was allowed between successive 20 min. periods of stimulation. Under these conditions the preparation could sustain up to eight successive 20 min. periods of stimulation before there was any appreciable fall in the amount of acetylcholine released.

In experiments to determine the recovery of acetylcholine at different concentrations of anticholinesterase, the initial equilibration was made at a low concentration of neostigmine, and the samples from two successive periods of stimulation collected. Subsequent samples were collected at gradually increasing concentrations of neostigmine.

When the effect of changing the frequency of stimulation on release was studied, the frequency of

stimulation in successive 20 min. periods was varied in a random order, so that there were two samples at each frequency. When the effect of duration of tetanus on release was studied, 20 min. periods of stimulation were alternated with 10 min. periods at the same frequency, so that there were three samples for each duration.

In experiments on the effect of temperature on acetylcholine release, the initial equilibration was always at 37° ; samples were either collected at this temperature and at successively lower temperatures ; or the muscle was cooled and samples collected at stages in the course of subsequent rewarming. Samples were withdrawn from the bath within a minute of the end of stimulation, acidified to about *p*H 4 with one drop of 5 N hydrochloric acid and frozen to −20° until required for assay.

The Estimation of Acetylcholine

The activity of small volumes of the bath fluid was matched against known concentrations of acetylcholine in Krebs solution containing neostigmine on the rat blood pressure preparation, as described by Straughan (1958 ; 1959). A male albino rat of 250 g. was anaesthetized with urethane 40 mg./100 g. and sodium pentobarbitone 3 mg./100 g. and allowed to cool to about 28° when a stable long-surviving preparation with a good blood pressure was obtained. Depressor artifacts which are normally a hazard of this preparation were almost completely avoided by carefully immobilizing the venous cannula after cutting the femoral nerves, and by ensuring that not more than 0.3 ml. of fluid was injected at one time. Sensitivity was highest in the winter months, when as little as 0.25 ng. acetylcholine base gave a measurable depressor response in the rat blood pressure.

The preparation also usefully discriminated between biologically active depressor substances ; equidepressor responses were seen with acetylcholine base 1 ng., choline base 10 μg., histamine 0.2 μg., adenosine triphosphate 1 mg., and potassium chloride 1 mg.

The identification of the depressor substance in the bath fluid as acetylcholine was based on the following observations.

1. The relative insensitivity of the assay preparation to other interfering biological substances.

2. The stability of the depressor substance in acid solution, and its destruction by boiling in an alkaline medium.

3. The absence of depressor activity in the bath fluid in the absence of adequate amounts of neostigmine (Table I).

4. The depressor activity of the bath fluid when present was abolished after atropine, and was enhanced by the previous administration of an anticholinesterase to the assay animal.

Krebs solution of the following composition was used (g./litre): NaCl 6.92, KCl 0.354, $CaCl_2$ 0.282, $NaHCO_3$ 2.1, KH_2PO_4 0.162, $MgSO_4,7H_2O$ 0.294, glucose 2.0.

TABLE I

ACETYLCHOLINE RECOVERY AND NEOSTIGMINE CONCENTRATION

Acetylcholine recovery in ng. base after 20 min. stimulation at 25/sec. in different concentrations of neostigmine methylsulphate. Each figure is the mean of two successive periods of stimulation.

Expt. No.	Neostigmine Concentration					
	Absent	1×10^{-7}	5×10^{-7}	1×10^{-6}	5×10^{-6}	1×10^{-5}
1	0	4	—	—	58	—
2	—	1	15	—	33	—
3	—	—	19	37	71	—
4	—	—	—	22	51	51

A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the solution. A correction was applied to the results for the small volume of fluid which remains in the diaphragm bath after draining and dilutes the subsequent addition of known volumes of fluid.

The resting release was measured in most experiments, but the results were not corrected for it, because of the larger errors involved.

RESULTS

The Effect of Anticholinesterase Concentration on the Recovery of Acetylcholine

The results from a number of experiments with the rat phrenic nerve diaphragm preparation showed that when neostigmine was omitted from the bath fluid no acetylcholine could be recovered

TABLE II

RESULTS FROM DIFFERENT DIAPHRAGMS SHOWING HOW THE RECOVERY OF ACETYLCHOLINE VARIES WITH THE CONCENTRATION OF NEOSTIGMINE

Means±S.E. are given. The numbers in parentheses indicate the number of diaphragms from which the means were derived. With each diaphragm two successive 20 min. periods of stimulation at 25/sec. were applied with the stated concentration of neostigmine.

Acetylcholine Recovery	Concentration of Neostigmine Methylsulphate
0 (4)	Absent
2±0·9 (3)	1×10^{-7}
14±1·9 (4)	5×10^{-7}
28±3·4 (6)	1×10^{-6}
52±3·6 (11)	5×10^{-6}
53±0·9 (3)	1×10^{-5}

after a 20 min. period of nerve stimulation at 25/sec. Little acetylcholine was recovered at 1×10^{-7} neostigmine methylsulphate, but at higher concentrations of neostigmine the recovery increased to become maximal at a 5×10^{-6} concentration of neostigmine salt. This is illustrated in Table I.

The collected results from these and other experiments (Table II) show that there was a statistically significant increase in acetylcholine recovery at each of the following concentrations of neostigmine methyl sulphate, 1×10^{-7}, 5×10^{-7}, 1×10^{-6}, and 5×10^{-6}. Thereafter further increase in the neostigmine concentration brought about no statistically significant increase in the recovery of acetylcholine after a period of stimulation. The possibility that neostigmine might increase the amount of transmitter released by each nerve impulse cannot be ignored. But Liley (1956a) has shown that 10^{-6} neostigmine did not alter the rate of the spontaneous quantal discharge of acetylcholine in the rat phrenic nerve diaphragm preparation.

The Effect of Changes in the Frequency of Stimulation on Acetylcholine Release in the Rat and Guinea-pig Diaphragms

The amount of acetylcholine released during a 20 min. collection from the rat diaphragm increased with the frequency of stimulation from 6/sec. to 25/sec. (Table III). But the amount of acetylcholine released did not increase in direct proportion to the number of stimuli applied, so that at 25/sec. it was less than half that expected (Fig. 1). This "failure" to release acetylcholine becomes even more marked at higher frequencies, and though the response of individual diaphragms was variable, in general the amount of acetylcholine released in each 20 min. period was steady within the range 25 to 100/sec. (Fig. 1).

TABLE III

ACETYLCHOLINE RELEASE AT DIFFERENT FREQUENCIES OF STIMULATION IN THE RAT AND GUINEA-PIG DIAPHRAGM PREPARATIONS

The acetylcholine release is given in ng. base per 20 min. stimulation. Each figure is the mean of two periods of stimulation applied at random to the particular diaphragm.

Expt.	Resting	Frequency of Stimulation				
		6/sec.	12/sec.	25/sec.	50/sec.	100/sec.
Rat						
1	5	33	41	59	—	—
2	10	34	43	61	—	—
3	7	—	44	56	38	50
4	10	—	51	69	69	55
5	—	—	25	29	48	55
Guinea-pig						
1	7	28	52	71	—	—
2	9	42	63	86	—	—
3	7	35	39	68	—	—
4	13	—	—	59	58	50
5	7	—	—	77	106	64
6	8	—	—	80	110	72

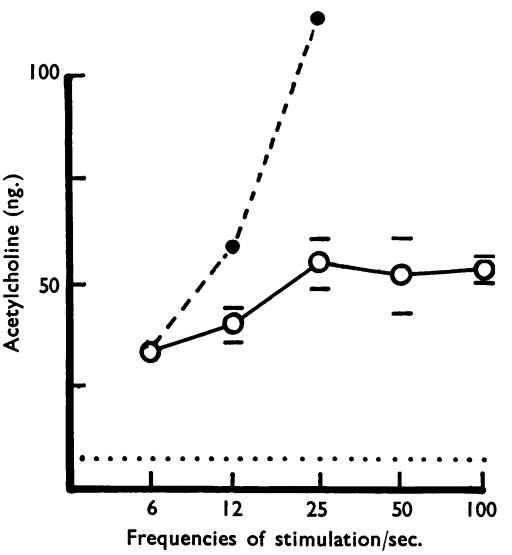

FIG. 1.—Variation in the amount of acetylcholine released from the isolated rat phrenic nerve diaphragm preparation at different rates of stimulation. Ordinate: ng. acetylcholine released per 20 min. period of stimulation in Krebs solution containing 5×10^{-6} neostigmine methylsulphate at 37°. Abscissa: rate of stimulation/sec. O——O : actual acetylcholine release. Each point is the mean ±S.E. from 2 to 6 diaphragms. ●– – –● : predicted acetylcholine release, calculated from the release at a stimulation rate of 6/sec. · · · · · : resting release of acetylcholine/20 min. collection period in the absence of stimulation.

In the guinea-pig diaphragm the amount of acetylcholine released per 20 min. period increased over the range 6 to 50 stimuli/sec. and thereafter declined (Table III and Fig. 2). "Failure" to release acetylcholine was not evident at 12/sec. and there was less "failure" at a frequency of 25/sec. than in the corresponding rat diaphragm, an observation which probably accounts for the relatively greater acetylcholine release in the guinea-pig diaphragm at this frequency.

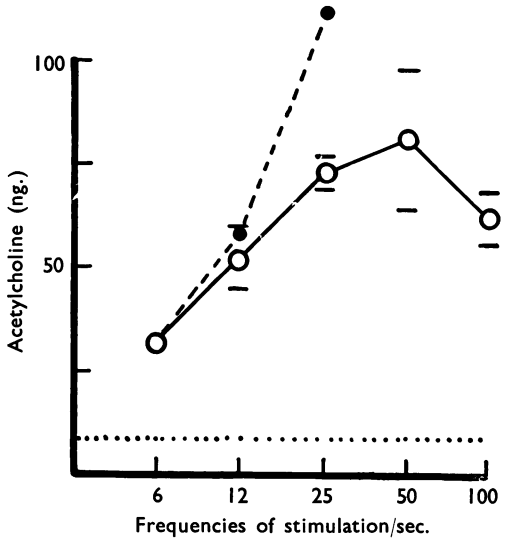

FIG. 2.—Variation in the amount of acetylcholine released from the guinea-pig phrenic nerve diaphragm preparation at different rates of stimulation. Ordinate: ng. acetylcholine base released per 20 min. period of stimulation in Krebs solution containing 5×10^{-6} neostigmine methylsulphate at 37°. Abscissa: rate of stimulation/sec. O——O : actual acetylcholine release. Each point is the mean ±S.E. from 2 to 6 diaphragms. ●- - -● : predicted acetylcholine release, calculated from the release at a stimulation rate of 6/sec. · · · · ·: resting release of acetylcholine 20 min. collection period in the absence of stimulation.

The Influence of the Duration of Stimulation on Acetylcholine Release in the Rat Diaphragm

The observed "failure" in the release of acetylcholine at some frequencies from the rat diaphragm might occur at the beginning of the period of stimulation, or might develop in intensity with continued stimulation. It is not practicable to measure the amount released each minute during a period of stimulation, so the acetylcholine released during 20 min. of stimulation at 25/sec. was compared with the acetylcholine released by 10 min. of continuous stimulation at the same frequency in each diaphragm.

In five experiments the release declined with continued stimulation during 20 min. more than 50% acetylcholine being released by 10 min. of stimulation (Table IV). In only one experiment was the acetylcholine release constant throughout 20 min. of stimulation, which suggested that uniform "failure" was present from the beginning of stimulation.

TABLE IV

DURATION OF STIMULATION AND ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

Acetylcholine release in ng. base. Each figure is the mean of three alternate periods of stimulation at 25/sec. in that experiment.

Expt. No.	Duration of Stimulation		
	(a) 10 min.	(b) 20 min.	$\frac{2a}{b}$%
1	24	40	120
2	39	50	158
3	25	39	128
4	45	54	166
5	23	39	120
6	25	51	98

Perry (1953) has shown that with the cat's superior cervical ganglion perfused with saline there is a sharp decline in acetylcholine release after the first few minutes during prolonged stimulation, even at low frequencies. In this respect acetylcholine release from preganglionic and motor nerve endings is comparable, though the actual mechanism of failure is probably not identical.

The Influence of Temperature Changes on Acetylcholine Release in the Rat Diaphragm

Acetylcholine release from mammalian motor nerve endings has not been directly measured at different temperatures, though there have been indirect observations from electrophysiological studies by Boyd and Martin (1956), Liley (1956a) and Li (1958). The results (Fig. 3 and Table V)

TABLE V

THE EFFECT OF TEMPERATURE ON ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

Release in ng. base per 20 min. at 25/sec. Each figure is the mean of two successive periods of stimulation.

Expt. No.	Temperature			
	20°	30°	37°	40°
1	6	22	31	—
2	9	17	20	—
3	6	26	37	—
4	5	17	22	—
5	—	—	63	69
6	—	—	37	38
7	—	—	63	46
8	—	—	39	29

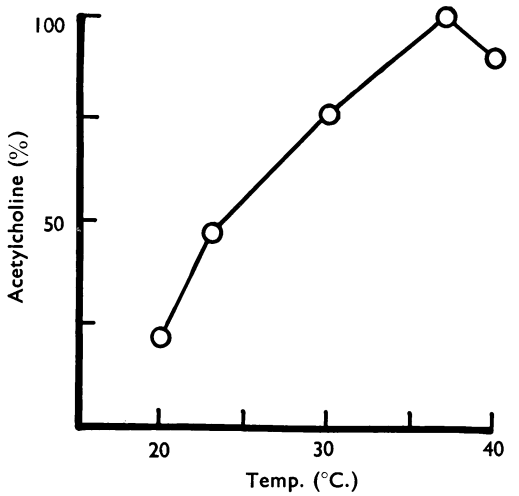

FIG. 3.—Variation in the amount of acetylcholine released from the rat phrenic nerve diaphragm preparation at different temperatures. 20 min. stimulation at 25/sec. in Krebs solution with 5×10^{-6} neostigmine methylsulphate. Ordinate: acetylcholine release as a percentage of the release at 37°. Abscissa: muscle temperature. O——O Each point is the mean release from 2 to 6 diaphragms.

showed that acetylcholine release varies directly with the temperature within the range of 20° to 37° whether acetylcholine release was studied after cooling or warming. The amount released at the two temperatures was significantly different at the 5% level of probability. The mean release of acetylcholine at 40° was less than at 37° (Table V), but this difference was not statistically significant at the 5% level of probability.

To provide a better basis for comparison between different diaphragms, the mean acetylcholine release in each experiment was plotted as a percentage of the release at 37°. The mean results from these different experiments are shown in Fig. 3.

The Effects of Choline on Acetylcholine Release in the Rat Diaphragm

The observation that the rat diaphragm preparation maintains a steady release of acetylcholine in response to repeated periods of stimulation suggests that, apart from the glucose in the Krebs solution, all the elements necessary for acetylcholine synthesis must be available in the preparation itself. Whether the absence of choline from the extracellular fluid imposed any restraint on the synthesis or release of acetylcholine was tested.

The results from four experiments (Table VI) showed that the presence of physiological amounts of choline (Bligh, 1952) brought about no significant increase in the amount of acetylcholine released by two 20 min. periods of stimulation at 25/sec.

TABLE VI

THE EFFECT OF CHOLINE ON ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

Acetylcholine release in ng. base/20 min./25/sec. Each figure is the mean of two successive periods of stimulation.

Expt. No.	Normal Krebs Solution	Krebs Solution + 1×10^{-6} Choline
1	46	60
2	51	41
3	37	32
4	38	42

The Resting Release of Acetylcholine from the Rat Diaphragm in the Absence of Nerve Stimulation

Brooks (1954) showed that there was a resting release of acetylcholine from the guinea-pig diaphragm preparation in the absence of nerve stimulation. A resting release has not yet been described in the isolated rat diaphragm preparation, though it was evident from the results of Burgen and others (1949) that a measurable release of acetylcholine still persisted in the isolated rat diaphragm preparation after paralysis with botulinum toxin; and Brooks (1956) has shown that any release of acetylcholine from motor nerve endings is abolished by this toxin.

The small amounts of acetylcholine involved in this resting release were measured in both rat and guinea-pig diaphragm by collecting samples over 20 min. collection periods at intervals of 1.5 hr. The mean resting release from 19 rat diaphragms was 7 ± 0.6 ng. (S.E.) acetylcholine base per 20 min.; and the mean resting release from 6 guinea-pig diaphragms (Table III) was 8.5 ± 0.6 ng. (S.E.) acetylcholine base. In both preparations the resting release appeared to be continuous and steady over many hours.

DISCUSSION

Acetylcholine Release and Frequency of Nerve Stimulation

If precautions are taken to prevent the destruction of acetylcholine, then variations in the amounts recovered after a period of nerve

stimulation under similar experimental conditions may be thought to have their origin in changes in the amounts set free at the nerve endings. This may occur in five principal ways.

1. By depletion of the stores of acetylcholine in the motor nerve terminals.

2. By altering the probability of quantal discharge in the terminal membrane. Liley (1956b) has shown that prolonged stimulation at high frequencies in the rat diaphragm has this effect, which probably accounts for the observation that less acetylcholine is released by the first period than by subsequent periods of tetanus, under identical conditions in the rat phrenic nerve diaphragm preparation.

3. By acting at some intermediate stage through which the nerve impulse co-ordinates the normal spontaneous discharge of quanta. Calcium and magnesium ions, particularly, appear to affect acetylcholine release by acting at this stage (Liley, 1956b ; Straughan, 1959).

4. By altering the level of depolarization produced by the nerve impulse in the terminals (Liley, 1956c). This is thought to be the mechanism by which small concentrations of procaine selectively reduce acetylcholine release (Straughan, 1959).

5. By altering the number of impulses invading the nerve terminals at particular synapses. Brooks (1954) suggested that high-frequency stimulation of motor nerves could cause a "blocking" reaction in the nerve terminals.

The most likely explanation for the observed "failure" to achieve the predicted release at frequencies above 6/sec. comes from the work of Krnjević and Miledi (1958b). They showed in the rat diaphragm preparation that, with stimulation of sufficient frequency and duration, there was an intermittent all-or-none failure of nerve impulse conduction, probably located in the narrow unmyelinated preterminal part of the nerve fibre. The observed decline in the amount of acetylcholine released during a 20 min. period of nerve stimulation is probably due to the fact that intermittent presynaptic failure is absent initially, and only appears and develops in degree with continued stimulation. The marked variation between different diaphragms in the amounts of acetylcholine which are released by nerve stimulation at rates above 25/sec. is perhaps due to the variation between different diaphragms in the time of onset, and degree of intermittent presynaptic failure.

It seems unlikely that there is any depletion of acetylcholine stores in the terminals during a tetanic period of stimulation even when there is complete neuromuscular block (Krnjević and Miledi, 1958b). The serial decline in the size of the end-plate potential in the curarized rat diaphragm with continued stimulation (Liley, 1956b) is due most likely to a gradual reduction in the acetylcholine sensitivity of the end plate (Krnjević and Miledi, 1958b) and an enhancement of the blocking activity of curare (Chou, 1947).

During a period of tetanus, therefore, there is a tendency for certain nerve impulses to be blocked intermittently, though when a nerve impulse does invade the terminals it will cause the normal amount of acetylcholine to be released from the terminals. This intermittent failure of conduction probably forestalls exhaustion of the terminal stores of acetylcholine (Castillo and Katz, 1956).

The Effects of Temperature on Acetylcholine Release

The most likely explanation for the observed decline in release with cooling over the range 37° to 28° is that cooling acts directly on the terminal membrane and reduces the probability of quantal discharge ; for the results are in fairly close agreement with the figures of Liley (1956a) and Li (1958), who measured the frequency of the spontaneous discharge in the rat diaphragm at these temperatures. These workers showed that with continued cooling to 20° there was a decrease and later an increase in the spontaneous discharge frequency, but Fig. 3 shows that the amount of acetylcholine released by a period of tetanus in fact decreased with continued cooling to 20°. This anomaly would be explained if, at and around room temperature, some further additive process were taking place, such as an increase in intermittent presynaptic failure at room temperature compared with 37° (as proposed by Krnjević, 1958), or possibly a reduced rate of acetylcholine resynthesis with cooling as suggested by Kostial and Vouk (1956) for the cat perfused superior cervical ganglion.

A decline in the amount of acetylcholine released with cooling over this range of temperature occurs in other nerve-muscle preparations. Boyd and Martin (1956) showed, in the curarized tenuissimus of the cat, that the amplitude of the end plate potential declined with cooling, while Bigland, Goetzee, Maclagan and Zaimis (1958) have stated that the blocking activity of curare was reduced by cooling. From their observation that the amplitude of the end plate potential in the cat tenuissimus blocked with magnesium increased on cooling, Boyd and Martin suggested that acetylcholine release was increased by cooling. But this last observation could be attributed more

easily to decreased curare-like and decreased depressant actions on acetylcholine release of magnesium ions at low temperatures.

Estimation of Acetylcholine Release per Synapse in the Rat Diaphragm

The figures in Table III show that about 2.5×10^{-8} g. of acetylcholine base is released from the rat diaphragm preparation by a 20 min. period of stimulation at 6/sec. after corrections are made for the resting release. Intermittent presynaptic failure which is thought to be precipitated by anoxia in the intramuscular portions of the motor nerve is minimal at this rate of stimulation (Krnjević and Miledi, 1958b, 1959); yet the post-synaptic changes observed in this preparation during stimulation at lower rates suggested that the deepest muscle fibres were anoxic (Creese, Hashish and Scholes, 1958). The use of neostigmine in the present experiments caused the rapid development of neuromuscular blockade, so by the end of the first minute of stimulation there was very little tension developed in the muscle. The absence of muscle activity during the greater part of stimulation thus makes it seem unlikely that severe anoxia occurred in the present experiments. If it is accepted that there is little or no "failure" in release at this frequency, then a single motor nerve volley released about 3.5×10^{-12} g. acetylcholine base. Accepting the calculations of Krnjević and Miledi (1958a) that there are 10,000 synapses in the rat diaphragm, it may be calculated that 3.5×10^{-16} g. of acetylcholine base will be released at a single synapse by a single maximal motor nerve volley in the rat diaphragm at 37° in Krebs solution. This is about three times as much as Acheson (1948) calculated was released at a nerve ending in the cat tongue. The difference may be a genuine species difference, or may arise from experimental errors.

Recent work by Liley (1956b) suggests that an individual quantum of acetylcholine in the rat diaphragm is between 1 and 2% of the amount normally released by a single nerve volley at the neuromuscular junction. From the figures presented above it would follow that each quantum would contain about 3.5×10^{-18} g. base or about 15,000 molecules of acetylcholine. This is higher than the revised estimate of 900 molecules per quantum for cat motor nerves given by MacIntosh (1959a).

It seems reasonable to suppose that most of the acetylcholine released at the nerve terminals is recovered under the conditions of the experiments, since the amounts needed to evoke substantial end plate potentials in the rat diaphragm (calculated from Krnjević and Miledi, 1958c) at 37° are only a hundred times greater than the release per synapse calculated from this present work. This discrepancy would probably disappear if it were possible to bring acetylcholine in as close proximity to the end plate as it is when naturally released.

Resting Release of Acetylcholine

The origin of the resting release was not clear; only a very small proportion of it could be accounted for by acetylcholine leakage from the cut end of the phrenic nerve, 0.6 ng. base/20 min. in one experiment; or by the spontaneous quantal discharge of acetylcholine estimated as 0.5 to 0.1 ng. base/20 min. by Straughan (1959). No great difference was apparent either between the resting release from the 7-day chronically denervated side of a diaphragm and the control acutely denervated side, in two preliminary experiments.

It would seem therefore that the resting release of acetylcholine originates in some non-nervous structure, perhaps the muscle fibres themselves. For MacIntosh (1959b) has pointed out that only half the preformed acetylcholine is lost from cat leg muscles after careful denervation.

It seems that the actual mechanisms of acetylcholine release from preganglionic and motor nerve terminals are not identical, and we must be cautious about extending results and knowledge from the one to the other. For instance, the release from motor nerve endings varies with the rate of stimulation in an almost identical manner to that observed in the cat superior cervical ganglion perfused with plasma (Birks and MacIntosh, 1957). But, in the ganglion perfused with saline the release declines sharply after the onset of stimulation so that there is little change in the amounts of acetylcholine released at different frequencies (Perry, 1953). It seems therefore that motor nerve endings maintain a high enough intracellular concentration of acetylcholine precursors to be independent of the organic composition of the extracellular fluid (apart from glucose), while the preganglionic nerve endings need choline and labile plasma factor for the optimal synthesis and release of acetylcholine (Birks and MacIntosh, 1957; MacIntosh, 1959a). It seems uncertain whether the mechanism of failure of acetylcholine release at high frequencies of stimulation in the ganglion perfused with plasma can be explained by intermittent presynaptic failure.

In contrast to motor nerve endings the acetylcholine release from the preganglionic nerve

endings of the cat superior cervical ganglion perfused with saline is not decreased by cooling (Kostial and Vouk, 1956). Thus it would appear that the acetylcholine release mechanism in preganglionic sympathetic nerve endings is not temperature-sensitive like the acetylcholine release mechanism in motor nerve endings. This implies that there is a fundamental difference between the terminal membranes of these two kinds of nerve endings. It will be of interest to see if acetylcholine release from preganglionic nerve endings is temperature-sensitive when the optimal conditions for synthesis and release are provided.

This work was included in a thesis submitted for the degree of Doctor of Philosophy in the University of London, in February, 1959, and part of it was communicated to the British Pharmacological Society in January and July, 1958. I wish to thank Professor G. Brownlee for his help and guidance throughout this work.

References

Acheson, G. H. (1948). *Fed. Proc.*, **7**, 447.
Barnes, J. M., and Duff, J. I. (1954). *Brit. J. Pharmacol.*, **9**, 153.
Bigland, B., Goetzee, B., Maclagan, J., and Zaimis, E. (1958). *J. Physiol. (Lond.)*, **141**, 425.
Birks, R. I., and MacIntosh, F. C. (1957). *Brit. med. Bull.*, **13**, 157.
Bligh, J. (1952). *J. Physiol. (Lond.)*, **117**, 234.
Boyd, I. A., and Martin, A. R. (1956). Ibid., **132**, 74.
Brooks, V. B. (1954). Ibid., **123**, 501.
—— (1956). Ibid., **134**, 264.
Brownlee, G. (1957). Ibid., **136**, 19P.
Burgen, A. S. V., Dickens, F., and Zatman, L. J. (1949). Ibid., **109**, 10.
Castillo, J. del, and Katz, B. (1956). *Progress in Biophysics*, J. A. V. Butler, **6**, 121. London: Pergamon Press.
Chou, T. C. (1947). *Brit. J. Pharmacol.*, **2**, 1.
Creese, R., Hashish, S. E. E., and Scholes, N. W. (1958). *J. Physiol. (Lond.)*, **143**, 307.
Dale, H. H., and Feldberg, W. (1934). Ibid., **81**, 39P.
—— —— and Vogt, M. (1936). Ibid., **86**, 353.
Emmelin, N., and MacIntosh, F. C. (1956). Ibid., **131**, 477.
Kostial, K., and Vouk, V. B. (1956). Ibid., **132**, 239.
Krnjević, K. (1958). Personal communication.
—— and Miledi, R. (1958a). *J. Physiol. (Lond.)*, **140**, 427.
—— —— (1958b). Ibid., **140**, 440.
—— —— (1958c). *Nature, Lond.*, **182**, 805.
—— —— (1959). *J. Physiol. (Lond.)*, **149**, 1.
Li, C.-L. (1958). *Amer. J. Physiol.*, **194**, 200.
Liley, A. W. (1956a). *J. Physiol. (Lond.)*, **132**, 650.
—— (1956b). Ibid., **133**, 571.
—— (1956c). Ibid., **134**, 427.
MacIntosh, F. C. (1959a). *Canad. J. Biochem. Physiol.*, **37**, 343.
—— (1959b). Personal communication.
Perry, W. L. M. (1953). *J. Physiol. (Lond.)*, **119**, 439.
Straughan, D. W. (1958). *J. Pharm. Pharmacol.*, **10**, 783.
—— (1959). Ph.D. thesis, University of London.