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PRESYNAPTIC ACTION OF HEMICHOLINIUM AT THE NEUROMUSCULAR JUNCTION

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The drug hemicholinium no. 3 (HC-3), which was described by Schueler in 1955, has been shown to inhibit the synthesis of acetylcholine (ACh) in intact nervous tissues, and also to block neuromuscular transmission (cf. Schueler, 1960). With low doses of HC-3 the neuromuscular blockade is delayed in onset and occurs only if repetitive nerve stimulation is continued for some time. High doses of the drug, on the other hand, cause a prompt neuromuscular blockade which is independent of the intensity of nerve stimulation. The latter effect is clearly post-synaptic (Reitzel & Long, 1959; Schueler, 1960), and investigations using intracellular recording from the end-plate region of single muscle fibres have been reported to reveal only this curare- or procaine-like post-synaptic activity of the drug (Martin & Orkand, 1960, 1961; Thies & Brooks, 1961).

Experiments using perfused sympathetic ganglia have shown that HC-3 has no obvious effect on the output of ACh until the total quantity of ACh released amounts to about one third of the total initial content of the ganglia (Birks & MacIntosh, 1961; R. Birks and D. M. J. Quastel, unpublished observations). Only subsequently does the rate of ACh release decline, as the stores become emptied, this being related to a parallel decline of the assayed ACh content of the ganglia. A similar depletion of the ACh stocks of intact striated muscle in the presence of HC-3, as a result of stimulation and consequent release of transmitter, has been reported by Thies (1962). This would suggest that the drug acts at the neuromuscular junction in the same way as in sympathetic ganglia and that reduction of ACh release should occur, as a result of store depletion, provided the nerve terminals are stimulated sufficiently in the presence of the drug. To study the action of HC-3 at the neuromuscular junction we have therefore examined the behaviour of end-plate potentials (e.p.p.s) and miniature end-plate potentials (m.e.p.p.s) during and after prolonged activation of the neuromuscular junction, using either nerve stimulation or raised potassium ion concentration. A presynaptic effect of the drug, attributable

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to blockade of acetylcholine synthesis, was readily demonstrable. Perhaps surprisingly, it was manifest as a progressive diminution of quantum size as stimulation was prolonged, without evidence of any effect on the number of quanta released. Some of our results have been briefly presented earlier (Elmqvist, Quastel & Thesleff, 1963).

METHODS

In most experiments the phrenic diaphragm preparation from rats weighing 100–200 g was used; human intercostal muscle obtained during thoracotomy was also sometimes employed, prepared as previously described (Elmqvist, Johns & Thesleff, 1960). Techniques for nerve stimulation and intracellular recording with KCl-filled glass capillary microelectrodes were conventional (Fatt & Katz, 1951). Potentials were recorded both by photographing oscilloscope traces and on paper, using the Mingograf 81, which is an ink-jet recorder with a flat frequency response up to 500 c/s. Simultaneous recording of e.p.p.s and m.e.p.p.s on film and paper showed no difference in amplitude measurements.

The bathing fluid had the following composition (mM): NaCl 135, $NaHCO_3$ 15, Na_2HPO_4 10, KCl 5·0, $CaCl_2$ 2·0, $MgCl_2$ 1·0, glucose 11·0, and was bubbled with 95 % O_2 and 5 % CO_2 with a resulting pH of 7·2–7·4.

Changes in $MgCl_2$ and $CaCl_2$ concentrations were often used to block transmission and were accompanied by appropriate alterations of NaCl concentration to keep the solutions isomolar. When KCl concentration was increased, in order to accelerate spontaneous quantal release of ACh, the NaCl concentration was not changed. The HC-3 preparation used was HC-3 bromide, and was kindly supplied by Dr F. W. Schueler. In all experiments in which stimulation was applied in the presence of HC-3, the drug was present for at least 30 min before starting stimulation.

Experiments were performed usually at a temperature of 32° C, since at higher temperatures failure of nerve conduction tended to be more frequent. All values for e.p.p. and m.e.p.p. amplitudes were corrected for non-linearity of the post-synaptic response according to the formula of Martin (1955) before being used in calculations. A transmitter equilibrium potential of 15 mV inside negative was assumed.

RESULTS

The immediate effect of HC-3 at concentrations of $4–20 \times 10^{-6}$ M was to depress the amplitude of intracellularly recorded miniature end-plate potentials (m.e.p.p.s) in rat diaphragms by 10–60 % depending upon the concentration of HC-3. This effect, presumably post-synaptic (Martin & Orkand, 1960, 1961; Brooks & Thies, 1962), was evidently maximal within a few minutes. If the preparation was then simply allowed to rest in the HC-3-containing solution there was no subsequent change of m.e.p.p. size for periods of at least 4 hr. If the phrenic nerve was stimulated the response of the muscle was a vigorous contraction and with high frequency stimulation the preparation sustained a tetanus well for several minutes. However, as stimulation was continued a progressive block of transmission soon became obvious.

With stimulation at a very low frequency, 0·1/sec, for example, the

onset of blockade was much delayed, but at stimulation rates of 5/sec or greater all fibres in the preparation became blocked within 10–15 min. When the block was complete, intracellular recording from end-plates showed sometimes no end-plate potentials (e.p.p.s) at all but most often there were very small evoked responses with an amplitude of about 1 mV or less, without the large amplitude variation seen in magnesium blocked preparations. Invariably, in fibres where e.p.p.s were recorded no m.e.p.p.s could be found, while at end-plates in which there were no responses to nerve stimulation m.e.p.p.s were not reduced in size and were

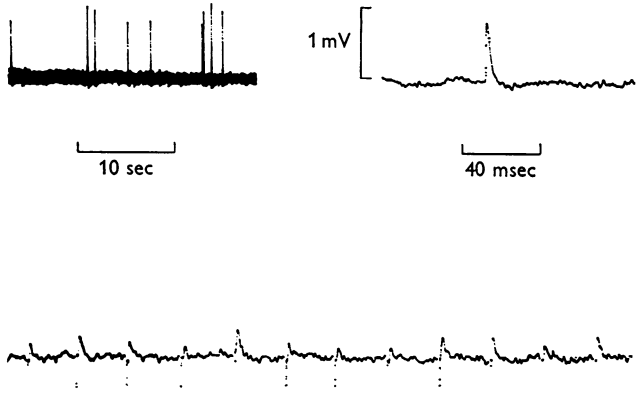

Fig. 1. Rat phrenic-diaphragm preparation in normal bathing solution containing HC-3 4×10^{-6}M. Top records: typical m.e.p.p.s intracellularly recorded at two different paper speeds at a junction, 30 min after the addition of HC-3 and before stimulation was started. Resting potential, 72 mV. Lower record: e.p.p.s recorded at another junction 15 min after starting indirect stimulation at 40/sec. Resting potential, 67 mV.

of normal frequency. Presumably the latter junctions were those to which nerve impulse transmission had failed (Krnjević & Miledi, 1958, 1959). Figure 1 shows typical m.e.p.p.s recorded in a rested preparation after 30 min in the presence of HC-3 (4×10^{-6}M) and e.p.p.s recorded from a fibre in the same preparation after 15 min continuous nerve stimulation at a frequency of 40/sec. Although the small amplitude of the e.p.p.s may in part have been due to low quantum content (del Castillo & Katz, 1954*b*; Brooks & Thies, 1962), the fact that the e.p.p.s in most fibres were smaller than most m.e.p.p.s before stimulation was begun indicates that the quantal units composing the end-plate potentials had become greatly reduced.

Potassium stimulation

Activation of ACh release by raising the concentration of potassium ions was also effective in causing reduction of quantum size, provided HC-3

was present. With this method it was possible to follow the continuous change of quantum size at single junctions. The results of such an experiment are shown in Fig. 2. The addition of 20 mM-KCl to the bathing solution caused a gradual increase of m.e.p.p. frequency from about 2/sec to about 100/sec. This behaviour was also observed in control experiments in

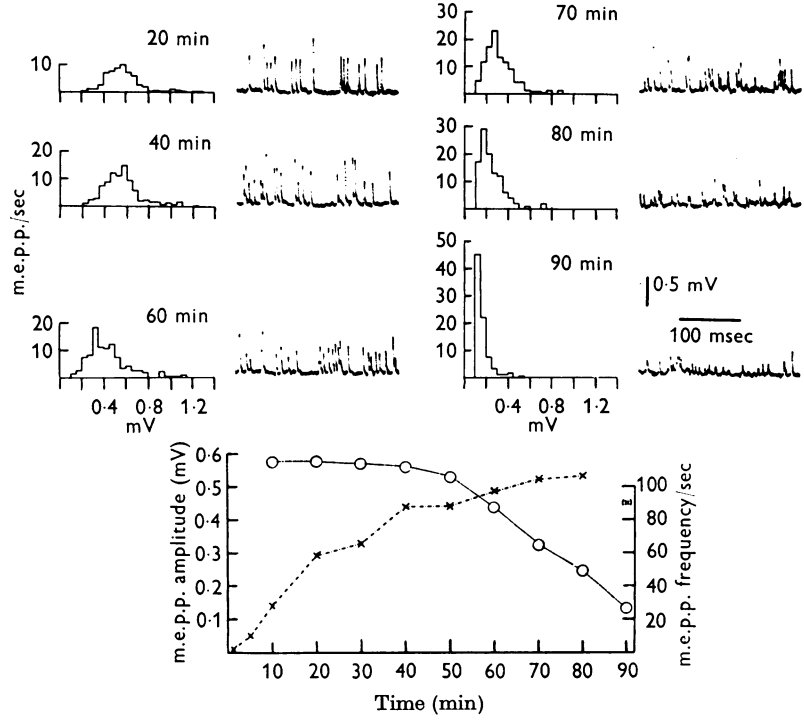

Fig. 2. Intracellularly recorded m.e.p.p.s from a neuromuscular junction in a rat diaphragm preparation. HC-3 4×10^{-6} M was present 30 min before the potassium concentration was increased. The histograms show m.e.p.p. amplitude distributions at different times after the addition of 20 mM-KCl. Examples of the records from which the amplitude distributions are derived are also shown. In the lower curves mean m.e.p.p. amplitude (O) and m.e.p.p. frequency (×) are plotted against time after the addition of KCl. The apparent low value for m.e.p.p. frequency at 90 min, (×), is probably an artifact as some m.e.p.p.s certainly were lost in the base line noise at this time.

which no HC-3 was used. No explanation for the gradual increase in m.e.p.p. frequency was found. It is unlikely to have been caused by a slow increase in potassium ion concentration, as the bath only contained 3 ml. of solution and this was changed at a rate of about 1 ml./min, and it may be noted that the muscle membrane depolarization was completed in

about 10 min after switching to the solution containing 20 mM-KCl. In the presence of HC-3 the amplitude of the m.e.p.p.s did not remain constant but after some time gradually diminished, until eventually they became so small as to be lost in the base line noise. This phenomenon did not take place in the absence of the drug.

With raised potassium ion concentration, as with nerve stimulation, the extent of the depression of m.e.p.p. size and the speed at which it took place were related not only to the HC-3 concentration but also to the intensity of stimulation. For example, with 2×10^{-6}M-HC-3, release of quanta at a rate of about 100/sec, by 20 mM-KCl, had no apparent effect on m.e.p.p. amplitude; with double this concentration or more of HC-3 after 90 min of stimulation with 20 mM-KCl it was difficult to find m.e.p.p.s at the end-plates and where found they were very small (Table 1). Conversely, with 40 mM-KCl, which produced a m.e.p.p. frequency so high as to be unmeasurable (more than 500/sec), m.e.p.p.s soon became so small that no junctional activity at all could be recorded, even with the HC-3 at a concentration of 2×10^{-6}M.

Nerve stimulation

By blocking neuromuscular transmission with raised Mg^{2+} and/or lowered Ca^{2+} concentrations, it was possible to follow simultaneously e.p.p. and m.e.p.p. sizes during prolonged stimulation of the motor nerve in the presence of HC-3. The results of one of these experiments are shown in Fig. 3. In this experiment HC-3 was used at a concentration of 4×10^{-6}M, a concentration which seemed to have a virtually complete presynaptic effect but little enough post-synaptic action that m.e.p.p.s could still be recorded when they became less than half the initial size. E.p.p. amplitude increased for about 20 min after starting stimulation. This was evidently due to a rise in quantum content since m.e.p.p.s, after a latency of about 5 min, progressively declined in size.

The histograms of m.e.p.p. amplitude distributions were similar to those found with potassium stimulation (Fig. 2). They show not only the gradual change in mean size but also that there was an increase in relative amplitude variation. This is shown in more detail in Fig. 4*A*. In this experiment a fourfold reduction in m.e.p.p. size was associated with a doubling of the coefficient of variation. Only a small part of the increased variability of the m.e.p.p.s could have been due to measurement error resulting from noise.

As shown by del Castillo & Katz (1954*a*) and confirmed by Liley (1956) and Boyd & Martin (1956) the distribution of the number of quanta in e.p.p.s follows a Poisson distribution when neuromuscular blockade is effected by a raised magnesium concentration. On the assumption of

a Poisson distribution the following theoretical relationship may be derived:

$$q = \text{mean quantum} + \frac{\text{variance of quanta}}{\text{mean quantum}}.$$

where q is defined as the quotient (variance of e.p.p.s)/(mean e.p.p.) (cf. appendix by Brown in Edwards & Ikeda, 1962; Blackman, Ginsborg & Ray, 1963). If the quanta in e.p.p.s were identical with the m.e.p.p.s, then the following relation would hold:

$$q = \text{mean m.e.p.p.} + \frac{\text{variance of m.e.p.p.s}}{\text{mean m.e.p.p.}}.$$

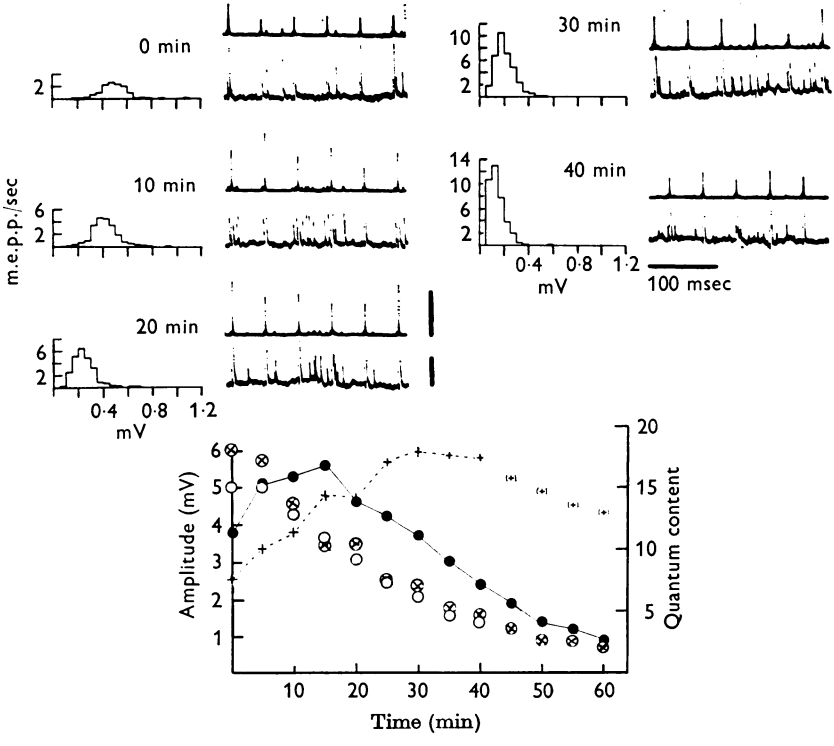

Fig. 3. Intracellularly recorded e.p.p.s and m.e.p.p.s from a magnesium-blocked rat phrenic-diaphragm preparation (Mg^{2+} 15 mM, Ca^{2+} 3 mM). HC-3 4×10^{-6} M was present 30 min before nerve stimulation (10·5/sec) was begun. The histograms show m.e.p.p. amplitude distributions at different times after stimulation was started. Examples of the records, at two different amplifications, from which the data were obtained are also shown. In the lower curves mean e.p.p. amplitude (●), mean m.e.p.p. amplitude (○), calculated q (⊗) and mean quantum content of the e.p.p.s (+) calculated as (mean e.p.p. amplitude)/(mean m.e.p.p. amplitude) and when m.e.p.p.s became too small to measure as (mean e.p.p. amplitude)/q, are plotted against time after starting the indirect stimulation. M.e.p.p.s and q are plotted at 10 times their actual value.

Figure 4*B* shows the observed correlation between these two values, the data being from the same experiment as illustrated in Fig. 3. At 5 min intervals samples of 200 sequential e.p.p.s were taken and divided into five equal groups from each of which an estimate of q was obtained. From each group of five estimates of q the mean and standard errors plotted in the graph were calculated. The values for mean m.e.p.p. and variance of

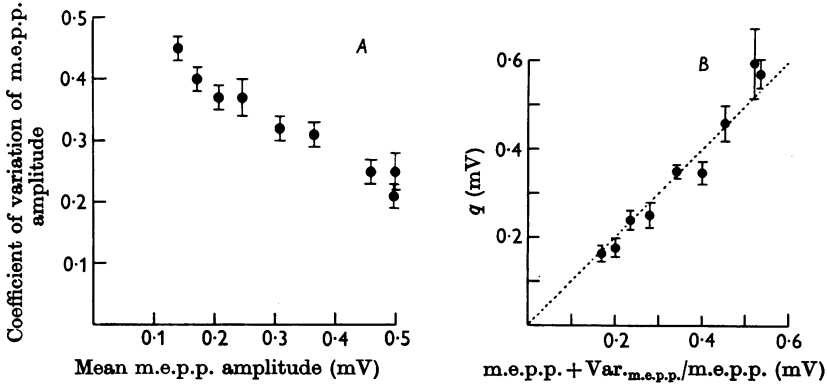

Fig. 4. Data from the same experiment shown in Fig. 3. *A*. Coefficient of variation of m.e.p.p. amplitude plotted against mean m.e.p.p. amplitude. *B*. q calculated as (variance of e.p.p.s)/(mean e.p.p.) plotted against (mean m.e.p.p.) + (variance of m.e.p.p.)/(mean m.e.p.p.) (see text). Interrupted line shows perfect agreement between the two values. Vertical lines represent $\pm$ S.E. of q.

m.e.p.p. were derived from samples of about 200 m.e.p.p.s taken at corresponding times. Despite the high sampling error of q, a very good agreement is apparent. It may be concluded that the quantal components of the e.p.p.s were the same as the m.e.p.p.s even when they became reduced as a result of stimulation in the presence of HC-3. In Fig. 3 the values plotted for quantum content after m.e.p.p.s became too small to measure were derived as (e.p.p. size)/q without compensation for variation of quantum size. A continued relative increase of the variation would account completely for the apparent decline of quantum content.

When neuromuscular transmission is blocked by tubocurarine, m.e.p.p.s cannot of course be observed, and even in the absence of HC-3 there is a progressive reduction in e.p.p. amplitude during prolonged stimulation at frequencies of 5/sec or greater (Brooks & Thies, 1962). However, it was possible to demonstrate the effect of HC-3 in curare blocked preparations, by using stimulation frequencies so low that quantum content was well maintained. With stimulation at 1/sec, for example, e.p.p.s progressively declined in amplitude in the presence of 4×10^{-6}M-HC-3 (Fig. 7*C*). Because of the relatively high contribution of noise to the measured variance of the e.p.p.s it was usually difficult to demonstrate unequivocally that under

these conditions the effect of HC-3 was on quantum size rather than on quantum content.

Post-synaptic sensitivity

To test whether changes of post-synaptic chemosensitivity could have been responsible for the reduction in quantum size produced by activation of transmitter release in the presence of HC-3, experiments were made using bath application of carbachol. Figure 5 shows the results of injecting carbachol into the bath shortly after starting stimulation at 40/sec of a magnesium-blocked preparation, in the presence of 4×10^{-6}M-HC-3, and

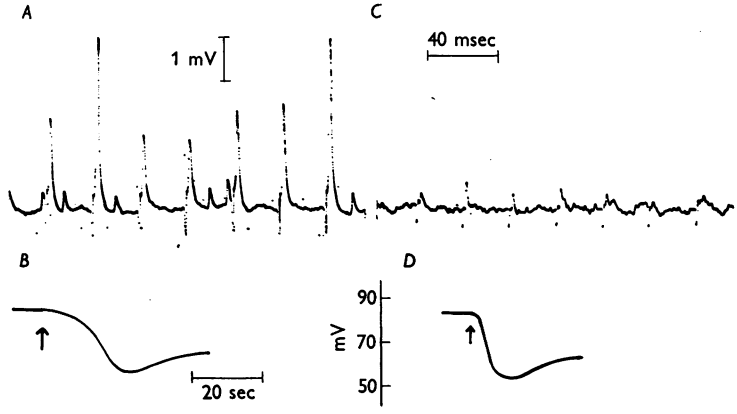

Fig. 5. Rat phrenic diaphragm preparation blocked by 8 mM-Mg^{2+} with HC-3 4×10^{-6}M present. Continuous indirect stimulation 40/sec. Upper record: intracellular records of e.p.p.s and m.e.p.p.s. Lower record: membrane potential graphed on a slower time scale. At arrows carbachol was injected into the bath to give a final concentration of 5×10^{-5} g/ml. Record *A* was obtained immediately before the first injection of carbachol. The depolarization it caused is depicted in *B*. Twenty minutes later record *C* was obtained and then the same amount of carbachol was again applied causing the membrane depolarization depicted in *D*.

20 min later, when the e.p.p.s had become small. The depolarization was about the same at both times. The dose of carbachol (5×10^{-5} g/ml.) was chosen as it gave rise to depolarizations great enough to be clearly measurable but still submaximal, as greater doses of carbachol gave much greater depolarizations. Because nerve impulse propagation usually failed in many nerve fibres in a preparation, with the consequence that the quanta did not become small at all end-plates, this test could only be applied to those fibres in which the rundown had been followed, i.e. one fibre in each preparation; however, the result could be considered valid only if the membrane potential was the same before both tests. For these reasons an alternative procedure was preferred. In preparations exposed to raised KCl concentrations m.e.p.p.s became small at all end-plates. It was then

valid to test for an effect on post-synaptic sensitivity by recording membrane potentials at end-plate regions before and after changing the bathing solution to one containing carbachol, the experiment being done shortly after the addition of KCl and again when the m.e.p.p.s had become small. Previous experiments showed that this is a sensitive method for detecting a depression of post-synaptic sensitivity (Elmqvist, Hofmann, Kugelberg & Quastel, 1964).

The pooled results of two such experiments are shown in Table 1. The response to carbachol was evidently the same shortly after starting stimulation with potassium and 2 hr later, when m.e.p.p.s were smaller by at least 65%.

TABLE 1. End-plate depolarization caused by carbachol (5×10^{-5} g/ml.) in the presence of 20 mM-KCl and 4×10^{-6} M-HC-3

Time (min)	Carbachol	M.e.p.p. amplitude (μV ± S.E. of mean)	Membrane potential (mV ± S.E. of mean)
1–15	–	239 ± 14 (50)*	57·1 ± 0·4 (56)
16–22	+	—	45·8 ± 0·4 (65)
90–115	–	75 ± 8 (39)*	57·5 ± 0·5 (79)
116–122	+	—	46·0 ± 0·4 (67)

Number of fibres in parentheses.

* In the other 6 and 40 fibres m.e.p.p. could not be seen. In most fibres only m.e.p.p.s less than 50 μV in size could not have been distinguished because of noise.

The preparations were in HC-3 4×10^{-6} M for 30 min before the application of KCl. Carbachol was present only during periods of time 15–22 min and 115–122 min after the start of the experiment.

Time course of quantum size change and estimates of presynaptic store

In view of the above results it must be considered highly unlikely that the progressive change in m.e.p.p. and e.p.p. amplitudes could have been due to a change of post-synaptic sensitivity. It would seem more likely that the phenomenon is related to the ability of HC-3 to block ACh synthesis, quanta becoming smaller as the presynaptic store of transmitter becomes depleted. If this was the case then one would expect that quantum size should diminish more rapidly the faster the transmitter happened to be released. Conversely, the time taken for a certain reduction of quantum size should be inversely proportional to the rate of release. Figure 6 shows the relation between the inverse of the time taken for 50% reduction of quantum size and the average rate of quantal release during this time in fourteen fibres from rat diaphragm preparations. The correlation was highly significant ($r = 0{\cdot}952$, $P < 0{\cdot}001$). The total number of quanta which had been released when quantum size was reduced by 50% was not very different from one fibre to another; the mean was 243,000, S.D. 54,000.

In ten fibres in which quantum size could be followed down to 25% of the initial the correlation between the inverse of the time taken and the rate of quantal release was again highly significant ($r = 0{\cdot}935$, $P < 0{\cdot}001$). The number of quanta released was 366,000 ± 92,000 (mean ± S.D.). There was no correlation between the number of quanta released up to the time of 50 or 75% reduction in quantum size and either the rate of release or the time necessary. This suggests that the blockade of ACh synthesis in these experiments was virtually complete, or that residual synthesis was counterbalanced by an undetected loss of ACh from the nerve endings.

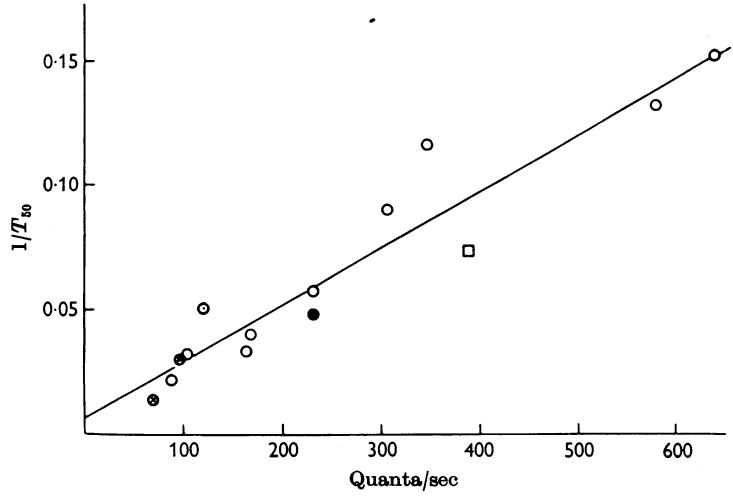

Fig. 6. Correlation between the average rate of quantal release and the inverse of the time taken for a 50% reduction of quantum size ($1/T_{50}$) after block of ACh synthesis at fourteen junctions in rat phrenic diaphragms. ○, Nerve stimulation; n-m transmission blocked by magnesium HC-3 10^{-6} M. ●, Nerve stimulation; n-m transmission blocked by magnesium HC-3 4×10^{-6} M. ⊙, Nerve stimulation; n-m transmission blocked by dTC 7×10^{-7} g/ml. HC-3 4×10^{-6} M. ⊗ KCl (20 mM) stimulation; HC-3 4×10^{-6} M. □, Nerve stimulation; n-m transmission blocked by magnesium; triethylcholine 3×10^{-4} g/ml. The line drawn is fitted by the method of least squares ($r = 0{\cdot}952$, $P < 0{\cdot}001$).

It may be assumed that the size of a m.e.p.p. or an e.p.p., compensated for non-linearity of the end-plate response as the depolarization approaches the equilibrium potential for the transmitter (Martin, 1955), is a linear measure of the amount of transmitter released from the nerve terminal. Then, in these experiments in which synthesis was apparently negligible, the amount of ACh in a nerve terminal at any time was the amount initially present minus the sum of the amounts of ACh released to elicit all e.p.p.s and m.e.p.p.s recorded after the application of HC-3. By extrapolation of the curve of quantum size against the sum of what had come

out from a nerve ending it should therefore be possible to estimate the amount of releasable transmitter which had been present initially.

Figure 7*A* shows, plotted in this way, the results of the experiment presented previously in Fig. 2. The extrapolation of m.e.p.p. size gives a total initial content of $2{\cdot}1 \times 10^5$ mV or 370,000 times the amount in a single quantum of initial size. The extrapolation of e.p.p. amplitudes should be equally valid provided their diminution can be attributed to change in quantum size. This was generally the case in experiments using magnesium

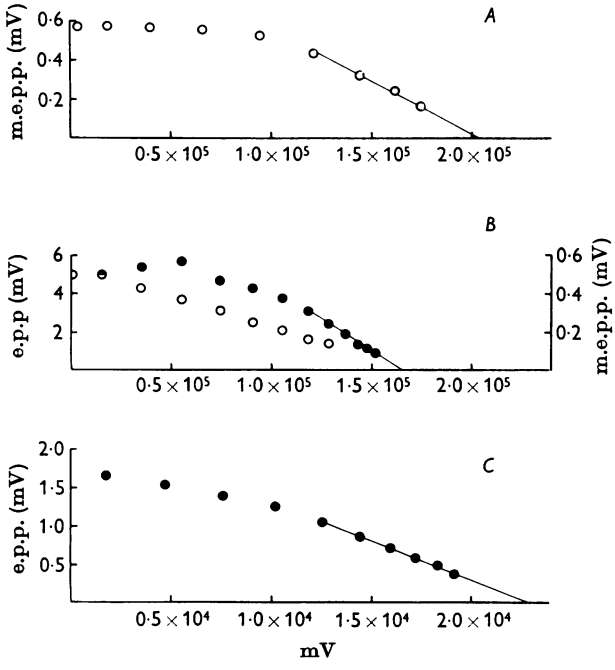

Fig. 7. E.p.p. and m.e.p.p. amplitude plotted against the sum of e.p.p.s and m.e.p.p.s previously recorded. *A*. M.e.p.p.s released by KCl (20 mM) in the presence of HC-3 4×10^{-6} M (the same experiment as in Fig. 2). *B*. Indirect stimulation (10·5/sec) in the presence of 15 mM Ca^{2+}, 3 mM Mg^{2+}, and 4×10^{-6} M HC-3 (data from the same experiment as in Fig. 3). *C*. Indirect stimulation (1·1/sec) in the presence of 6×10^{-7} g/ml. dTC and 10^{-5} M HC-3. The terminal parts of the curves have been extrapolated to the abscissae by eye.

to block neuromuscular transmission, or, if tubocurarine was used, when stimulation was at a frequency of less than 5/sec. Because of the difficulties involved in measuring quantum size when m.e.p.p.s were close to noise level, or quantum size became highly variable, this was usually preferred. Examples are shown in Fig. 7*B* and *C*, the data in the former being from the experiment also shown previously in Fig .3, from a magnesium-blocked preparation. The estimate of the initial store, $1{\cdot}7 \times 10^5$ mV, corresponds to

330,000 times the amount in a quantum initially. In the experiment shown in Fig. 7*C*, in which tubocurarine 10^{-6} g/ml. was used to block transmission, the quantum content derived from the coefficient of variation of the e.p.p.s appeared to be steady throughout the experiment at about 120 quanta per impulse. The total initial store, $2{\cdot}3 \times 10^4$ mV, therefore corresponded to 170,000 initial quanta.

In this way estimates of the total store were obtained for fourteen fibres. The average was $271{,}000 \pm 71{,}000$ (mean $\pm$ S.D.) expressed in terms of initial quantum size. This mode of expression makes the estimates independent of differences in post-synaptic sensitivity depending upon muscle fibre dimensions or membrane potential (Katz & Thesleff, 1957), or the presence of post-synaptically depressing substances such as Mg^{2+}, K^+, tubocurarine or HC-3.

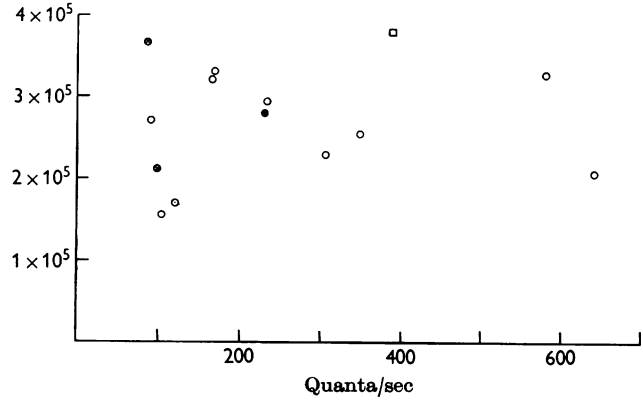

Fig. 8. Relation between the estimated size of the presynaptic store of ACh and the rate of release of transmitter ($r = 0{\cdot}115$; $0{\cdot}7 > P > 0{\cdot}6$). The symbols are the same as in Fig. 6.

The store estimates appeared to be quite independent of how rapidly the nerve terminals were emptied (Fig. 8); the coefficient of correlation of store estimates against rate of release (r) was 0·115 ($0{\cdot}7 > P > 0{\cdot}6$). The same method of determining presynaptic stores has also been used on human intercostal muscle, with similar results (Elmqvist *et al.* 1964).

Recovery of quantum size

The assumption of complete blockade of ACh synthesis by the concentrations of HC-3 employed is probably not completely justified. It is true that under favourable recording conditions a depression of e.p.p. and quantum size to less than 10% of the initial could usually be observed clearly, which would indicate an inhibition of synthesis to less than 10% of

that necessary to sustain release at a rate far below that which can be elicited with vigorous stimulation in normal bathing solution. That slow release of transmitter did not result in any increase of the apparent initial store could also indicate that synthesis was not significant. However, in some experiments in which it was possible to rest the preparation for a time after a long period of stimulation and then again record e.p.p.s the result was some recovery of e.p.p. and quantum size following the period of rest. Figure 9 shows the data from such an experiment, using human

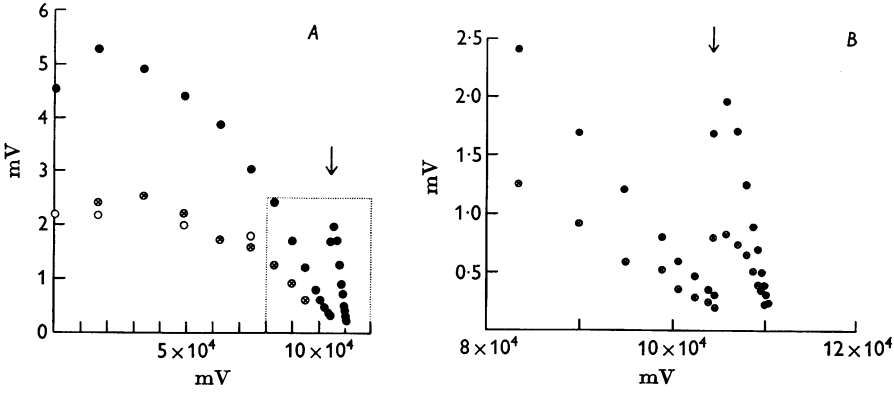

Fig. 9. Human intercostal muscle neuromuscular transmission blocked by Mg^{2+} 8 mM and Ca^{2+} 1·33 mM in the presence of HC-3 10^{-5} M. Nerve stimulation 11·1/sec. At arrow, preparation was rested for 15 min. E.p.p. (●), m.e.p.p. (○) and estimated quantum size (⊗) plotted against the sum of previously recorded e.p.p.s and m.e.p.p.s. M.e.p.p. and quantum size plotted as 10 times their actual value. The portion enclosed by dotted line in *A* is shown enlarged in *B*.

muscle, where m.e.p.p. frequency is very low and stopping nerve stimulation effectively terminates release. Extrapolation of the final parts of the two curves suggests that there was a small increment in the presynaptic store during the 15 min rest. An alternative explanation is that this extra transmitter was already present in the nerve terminal and made available for release during the rest period, rather than newly synthesized. The striking feature is the great rise in quantum size resulting from such a small increase of available ACh and its extremely rapid rundown with renewed release. It must be concluded that when synthesis of ACh is inhibited the relation between quantum size and the amount of transmitter in the nerve terminal is not a simple one, and depends upon the previous history of stimulation and rest.

Recovery of m.e.p.p. amplitude after rundown with KCl stimulation, in the continued presence of HC-3 ($4–20 \times 10^{-6}$ M) could also be observed, although it was too slow to follow at single junctions. When a strip of rat

diaphragm, which had been immersed for 2 hr in 20 mM-KCl, and 2×10^{-5}M-HC-3, was placed in a solution still containing 2×10^{-5}M-HC-3, but with only 5 mM-KCl, m.e.p.p. continued to be indetectable for 2 hr. After 4 hr, however, small m.e.p.p.s could be found at most end-plates. With smaller doses of HC-3 ($2–4 \times 10^{-6}$M), recovery was hardly noticeably faster, it being a matter of hours before m.e.p.p.s regained their full size.

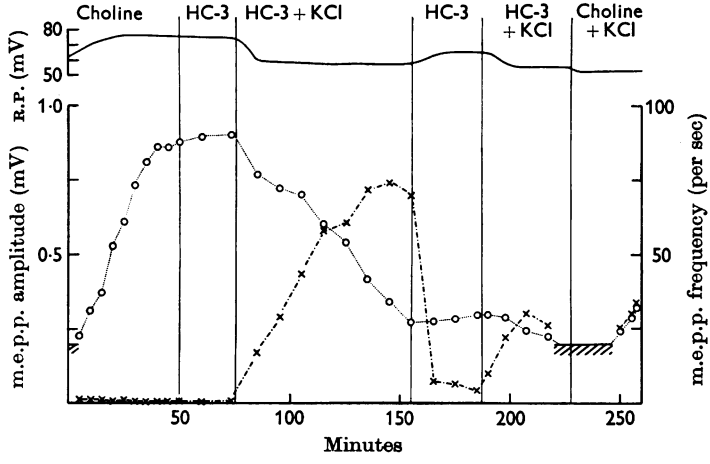

Fig. 10. Plots of membrane potential (R.P.), m.e.p.p. amplitude (○), and frequency (×) continuously recorded at a neuromuscular junction in a rat diaphragm preparation previously soaked for 4 hr in a solution containing HC-3 and 20 mM-KCl to run down quantum size. During the experiment the bathing solution was changed as listed at top of diaphragm. The concentration used were HC-3 4×10^{-6}M, choline 7×10^{-5}M and KCl 20 mM. //// indicates that m.e.p.p.s were lost in the base line noise.

It was not possible in these experiments unequivocally to demonstrate any action of choline to antagonize the effect of HC-3, but in some cases it appeared that recovery of m.e.p.p. size was a little faster in the presence of added choline (7×10^{-5}M). Recovery in the absence of HC-3 was faster than if HC-3 was still present, and if choline was added it was faster still. Figure 10 shows the time course of recovery of m.e.p.p. amplitude, after transfer to a HC-3-free solution containing 7×10^{-5}M choline, at a junction in a rat diaphragm whose quantal size had previously been reduced by immersion in 20 mM-KCl and 4×10^{-6}M-HC-3 for about 4 hr. Renewed stimulation by KCl in the presence of HC-3 again produced a reduction in m.e.p.p. amplitude and there was very little, if any, recovery when the KCl alone was removed. Judging from such experiments it seems likely that full restoration of the presynaptic store is possible after depletion, although this is perhaps not necessary for full recovery of quantum size.

Choline deficiency

In perfused cat ganglia it has been shown that it is necessary to supplement the perfusion fluid with choline to avoid a decreasing output of ACh with prolonged stimulation even in the absence of HC-3 (Birks & MacIntosh, 1961). In experiments with rat-diaphragm preparation we have not observed any change in quantum size in the absence of HC-3, even with prolonged high frequency stimulation. However, we have sometimes found a reduction of quantum size by as much as 60% with prolonged high-frequency indirect stimulation of magnesium-blocked human intercostal muscle preparations. When the bathing fluid was supplemented with choline ($3{\cdot}5 \times 10^{-5}$ M) the phenomenon was never observed. The difference between rat diaphragm and human intercostal muscle may be related to the fact that the human muscle preparations consisted of small fascicles of only about 60–100 fibres spread out in a thin layer only a few fibres thick and these fibres were presumably better washed by the bathing fluid than even the surface fibres in rat diaphragms.

Triethylcholine

The triethyl analogue of choline (triethylcholine) has pharmacological actions resembling those of HC-3 (Bowman & Rand, 1961; Bowman, Hemsworth & Rand, 1962). It produces a delayed neuromuscular blockade, the onset and degree of which depends on the rate of nerve stimulation. Like HC-3 it has been shown to block ACh synthesis in intact nervous tissue (Bull & Hemsworth, 1963). Figure 11 shows the result of an experi-

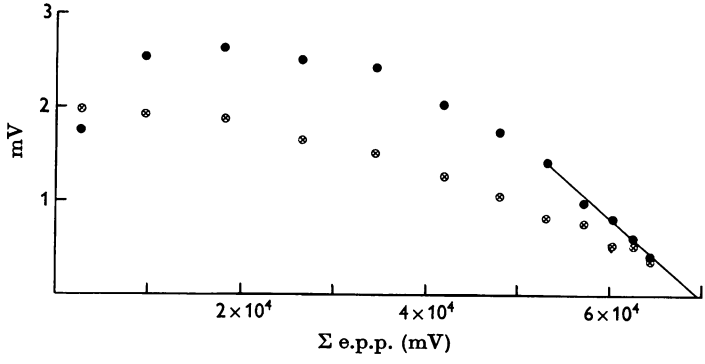

Fig. 11. Plots of e.p.p. amplitude (●) and estimated quantum size (⊗) against the sum of foregoing e.p.p.s, as in Fig. 7, obtained from a rat phrenic–diaphragm neuromuscular junction indirectly stimulated at a frequency of 27/sec. Mg^{2+} 12 mM and Ca^{2+} 1·33 mM were used to block neuromuscular transmission. Triethylcholine 3×10^{-4} g/ml. was present to inhibit ACh synthesis.

ment in which e.p.p.s were followed for about 25 min during continuous nerve stimulation at about 20/sec in the presence of $1{\cdot}66 \times 10^{-3}$M-triethylcholine and with raised magnesium-ion concentration to block transmission. The results were very much the same as those obtained with HC-3; e.p.p.s declined in amplitude, the change evidently being due to decreasing quantum size. Estimation of the presynaptic store of releasable ACh in this experiment gave a value corresponding to about 350,000 quanta of initial size, which is within the range indicated by the HC-3 experiments. The result of this experiment has therefore been included with the HC-3 experiments in Figs. 6 and 8 and in the average of the store estimates already presented.

DISCUSSION

The main interest of the results obtained from these experiments using HC-3 is of course the light they may throw on some of the presynaptic mechanisms involved in synaptic transmission.

To begin with it seems possible to use the results to estimate the size of the presynaptic store of releasable transmitter. Experimentally this is rather simple; one need only follow, at a junction, e.p.p. or m.e.p.p. size until it becomes very small, and have some reasonable estimate of quantum size at the time when stimulation was begun. However, there are probably some sources of regular error in such estimates. The first is the possibility of residual synthesis. Although we could usually not see any eventual levelling off of quantum size as stimulation was continued it is possible that this did occur at a low level, perhaps 5–10% of the initial. If there were residual synthesis to about this extent our estimates of the presynaptic store would be too high by about 10–20%. On the other hand, if there was release of ACh from the nerve terminals in a form other than that recordable as m.e.p.p.s or e.p.p.s, as suggested by assays of ACh released from resting rat diaphragm (Krnjević & Mitchell, 1961), our estimates would be too low. We have also neglected the release of ACh as quanta between the application of HC-3 and the starting of stimulation and recording, which was usually 30–60 min. This would lead to an underestimate of the size of the store by at most 10%. It would therefore seem unlikely that the estimates of the releasable store are in error by more than 25% in either direction.

Since the total amount of bound ACh in a rat hemidiaphragm is about 300 pmole and in the hemidiaphragm there are about 10,000 nerve endings, the total ACh per ending is about $1{\cdot}8 \times 10^{10}$ molecules (Krnjević & Mitchell, 1961). The average size of the presynaptic store in rat phrenic nerve terminals, according to the experiments with HC-3 and with triethylcholine, corresponded to 270,000 initial or full quanta. If all the bound ACh in a nerve-ending is releasable this gives a value of 76,000 molecules of ACh in

a normal quantum. If as in the cat superior cervical ganglion, only 85% of the total bound ACh is releasable (Birks & MacIntosh, 1961), a value of 57,000 molecules of ACh per quantum is obtained. These figures may be compared with the figure of 62,000 molecules which is the most that could be contained in a vesicle of 500 Å diameter (Canepa, 1964).

These values are in agreement with Krnjević & Mitchell's (1961) finding of 0·12 pmole per impulse in a rat hemidiaphragm stimulated at 1–5/sec, if the number of quanta released per impulse at this stimulation frequency is about 100 (Liley, 1956). These figures give a value of 72,000 molecules per quantum.

The change of quantum size consequent upon stimulation appeared not only in the presence of HC-3 but also in the presence of triethylcholine and sometimes in the absence of any drug, presumably because of choline deficiency. It was found that after the quanta had been run down by stimulation there was an increase of quantum size with rest, probably more than could be accounted for simply by synthesis. Therefore it can be concluded that both the amount of ACh in the nerve terminal and its distribution determine quantum size, hemicholinium or triethylcholine making this relation demonstrable.

It has been suggested that triethylcholine and HC-3 might in themselves or after acetylation compete with ACh for presynaptic storage sites, later being released by the nerve terminals as false transmitters (Burgen, Burke & Desbarats-Schönbaum, 1956; Bowman & Rand, 1961; MacIntosh, 1961). Our results cannot be said to support this notion, but, on the other hand, they do not exclude it. The lack of any detectable change in post-synaptic sensitivity, at a time when, by this hypothesis, the post-synaptic membrane would be heavily bombarded with depressant agents, speaks somewhat against it; the other data could well be interpreted to fit the hypothesis. The simpler hypothesis, that the decrease in quantum size produced by stimulation in the presence of these drugs is the result of their inhibiting action of ACh synthesis, would appear to be completely sufficient to explain the results and is therefore preferable.

The question naturally arises what models of ACh storage in the nerve terminal are compatible with the data. To begin with, the concept of a presynaptic store of ACh contained solely in rather uniform quanta, which stay unchanged from the time of formation until the moment of release, can be excluded. This model predicts that the size of the newly formed quanta should be determined by the rate of synthesis. If synthesis is completely blocked the nerve terminal should put out at first full quanta and then only empty ones. With a lesser degree of inhibition of synthesis, the quanta put out would either be full sized or small, the size of the small ones being constant and only dependent on the rate of synthesis. All

quanta of less than initial size, by this model, could necessarily contain only newly synthesized ACh, i.e. a large fraction of the ACh released would have been newly synthesized. However, in perfused cat ganglia, the total output of ACh in the presence of HC-3 (2×10^{-5}M) is almost completely accounted for by depletion of the ganglion content of ACh (Birks & MacIntosh, 1961; R. Birks and D. M. J. Quastel, unpublished observations), there being very little synthesis. The finding of quanta of slowly diminishing ACh content therefore rules out this hypothesis. A modification of the model, putting only a part of the presynaptic store into a pool of such immutable quanta and the remainder into a pool from which new quanta are constantly being formed with an ACh content depending on the amount of ACh in this store, would suffice to explain the shape of the rundown curve. However, to explain the rapid increase of quantum size with new synthesis, without effective turnover of the preformed pool, would require a further modification, that quanta once formed may acquire synthesized ACh and perhaps even have a privileged position in this respect.

Once it is assumed that the preformed quanta are not immutable, which is necessary if we are to suppose that there are any preformed quanta at all, then the above picture may be simplified. It could be that, to begin with, there are in the nerve terminal quanta containing the complete range of ACh content from nothing to saturated, with the fullest being most readily released. Again it would have to be supposed that the most readily releasable, perhaps those closest to the presynaptic membrane, are those most rapidly filled as new synthesis takes place. Of course, the case could also be that, to begin with, all stored quanta are filled but, as new quanta are formed without corresponding synthesis of ACh, there is a redistribution of ACh between the quanta setting up a situation similar to the previous one.

It should be emphasized that there is nothing in these results to contradict an hypothesis which assumes that quanta are formed only as a part of the release process, there being no such thing as preformed quanta in the nerve ending. On this view synaptic vesicles might represent presynaptic reservoirs of ACh without actually being quanta. However, the concept of a pool of preformed quanta is useful to explain changes of quantum content with tetanic stimulation (Brooks & Thies, 1962; Elmqvist & Quastel, 1965) and is therefore rather attractive.

The increased variability of quantum size as quanta become small is most readily explained by heterogeneity of different parts of the nerve terminal. Alternatively it could reflect the co-existence, in a preformed pool, of quanta formed at different stages of depletion of a store of ACh from which quanta are formed.

The experiments with HC-3 have also provided some information on a closely related problem. It has now become well established that the quantal units composing e.p.p.s are of the same amplitude as those recorded as m.e.p.p.s (del Castillo & Katz, 1954*a*, *b*; Liley, 1956; Boyd & Martin, 1956). That the same relation seems to hold even when m.e.p.p.s become small as a consequence of stimulation in the presence of HC-3 is strong evidence that the quantal units of the e.p.p.s are always identical to the quanta causing m.e.p.p.s and either formed from or representative of the same presynaptic pool of transmitter.

SUMMARY

1. By the use of intracellular electrodes a presynaptic effect of HC-3 has been shown at the mammalian neuromuscular junction.

2. This effect was demonstrable only when transmitter was released from the motor nerve terminals at a high rate and consisted of a progressive decline in the size of the miniature end-plate potentials and in the quantal components of the end-plate potentials as transmitter was released.

3. Releasing the transmitter by nerve stimulation or by increased potassium ion concentration were equally effective in eliciting the phenomenon.

4. Post-synaptic chemosensitivity as measured by bath application of carbachol was the same before and after the quantal units had become small as a consequence of stimulation of transmitter release in the presence of HC-3.

5. The effect is attributed to an inhibition of ACh synthesis by HC-3, the quanta becoming smaller in size as the presynaptic stores of ACh become depleted.

6. It is suggested that the amount of releasable ACh in the motor nerve terminals can be estimated from the sum of all e.p.p.s and/or m.e.p.p.s that can be elicited after blockade of ACh synthesis.

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REFERENCES

Birks, R. & MacIntosh, F. C. (1961). Acetylcholine metabolism of a sympathetic ganglion. *Canad. J. Biochem. Physiol.* **39**, 787–827.

Blackman, J. G., Ginsborg, B. L. & Ray, C. (1963). On the quantal release of the transmitter at a sympathetic synapse. *J. Physiol.* **167**, 402–415.

Bowman, W. C. & Rand, M. J. (1961). Actions of triethylcholine on neuromuscular transmission. *Brit. J. Pharmacol.* **17**, 176–195.

BOWMAN, W. C., HEMSWORTH, B. A. & RAND, M. J. (1962). Triethylcholine compared with other substances affecting neuromuscular transmission. *Brit. J. Pharmacol.* **19**, 198–218.

BOYD, I. A. & MARTIN, A. R. (1956). The end-plate potential in mammalian muscle. *J. Physiol.* **132**, 74–91.

BROOKS, V. B. & THIES, R. E. (1962). Reduction of quantum content during neuromuscular transmission. *J. Physiol.* **162**, 298–310.

BULL, G. & HEMSWORTH, B. A. (1963). Inhibition of biological synthesis of acetylcholine by triethylcholine. *Nature, Lond.*, **199**, 487–488.

BURGEN, A. S. V., BURKE, G. & DESBARATS-SCHÖNBAUM, M. L. (1956). The specificity of brain choline acetylase. *Brit. J. Pharmacol.* **11**, 308–312.

CANEPA, F. G. (1964). Acetylcholine quanta. *Nature, Lond.*, **201**, 184–185.

DEL CASTILLO, J. & KATZ, B. (1954*a*). Quantal components of the end-plate potential. *J. Physiol.* **124**, 560–573.

DEL CASTILLO, J. & KATZ, B. (1954*b*). Statistical factors involved in neuromuscular facilitation and depression. *J. Physiol.* **124**, 574–585.

EDWARDS, C. & IKEDA, K. (1962). Effects of 2-PAM and succinylcholine on neuromuscular transmission in the frog. (Appendix by B. W. Brown.) *J. Pharmacol.* **138**, 322–328.

ELMQVIST, D., JOHNS, T. R. & THESLEFF, S. (1960). A study of some electrophysiological properties of human intercostal muscle. *J. Physiol.* **154**, 602–607.

ELMQVIST, D., HOFMANN, W. W., KUGELBERG, J. & QUASTEL, D. M. J. (1964). An electrophysiological investigation of neuromuscular transmission in myasthenia gravis. *J. Physiol.* **174**, 417–434.

ELMQVIST, D. & QUASTEL, D. M. J. (1965). A quantitative study of end-plate potentials in isolated human muscle, *J. Physiol.* (In the Press.)

ELMQVIST, D., QUASTEL, D. M. J. & THESLEFF, S. (1963). Prejunctional action of HC-3 on neuromuscular transmission. *J. Physiol.* **167**, 47–48*P*.

FATT, P. & KATZ, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

KATZ, B. & THESLEFF, S. (1957). On the factors which determined the amplitude of the 'miniature end-plate potential'. *J. Physiol.* **137**, 267–278.

KRNJEVIĆ, K. & MILEDI, R. (1958). Failure of neuromuscular propagation in rats. *J. Physiol.* **140**, 440–461.

KRNJEVIĆ, K. & MILEDI, R. (1959). Presynaptic failure of neuromuscular propagation in rats. *J. Physiol.* **149**, 1–22.

KRNJEVIĆ, K. & MITCHELL, J. F. (1961). The release of acetylcholine in the isolated rat diaphragm. *J. Physiol.* **155**, 246–262.

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

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

MARTIN, A. R. (1955). A further study of the statistical composition of the end-plate potential. *J. Physiol.* **130**, 114–132.

MARTIN, A. R. & ORKAND, R. K. (1960). Postsynaptic action of HC-3 on neuromuscular transmission. *Fed. proc.* **20**, 579–582.

MARTIN, A. R. & ORKAND, R. K. (1961). Postsynaptic effects of HC-3 at the neuromuscular junction of the frog. *Canad. J. Biochem. Physiol.* **39**, 343–349.

REITZEL, N. L. & LONG, J. P. (1959). The neuromuscular blocking properties of α, α', dimethylethanolamino 4, 4′ biacetophenone (Hemicholinium). *Arch. int. Pharmacodyn.* **119**, 20–30.

SCHUELER, F. W. (1955). A new group of respiratory paralyzants I. The 'Hemicholiniums'. *J. Pharmacol.* **115**, 127–143.

SCHUELER, F. W. (1960). The mechanism of action of the hemicholiniums. *Int. Rev. Neurobiol.* **2**, 77–97.

THIES, R. E. (1962). Depletion of acetylcholine from muscles treated with HC-3. *Physiologist*, **5**, 220.

THIES, R. E. & BROOKS, V. B. (1961). Postsynaptic neuromuscular block produced by hemicholinium no. 3. *Fed. Proc.* **20**, 569–578.