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THE ACTION OF BOTULINUM TOXIN ON MOTOR-NERVE FILAMENTS

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Burgen, Dickens & Zatman (1949) suggested that botulinum toxin produces neuromuscular block by specifically affecting peripheral parts of motor nerves. They put forward two main alternative hypotheses to account for this: either 'transmission of the nerve impulse through the fine unmyelinated nerve fibres to the acetylcholine-releasing region in apposition to the end-plates may be blocked ...' or '... the synthesis of acetylcholine by the nerve endings is inhibited.' Their data favoured the first solution. The present experiments were therefore undertaken in an attempt to settle this question. The results suggest that the site of block produced by type A toxin is situated far peripherally in motor nerve branches; the release mechanism of acetylcholine does not appear to be inactivated by any direct effect upon it, but merely because impulses from the nerve trunk cannot reach it. A preliminary account of some of the experiments has already appeared (Brooks, 1953).

METHODS

Types of toxin

Both crude and crystalline toxins (Type A) were used. The crude toxin was kindly supplied by Prof. G. B. Reed, Department of Bacteriology, Queen's University, Kingston, Ontario. The material was the acid precipitate of the bacterial culture medium, and was obtained either as powder, or as a brown sludge at pH 6·6. Buffered dilutions of these materials were prepared either with 0·15 M sodium phosphate or with 0·12 M sodium acetate, containing a final concentration of 0·2 % gelatin. The toxicity ranged from 10^6 to 10^7 mouse lethal doses (LD_{50}) per g of dried material. Crystalline toxin was kindly provided by Dr E. J. Schantz, Frederick, Maryland, U.S.A. The colourless stock solution was maintained at pH 3·8 in 0·05 M acetate buffer, and contained a final concentration of 0·2 % gelatin. The usual toxicity was about 10^8 LD_{50}/mg nitrogen. All solutions were stored at 3° C. Aliquot portions of stock solutions were suitably diluted with Ringer-Locke's fluid just before use.

Recording of muscle potentials

Action potentials of groups of muscle fibres were recorded from the surface of the gracilis anterior muscle in the cat anaesthetized with chloralose (80 mg/kg i.v., Hoffmann-La Roche). The motor nerve was stimulated with supramaximal rectangular waves of constant voltage,

lasting 0·3 msec, at frequencies ranging from 6 to 60/min. The parameters of the pulses were electronically controlled. End-plate regions were localized by finding sites from which end-plate potentials of maximal amplitude and minimal latency could be recorded after intravenous injection of 0·2 mg/kg D-tubocurarine (crystalline, Burroughs-Wellcome) or of 0·05 mg/kg decamethonium-iodide (Burroughs-Wellcome). The surface of the muscle was covered with mineral oil. The preparation and method have been described by Brown & Burns (1949) and by Burns & Paton (1951). A few experiments were performed with the guinea-pig's excised serratus anterior muscle, recording from aneural parts of muscle fibres (Brooks, 1951).

Potentials of groups of fibres could conveniently be picked up from the surfaces of muscles with blunt-tipped, springy, platinum-wire recording electrodes, each touching the muscle over approximately 0·25 mm^2. Action potentials of a few fibres only were recorded from the surface of the serratus muscle with smaller electrodes. The tips of the recording leads were about 200 μ apart in both the horizontal and the vertical planes (Fig. 4M). The lower lead was either a sharp steel needle, varnished to its tip, which had a diameter of about 0·01 mm, or a platinum wire of approximately the same diameter, sealed in a glass capillary flush with the tip, which was then ground flat. The upper electrode was made of varnished copper wire of 0·08 mm diameter, cut off square at its lower end.

The recording equipment consisted of a 4-stage push-pull amplifier, connected through capacities of variable time-constants to the amplifiers of an oscilloscope (Cossor, model 1049). When high-impedance electrodes were used, a cathode-follower input stage was inserted between recording leads and amplifier. The frequency response of the system without the input cathode-follower was flat from 10 to 5000 c/s, which was adequate for the comparisons of slow and fast potentials made in those experiments. The high-frequency base-line noise was about 15 μV.

Maintenance of the experimental preparations

All cats received artificial respiration from a Palmer pump throughout the experiments, before and after injections of paralysing drugs. The pool of oil covering the exposed gracilis muscle was kept between 34 and 37° C by radiation from a lamp. Excised guinea-pig muscle was immersed in modified Ringer-Locke's solution (Table 1) in baths made from Petri dishes or Buchner funnels. Solutions were oxygenated by blowing oxygen through them either from small gas bubblers or through the stems of the funnels.

TABLE 1. Modified Ringer-Locke's solution

NaCl	149·00 mM
KCl	5·60 mM
$CaCl_2$	2·17 mM
Na_2HPO_4 3·9 / NaH_2PO_4 1·0	6·00 mM (Na)
Glucose	11·00 mM

Assays of acetylcholine output

The guinea-pig diaphragm muscles were dissected out with their costal attachments and phrenic nerves, after the animals had been stunned by a blow on the head. By choosing small animals and by trimming the ribs as closely as possible to the diaphragm, the amount of intercostal muscle included in the preparation was reduced to a minimum. This point was of importance in the comparison of the amounts of acetylcholine released by stimulation of the phrenic nerves and by sending current pulses through the bath. The phrenic nerves were stimulated supramaximally at 30/sec through platinum wire electrodes. The muscle fibres and nerve endings were excited directly at 30/sec by discharges from the secondary coil of an inductorium, led into the bath through chlorided silver plates. The baths, containing 10^{-5} eserine sulphate (Merck) in Ringer-Locke's solution, were maintained between 35 and 38° C by immersion in a thermostatically controlled water tank. The amounts of acetylcholine released were assayed by measurements of the depression

of the arterial pressure of chloralosed cats (80 mg/kg i.v.). Samples (2 ml.) of the bath fluid were injected into the jugular vein, usually within 15 min after removal from the bath, and their effects were compared with those of known doses of acetylcholine (Hoffmann-LaRoche) injected in total volumes of 2 ml. The sensitivity of the cats to acetylcholine was increased by intravenous injection of 0·1 mg/kg eserine sulphate. The cats were usually eviscerated.

RESULTS

Botulinum toxin does not impair conduction in motor-nerve trunks (Bishop & Bronfenbrenner, 1936; Guyton & MacDonald, 1947). This was confirmed for A, B and C fibres of the cat's excised phrenic, sciatic, and thoracic vagus nerves, when exposed to 10^4 LD_{50}/ml. of toxin for up to 5 hr; the perineurium of the sciatic nerve was removed. Both action potential amplitude and conduction velocity remained unchanged.

Similarly, reports of normal direct excitability of muscle fibres paralysed to indirect excitation with toxin were confirmed. The thresholds to direct excitation of the guinea-pig's excised serratus muscle were tested by applying shocks with platinum wire electrodes to the surface of the muscle after the preparation had been paralysed with D-tubocurarine. Thresholds did not change after subsequent addition of toxin, although changes of 3% could have been measured.

Action potentials of muscle fibres

The simplest procedure for detecting whether nerve impulses reach the motor-end-plates, when neuromuscular conduction has been blocked with toxin, is to record action potentials of muscle fibres from an end-plate zone. The most distal end-plate group of the cat's gracilis muscle was localized, as described in 'Methods', and the recording electrodes were left in place while the effects of curare or of decamethonium wore off; when maximal nerve volleys again produced action potentials of normal size, 10^7–10^8 LD_{50} of botulinum toxin were injected intravenously. Within 10–30 min the muscle action potentials started to decline; the decline continued until they disappeared about 1–2 hr after administration of the toxin. The total time to paralysis depended on the state of the circulation of the muscles. Since end-plate potentials were not seen in any experiment, the transmission of nerve impulses must have been arrested peripherally to the nerve-trunk, but proximally to the end-plates.

In muscles from cats and guinea-pigs Masland & Gammon (1949) found, during partial block with botulinum toxin, that the second of two maximal motor nerve volleys, when separated from the first by intervals of between 10 and 300 msec, elicited greater muscle potentials than the first volley alone. However, the maximum potentiation was only 20% (fig. 2, Masland & Gammon, 1949). A much greater increase was observed in the present experiments with the cat's gracilis and the guinea-pig's serratus muscles. The motor

nerve trunk was stimulated supramaximally with single and double shocks in alternation. Fig. 1 shows that as the amplitudes of potentials elicited by single volleys decline, the *relative* amplitudes of the second responses increase. After single volleys fail to excite any muscle fibres, double volleys can re-establish neuromuscular transmission to some fibres, for a period of 1–2 sec. The relation of the amplitude of the first and second responses is plotted in Fig. 2.

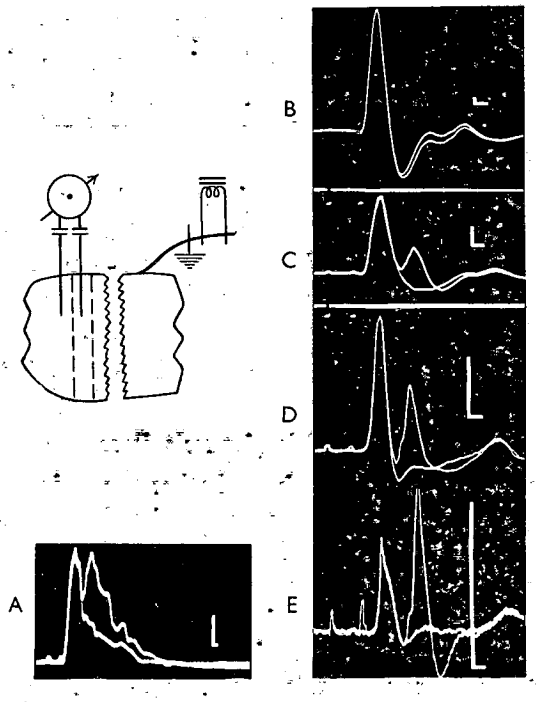

Fig. 1. Muscle action potentials during action of botulinum toxin. Cat, 2·5 kg, 200 mg chloralose i.v., gracilis preparation, recording from fixed position on surface of most distal end-plate zone, as shown in inset diagram. Time constant of recording system = 0·2 sec. Nerve trunk stimulated supramaximally 12/min, alternately with single and double shocks. Each frame (A–E) represents double exposures of frames to successive single and double volleys for comparison. A: end-plate potentials, 20 min after intravenous injection of 0·12 mg decamethonium. B: action potentials after recovery from decamethonium but before injection of toxin. C: 52 min after intravenous injection of 10^8 LD_{50} of crude botulinum toxin. D: 68 min after injection of toxin. E: 113 min after injection of toxin. Calibrations: 0·5 mV; 1·0 msec.

The facilitation observed with double volleys provides another opportunity of testing for the appearance of end-plate potentials. If the success of the second volley were due to summation at the post-synaptic face of the neuromuscular junction, then the first unsuccessful volley should produce an end-plate potential from which the muscle action potential due to the second volley

should arise. The records C–E in Fig. 1 show that this is not the case; the second as well as the first response is a rapidly rising diphasic potential, even when its amplitude has decreased to less than one-fifth of the amplitudes of end-plate potentials recorded in the presence of curare or decamethonium. This again confirms the pre-synaptic locus of the block produced by toxin, and the essential difference between the actions of botulinum toxin and those of curare or of decamethonium. Sometimes monophasic potentials were seen as responses

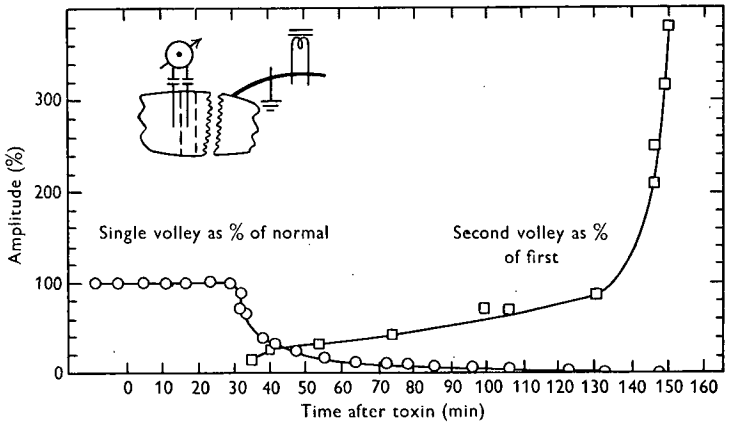

Fig. 2. Time course of changes of muscle potential during action of botulinum toxin. Cat, gracilis preparation, data from same experiment as Fig. 1. Arrangements shown in inset diagram. ○: amplitudes of muscle potentials at various times after administration of toxin, in response to single maximal nerve volleys at 12/min, plotted as percentage of normal amplitude (after recovery from effects of decamethonium, but before injection of toxin). □: amplitudes of muscle potentials in response to second of double maximal nerve volleys, separated by 1·8 msec, *plotted as percentage of responses to first volleys recorded at that time after injection of toxin.*

to single nerve volleys. In all instances they had shorter durations than end-plate potentials, and did not disappear when the recording electrodes were moved 1–2 mm in either direction from the end-plate zone. Most likely they originated from fibres distant to the recording leads, after the fibres immediately beneath the electrodes had been paralysed.

The duration of the potentiation created by a maximal volley, upon one following after various intervals of time, was tested repeatedly after injection of toxin. A plot of a representative experiment is shown in Fig. 3. The shift of the 'recovery curve' to the left (and to values exceeding 100%) during the action of botulinum indicates that double nerve volleys activate previously idle muscle fibres. A few occasional observations showed that the potentiation extends to at least 160 msec, which was the longest interval between volleys tested. As the amplitudes of action potentials decrease, latencies of responses to single volleys increase (Table 2). Guyton & MacDonald (1947) observed a

mean increase of latency of action potentials of 0·3 msec in partly paralysed guinea-pig's gastrocnemius. Since conduction velocity in nerve trunks remains normal, the delay probably occurs in those nerve branches beginning to be

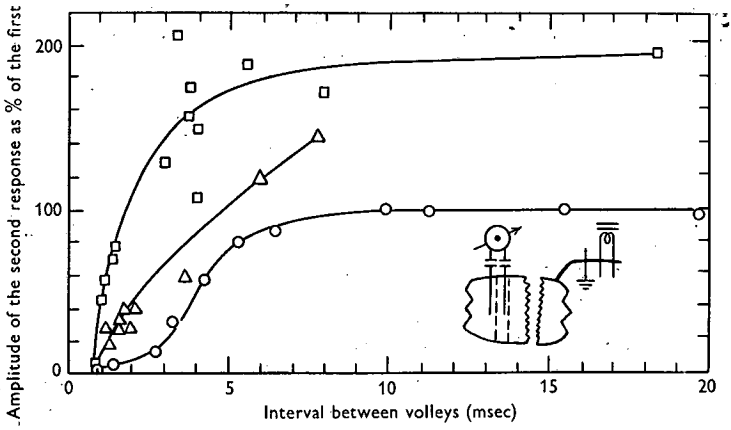

Fig. 3. Facilitation of muscle potentials during action of botulinum toxin. Cat, gracilis preparation, data from same experiment as Figs. 1 and 2. Inset diagram shows arrangements. Curves relating the amplitudes of the responses to the second of double maximal nerve volleys to the interval between the two stimuli (in msec); each value is expressed as a percentage of the response (not shown) to the first volley of that pair. ○ : normal preparations, recovered from effects of decamethonium, but before injection of toxin. △: 45–60 min after injection of toxin. □: 85–100 min after injection of toxin.

TABLE 2. Changes of latency during the action of botulinum toxin.

Preparation	Mean 'normal'* latency ±S.D. (msec)	No. of observations	Final latency (msec)	Increase of latency (msec)
	Cat's gracilis *in situ*			
Many fibres	2·80±0·0006	12	3·40	0·60
Many fibres	1·99±0·0033	10	3·20	1·21
	Excised guinea-pig's serratus			
Many fibres	5·25±0·016	10	6·50	1·25
Motor unit	1·75±0·095	25	2·01	0·26
Few fibres	1·82±0·0008	45	2·03	0·21
Few fibres	2·94±0·045	11	3·50	0·56
Mean increase for guinea-pig's serratus				0·57

* 'Normal' latency refers to last stable period after application of toxin before final rise of latency.

affected by the toxin. The deduction that previously idle fibres are discharged by the second volley is also borne out by the changes of latency. As the relative number of fibres discharged twice by double volleys decreases, the proportion of fibres discharged only by the second volley increases. Accordingly,

the latencies of the responses to second volleys decrease from values denoting relative refractoriness towards the same values as obtained with single volleys at that time after administration of toxin.

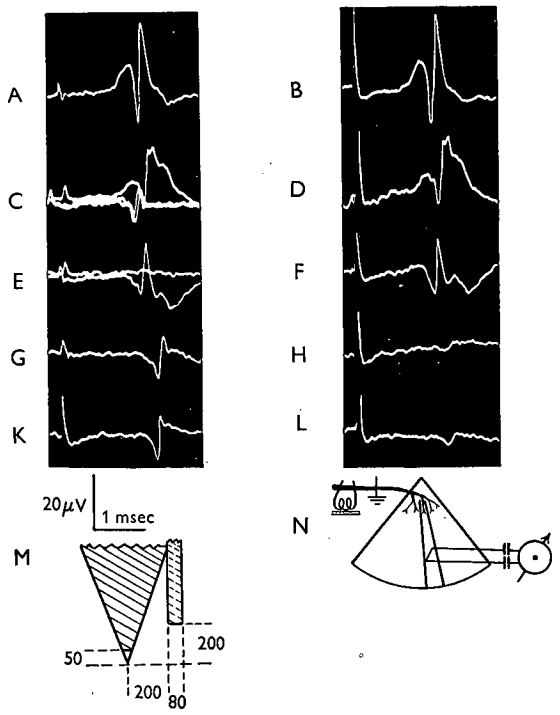

Fig. 4. Fractionation of a motor unit by botulinum toxin. Guinea-pig's excised serratus preparation. Action potentials of muscle fibres belonging to one motor unit, recorded from a fixed position on aneural parts of muscle surface (diagram N), in response to single maximal nerve volleys at 20/min. Time constant of recording system = 0·2 sec. Time after addition of 5×10^4 LD_{50} crude toxin to bath, and strengths of nerve stimuli: A: 48 min, 0·25 V; B: 47 min, 2·5 V; C: 49 min, 0·25 V; D: 50 min, 2·6 V; E: 56 min, 0·28 V; F: 57 min, 2·6 V; G: 62 min, 0·25 V; H: 64 min, 2·6 V; K: 64·5 min, 2·6 V; L: 65 min, 2·6 V; M: diagram of recording electrodes (see 'Methods'), dimensions in μ. Double exposures in C and E show that stimuli were just threshold.

Recording the activity of a single motor unit can demonstrate that botulinum toxin produces a gradual fractionation of the response to that unit. Fig. 4 illustrates the results of such an experiment on the guinea-pig's excised serratus preparation; the compound potential of the fibres within recording range (space constant = 0·3 mm) reacted in all-or-none fashion to shocks of all strengths. Comparable potentials in the two columns of Fig. 4 vary by about 5% from each other, owing to the progressive reduction of active muscle fibres by the toxin during the intervals between tests. The final intermittent responses originate from a very small number of fibres only.

Estimation of acetylcholine release

It follows from the results described in the previous sections that the cessation of acetylcholine release in response to nerve trunk stimulation after application of botulinum toxin, as described by Burgen, Dickens & Zatman (1949), may not be due to any inherent inability of the nerve endings to release acetylcholine. Instead, the foregoing results suggested that toxin might prevent nerve impulses from reaching the release sites or of activating them. This conclusion can be put to direct test by assaying the amounts of acetylcholine released by normal muscles when they are excited maximally (1) through the nerve trunk, and (2) by current pulses flowing through the bath fluid, and then repeating the experiment after indirect excitability has been abolished with toxin. The guinea-pig's excised diaphragm preparation was used for these experiments, because its nerve branches are oriented at random, making it unnecessary to align the muscle in any special way with reference to the plate-electrodes used for stimulation. Table 3 summarizes the results obtained with normal tissue, using the procedures outlined in 'Methods'. The data on release during stimulation are listed as 'Corrected Release' because it was found that under the conditions of the experiments, there was a small continuous release of acetylcholine even from resting muscles. In contrast to the experience of Dale, Feldberg & Vogt (1936) with muscles perfused *in situ*, this resting release did not cease after completion of arrangements for the tests, but instead continued without significant change for up to 5 hr, the longest period of observation. A large variation of output was observed in all experiments, both for resting release and for the production of acetylcholine in response to stimulation. In all cases the amounts released during rest and those in response to stimulation differed at better than the 1% level of significance. However, release due to stimulation of the trunk and of the nerve branches directly were of the same order of magnitude, although values of the latter tended to be higher. Analysis of variance showed that there is no constant relation between the resting release and that due to stimulation. The large scatter of values within and between experiments was neither due to changes in the tissues with time after dissection, nor to variation in bath temperatures, as can be seen from the values in Tables 3 and 4, in which experiments performed at the same time, with muscle chambers immersed in the same warming bath, are indicated by the letters *a*, *b* and *c*.

After application of paralysing doses of botulinum toxin, acetylcholine output due to nerve trunk tetanization fell to the 'resting' level when paralysis was complete (Table 4). The resting release continued without significant change after paralysis. Thus, in Expt. 2*b* (Table 4) the mean resting output (7 trials) was 2·3 mμg/min, with a standard deviation of 1·2 mμg/min. Five of these tests were made before paralysis and two after. The means and S.D.

TABLE 3. Acetylcholine output of the guinea-pig's excised diaphragm preparation.

Expt. no.	Method of stimulation	Time since dissection (min)	Period of stimulation (min)	Period of collection (min)	Observed output during collection (mμg)	Resting output* mean ± S.D. (mμg/min)	Corrected output†	
							Trunk (mμg/min)	Bath (mμg/min)
1	Trunk	100	5·0	5·0	41	1·7±0·6 (10)	6·5	—
	Trunk	190	5·0	5·0	68		11·9	—
	Trunk	245	2·0	2·0	18		7·3	—
	Trunk	362	2·0	7·0	45		16·6	—
	Trunk	437	2·0	7·0	30		9·0	—
2*a*	Trunk	208	2·0	12·0	90	2·0±0·6 (7)	33·0	—
	Trunk	247	2·0	12·0	45		10·5	—
	Bath	586	2·0	36·0	142		—	35·0
3*a*	Trunk	125	2·0	12·0	57	2·5±0·8 (3)	13·5	—
3*b*	Trunk	110	2·0	12·0	60	1·7±1·0 (3)	19·8	—
4*a*	Trunk	135	2·0	19·0	39	1·3±1·6 (6)	7·1	—
	Trunk	390	2·0	18·0	38		7·3	—
	Bath	378	2·0	19·0	48		—	11·7
4*b*	Trunk	100	2·0	19·0	39	0·9±0·3 (3)	10·9	—
5	Bath	154	2·0	15·0	98	1·2±0·7 (6)	—	40·0
	Total mean	—	—	—	—	1·6±0·9 (38)	12·8±7·1 (12)	28·9±15·1 (3)

* *Note.* The number of tests for 'resting output' is given in brackets beneath the mean and standard deviation (S.D.) of the value for 'resting output'.

† Sample calculation (Expt. 5): $\frac{98{\cdot}0 - (15 \times 1{\cdot}2)}{2{\cdot}0} = 40{\cdot}0$ mμg/min.

TABLE 4. Acetylcholine output of the guinea-pig's excised diaphragm preparation before and after the action of crystalline botulinum toxin.

Expt. no.	Method of stimulation	Response of preparation to stimulation of nerve trunk	Concentration of toxin (LD_{50}/ml.)	Time since dissection (min)	Period of stimulation (min)	Period of collection (min)	Observed output during collection (mμg)	Resting output* Mean$\pm$S.D. (mμg/min)	Corrected output† Trunk (mμg/min)	Corrected output† Bath (mμg/min)
2*b*	Trunk	Normal	50	238	2	12	97	2·3$\pm$1·2 (7)	34·7	—
	Trunk	Paralysed		550	2	12	34		3·2	—
	Bath	Paralysed		577	2	32	142		—	34·2
4*c*	Trunk	Normal	7000	135	2	19	41	0·8$\pm$0·5 (6)	12·9	—
	Trunk	Paralysed		340	2	18	20		2·8	—
	Bath	Paralysed		378	2	22	35		—	8·7

* *Note.* The number of tests for 'resting output' is given in brackets beneath the mean and standard deviation (S.D.) of the value for 'resting output'.

† Sample calculation (Expt. 2*b*): $\frac{97-(12\times 2\cdot 3)}{2}=34\cdot 7$ mμg/min.

values were respectively $2{\cdot}2 \pm 1{\cdot}8$ and $2{\cdot}1 \pm 0{\cdot}8$ mμg/min. Similarly, the values for Expt. 4*c* (Table 4) were: all 6 trials, $0{\cdot}8 \pm 0{\cdot}5$ mμg/min; 3 trials before paralysis, $0{\cdot}9 \pm 0{\cdot}4$ mμg/min and 3 trials after paralysis, $0{\cdot}6 \pm 0{\cdot}6$ mμg/min. Stimulation by current pulses through the bath, however, elicited releases of acetylcholine from the preparation comparable to those obtained by indirect stimulation before paralysis. Therefore the release mechanism of acetylcholine in the nerve endings is still capable of being activated by current flow in the bath at a time when the nerve filaments have been blocked by botulinum toxin.

Facilitation across the block

Fig. 3 shows graphically the duration of the facilitation remaining after a single nerve volley has reached nerve filaments under the influence of toxin. Such a state may be produced in nerve fibres for a few msec by local extrinsic potentials (Hodgkin, 1937*a*, *b*). Brown & Harvey (1938) described temporal summation in neuromuscular junctions of the fowl's sciatic-gastrocnemius preparation, occurring normally with intervals between volleys extending up to 160 msec. However, a facilitation of a hundred-fold duration follows supramaximal tetanization of the motor nerve trunk at 30–100/sec for 20–50 sec in cat's and guinea-pig's muscle poisoned with botulinum toxin. In the normal state such treatment decreases the amplitudes of muscle potentials slightly, as had been shown for cat's muscle by Brown & v. Euler (1938). Yet, when toxin has produced almost complete neuromuscular paralysis, the response to single volleys is potentiated for 1–2 min by such tetanization of the trunk. The exact time course of this post-tetanic potentiation (P.T.P.) was not obtained, but the following excerpt from a typical experiment establishes approximate values.

Experiment to demonstrate post-tetanic potentiation

Cat, 1·7 kg, 140 mg chloralose i.v. Gracilis muscle exposed *in situ*, surface covered with warm mineral oil. Record action potentials from surface of end-plate zone. Stimulate motor nerve trunk throughout experiment at 12/min alternately with single and double volleys. Double volleys refer to stimuli applied at 1·8 msec intervals.

2·14 p.m. 0·5 mg D-tubocurarine i.v. Localize end-plate zone.
4·13 p.m. Stimulate nerve trunk for 60 sec at 30/sec. No P.T.P. of subsequent single volleys.
5·10 p.m. Inject 10^7 LD_{50} crude botulinum toxin i.v.
6·31 p.m. Muscle responds only intermittently to maximal single motor-nerve volleys.
6·50 p.m. Double maximal volleys can potentiate responses.
6·54 p.m. No responses to double volleys.
6·55 p.m. Stimulate motor-nerve trunk supramaximally for 60 sec at 30/sec. Now muscle responds to single volleys; however, amplitude decreases continually.
6·56 p.m. Muscle responds intermittently to single volleys.
6·57 p.m. Muscle does not respond to single volleys, is potentiated by double volleys.
6·59 p.m. Stimulate motor-nerve trunk for 60 sec at 30/sec. Muscle responds now to single volleys, but amplitude decreases; within 30 sec only responds to double volleys.
7·01 p.m. Muscle does not respond to double volleys.
7·04 p.m. Stimulate motor-nerve trunk for 60 sec at 30/sec. No response to any form of nerve trunk stimulation.

The durations of both the facilitation following single volleys and that following trains of volleys make it very unlikely that they are produced by summation of local potentials. An alternative cause could be a more profound change of the ionic distribution in the filaments at the site of action of toxin, and/or beyond it, produced by action potentials impinging upon these sites.

DISCUSSION

The present experiments demonstrate that botulinum toxin renders motor nerve filaments inexcitable by impulses originating in nerve trunks, but leaves the release mechanism of acetylcholine responsive to electric current pulses passing through the surrounding medium. When thus activated, approximately normal amounts of acetylcholine are released.

During the action of toxin, the behaviour of motor nerve filaments is similar in several ways to that of partly depressed fibres of nerve trunks. Tasaki (1939) showed that single volleys facilitate succeeding test-volleys during the ensuing block produced at individual nodes of Ranvier by anelectrotonus, urethane, or cocaine, in the toad's excised sciatic nerve. However, the period of facilitation was only a few msec (cf. the present results with botulinum toxin). Similarly, Tasaki (1939) showed that nerve fibres, about to be blocked by depressing agents, respond intermittently and with increased latency to maximal volleys.

The time course of the P.T.P. created by trains of volleys in nerve filaments blocked by toxin is very similar to that of a phenomenon reported by Burns & Paton (1951). They observed that the end-plate regions of the cat's gracilis muscle remained electrically negative to neighbouring aneural parts of the muscle for several minutes after a direct cathodal current of 300 μA had been passed through the end-plate zone for 5 min. However, potentiation of single volleys through those junctions was not tested.

Since action potentials impinging repeatedly on the block produced by toxin restore conduction, rather than deepen paralysis, it is likely that botulinum toxin inactivates nerve filaments by hyperpolarizing their cellular membranes at the point of action, or by making them impermeable to ions. The effects of trains of volleys arriving at the block would be analogous to the action of a persistent cathode, discussed by Burns & Paton (1951).

An important species difference of the effects of botulinum toxin must be mentioned here. Burgen *et al.* (1949) did not observe any facilitation following maximal nerve volleys in poisoned excised rat's diaphragm. This may be due to a more rapid combination of toxin and its target-molecules in the rat's fibres, thus preventing the formation of a fringe of subliminally excitable nerve filaments. If this is true, it should be noted that rate of action on any one filament does not correlate with general sensitivity of species to toxin; the

duration of the minimal period between application of toxin and onset of action, as well as time to complete paralysis, are no shorter in the guinea-pig than in the rat, although the guinea-pig is far more sensitive to botulinum toxin than the rat.

Since the motor nerve filaments of the guinea-pig's serratus muscle are myelinated to within 1 μ of motor end-plates, conduction block may occur at nodes of Ranvier which have been shown to be primary targets of anelectrotonus and ionically imbalanced solutions (Blair & Erlanger, 1936; Tasaki, 1939) and of narcotics (Tasaki & Takeuchi, 1941). The increases of latency listed in Table 2 could likely be explained by block at a single node, judging by the time relations found for amphibian fibres by Tasaki & Takeuchi (1941). However, nodes of Ranvier in motor nerve filaments possess no known specialization which would make them exclusive targets for the action of botulinum toxin. Dun (1951) has suggested that the points of branching of motor nerves are most prone to attack by procedures depressing conduction, because the increased surface beyond the division lowers the safety factor for conduction across it. The present results could be explained by conduction block at peripheral points of branching, but the evidence does not prove it. Furthermore, that contention does not explain why only very distal branches are blocked by toxin, and even more critically, why cholinergic nerves are so very much more sensitive than other fibres to the toxin (Ambache, 1951). It would have to be assumed that the distal branches of cholinergic nerves have distinct molecular structures with which the toxin molecules combine. No information is available to-day to suggest what part of the cholinergic system in the filaments could be involved. The work of Burgen *et al.* (1949) rules out choline acetylase and cholinesterase. The months-long recovery of locally poisoned preparations, reported by Guyton & MacDonald (1947), implies a slow rebuilding of this hypothetical substance.

An alternative possible site of action of botulinum toxin, suggested by Dun's (1951) argument, and supported by the findings of Kuffler (1948, 1949), is the point of geometrical discontinuity between the motor nerve filament and the terminal arborization, constituting the pre-synaptic part of the neuromuscular junction. Kuffler (1948, 1949) showed that in the frog's nerve, current spread is greatly attenuated at this site. Both the selective peripheral action of toxin, and increased latency of muscle potentials could be explained satisfactorily on this basis, without having to assume chemical specialization at some of the more proximal nodes of Ranvier. However, the selective action of toxin on cholinergic nerves still remains unexplained.

SUMMARY

1. Botulinum toxin (type A) abolishes indirect excitability of nerve-muscle preparations; constituent fibres of motor units drop out in very small groups.

2. Motor nerve filaments of excised paralysed preparations remain excitable by current pulses passing through the muscle-bath, and when so excited release amounts of acetylcholine about equal to those released by supra-maximal stimulation of the nerve trunk of the same preparation before application of toxin.

3. Ineffective maximal nerve volleys facilitate the passage of succeeding test-volleys through the block for at least 160 msec.

4. Bursts of ineffective maximal nerve volleys (20–60 sec at 30–100/sec) facilitate conduction of succeeding test-volleys through the block for 1–2 min.

5. It is concluded that botulinum toxin blocks conduction in motor nerve filaments either near the point of final branching or at the point of geometrical discontinuity between filament and terminal ramifications in the pre-synaptic part of the end-plate.

6. It is further concluded that toxin likely acts either by rendering the filaments locally impermeable to ions or by hyperpolarizing their cellular membranes.

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Note added in proof. After this paper had been submitted for publication, a preliminary report on the persistence of end-plate potentials in frog's sartorius after poisoning with botulinum toxin was published by Stover, Fingerman & Forester (1953).