J. Physiol. (1949) 108, 127–141 612.017.4:612:842.4

THE PERIPHERAL ACTION OF *CL. BOTULINUM* TOXIN

By N. AMBACHE

From the Department of Physiology, University College, London

(*Received* 3 *February* 1948)

The nature of the paralysis which occurs in botulism does not appear to have been considered in the light of modern conceptions of humoral transmission at nerve endings. It is clear from the older work of Edmunds & Long (1923), and of Dickson & Shevky (1923) that the toxin of *Cl. botulinum* affects only those nerve-muscle and nerve-gland systems that are now described as cholinergic. These authors mapped out indirectly, with toxin, the distribution of cholinergic nerve fibres in the body, before this was done systematically by direct experiment. They thought that the essential action of the toxin was upon the motor-end plates of skeletal muscles, and, with other authors, have suggested a type of action similar to that of curare on striated, and of atropine on plain, muscle. Their experiments, however, only showed that the muscles in the paralysed limb were still excitable electrically, and did not prove the pharmocological action which they postulated; but they served to locate the paralysis at some point proximal to the muscle fibres. This question has therefore been re-examined by studying the local effects of the toxin (*a*) on the nerve-smooth muscle systems in the iris, after intraocular injections, and (*b*) on various voluntary muscles paralysed by local injections of toxin.

The results show that the toxin has no appreciable effect either on the sensory or on the adrenergic nerve fibres within the eye, but that it affects the cholinergic fibres specifically, and that this cholinergic paralysis is not due to the existence of a block between the transmitter and the effector cell.

METHODS

A powdered preparation of *Cl. botulinum* toxin type A, which was kindly supplied by Dr D. W. Henderson of the Microbiological Research Station, Porton, was used. Its mouse a.l.d. was 0·1 μg./kg. A small amount of it was dissolved in a sterile buffer of the following composition: 0·397% Na_2HPO_4, 0·3% powdered gelatin; pH adjusted to 6·6 (electrometrically) by adding 0·8–1 ml. N-HCl. To this solution an equal volume of pure glycerol was added. The glycerinated toxin, which had a final concentration of 10^{-3}, was stored in darkness in a refrigerator. Further dilutions of toxin in phosphate buffer were made up from this stock solution immediately before the injections. The pH of these dilutions was also 6·6 for the first few experiments, but it was later raised to 7·2, in order to avoid undesirable inflammatory reactions in the vitreous body.

Rabbits of mixed stock weighing 0·85–2·7 kg. were used in all the experiments on the eye. Because of the expense, an accurate determination of the m.l.d. for rabbits was not carried out but an approximate value for the m.l.d. (4 days) by the intravenous route (*c.* 0·05 μg./kg.) was arrived at from the five experiments quoted in Table 3 although the exact death-time was not measured, as the animals were used for experiment when they were moribund. For inoculation into the eye the rabbits were anaesthetized with nembutal (usual dose: 26 mg./kg. intravenously) and the toxin was injected: (*a*) into the anterior chamber of the eye, through the cornea. The technique of injection has been previously described in detail (Ambache, Morgan & Payling Wright, (1948); the only differences have been the omission of the local anaesthetic and the withdrawal of an equal volume of aqueous humour before the injections; (*b*) into the vitreous body; for this, the eyeball was rotated downwards and a puncture was made about 4–5 mm. behind the sclero-corneal junction, the needle being driven into the eye until it was visible in the vitreous cavity at a depth of 1–1·5 cm.; (*c*) subconjunctivally and into, or, more probably, around the m. rectus superior. The volume of all these injections was usually 0·02–0·05 ml. In a few experiments the toxin was instilled drop by drop into the conjunctival sac. In each animal only one eye received active toxin. The other eye, which served as a control, was either left without interference or received an identical volume of the same toxin solution, boiled for at least 2 min.

The horizontal diameters of the pupils were measured with a pair of calipers. Reaction to light was tested with a 60 W. lamp at 4–5 cm. distance from the eye, with a few exceptions in which bright sunshine was available on a few cloudless days in September. This was a more powerful stimulus and was used whenever possible. For faradic stimulation of the oculomotor nerves these were exposed in their cranial portion, after decerebration (under nembutal) and section of the optic nerves (Ambache, Morgan & Payling Wright, 1948). Stimuli of measured duration were delivered from a Du Bois-Reymond induction coil (turns ratio: 25/1) with a 2 V. battery in the primary.

For recording the contractions of the m. rectus superior, the conjunctiva was incised circularly round the sclerocorneal margin and reflected backwards. A thread was tied round the tendon of the superior rectus, which was severed: (*a*) from its insertion into the eyeball, (*b*) from the tendon of the superior oblique which adjoins it. The eyeball was freed, by dissection, from the other muscles which are inserted into it. Then, in order to minimize lateral friction on the superior rectus, the eyeball was either wholly eviscerated after preliminary ligation of the ophthalmic artery and optic nerve, or it was simply collapsed by making a circumferential cut and removing the anterior half of the eye together with the vitreous body in the posterior half. For myographic recording, the animal's head was held rigidly in a suitable head-clamp, and was further immobilized either by screwing the threaded end of a brass rod into a hole drilled in the occipital bone and fixing the rod to a metal bar on the operating table, or by means of a second clamp. The two superior recti muscles were connected in turn to the same sensitive torsion myograph for recording tensions. The common carotid arteries were needled for the arterial injections of acetylcholine.

Experiments on tibialis anticus. For these experiments cats were used. They were anaesthetized with ether and the skin was shaved over the tibialis anticus muscle on one or both sides. 5–50 μg. of toxin, in a total volume of 1 ml. saline, were injected on one side, in divided amounts, into the tibialis anticus muscle at 16–20 points down its whole length. The control muscle was injected in three out of seven of these experiments in exactly the same way with an identical amount of toxin boiled previously for 2 min.

Two to eight days later the animals were either decerebrated under ether or anaesthetized with nembutal (26–38 mg./kg. intraperitoneally) and the two tibialis muscles were prepared for myographic recording. The tendons of the extensor digitorum longus and of the peroneal muscles were cut. The peroneal nerve was dissected and prepared for stimulation where it curves round the head of the fibula, after placing a tight ligature round the whole sciatic nerve high up in the thigh. For stimulation neon discharges were led in through a transformer to shielded electrodes which were placed under the nerve. The frequency of stimulation varied between 1 in 10 sec. and 1 in 12·5 sec.

The paralysed muscle was then prepared for close arterial injection, the procedure described by Brown (1938) being followed closely. Acetylcholine was injected in doses of 2–20 μg., in a volume of 0·2–0·5 ml. saline.

RESULTS

Intraocular changes induced by the toxin

Loss of reaction to light. As shown in Table 1, which is summarized from more than forty measurements on 18 animals, the well-known botulinic effect on the iris is only obtained (within 24–48 hr. usually) with doses of toxin which approximate to or exceed the intravenous m.l.d. In order to abolish completely the reaction to light it was necessary to inject 0·05–0·1 μg. of toxin into the vitreous body and still larger doses into the anterior chamber. With amounts smaller than this a total paralysis was rare, although there were varying degrees of impairment of the light reflex, which was also more sluggish than in the control eye. Boiling the toxin for 2 min. for the control injections, which was done in five experiments, completely destroyed its activity in every case.

TABLE 1. Changes in the reaction to light in rabbits after intraocular injections of *Cl. botulinum toxin*

Dose of toxin (μg.)	Time of measurement after inoculation	Reduction in the horizontal diameter (mm.) of the pupil on illumination† (the number of observations is given in brackets wherever an average has been taken)	
		Control eye‡	Intoxicated eye
	A. Into the vitreous body		
0·01–0·05 (3 animals)	—	—	Weak reactions
0·1	48 hr.	2·5*	0*
0·1	72 hr.	1·5	0
0·2	68 hr.	2·25*	0*
0·5	19 hr.	2·5	3.5
	47 and 70 hr.	2·25 (2)	0 (2)
1	42 hr.	3*	0*
5	22 hr.	2	0·25
	B. Into the anterior chamber		
0·3–0·5 (4 animals)	—	—	Weak reactions
1	19 hr., 2, 3, 4 and 11 days	2·15 (5)	0 (5)
	16 days	2·25	1
1·2	72 hr.	1·5	1
1·5	45 hr.	4*	1·5*
2	24 hr.	1·5	0
2·5	20, 44 hr. and 3 days	2·5 (3)	0·4 (4)
	9 days	2·5	1·75

† For illumination a 60 W. lamp was used at a distance of 4–5 cm. from the eye, except for the measurements marked with an *asterisk*, which were made in direct sunlight.

‡ In five experiments the control eye received boiled toxin; in the others it was untreated.

Size of the pupil. Changes in pupillary diameter may or may not be concurrent with the loss of reaction to light in rabbits. All the measurements taken within 24 hr. of inoculation showed a difference in size between the two pupils,

with mydriasis present on the intoxicated side. Subsequently the pupils tended to become equal, although the mydriasis perisisted in a few experiments and a slight myosis on the intoxicated side was recorded twice.

Response to nerve stimulation; effect of the toxin on different types of nerve fibre

Cervical sympathetic. In all of five experiments stimulation of the cervical sympathetic nerve dilated the pupil on the intoxicated side approximately to the same extent as on the control side, even when the reaction to light was lost completely (Table 2).

TABLE 2. Effect of the toxin, injected into the vitreous body, on the pupillary response to stimulation of the oculomotor and cervical sympathetic nerves in rabbits. − indicates constriction; + dilation. Faradic stimulation of equal intensity and 5 sec. duration was applied to each side

Dose of toxin (μg.)	Time after inoculation	Reaction to light on intoxicated side	Nerve stimulated	Pupillary response in mm. (the number of observations is given in brackets wherever an average has been taken)	
				Control side	Intoxicated side
0·05	7 days	Sluggish	Third	—	−1, delayed (2)
0·1	72 hr.	Absent	Cervical sympathetic	+1·9 (2)	+1·9 (3)
			Third	−3	−0·75 (delayed 1 min.)
				−3·25	−0·5
				−3·75	
				−3	0
				−2·5	0
				−3	
0·2	68 hr.	Absent	Cervical sympathetic	+2·75 (2)	+1·6 (4)
0·5	70 hr.	Absent	Third	−3	−0·25
				−2·75	
				−0·5	
				0	0
				−1	0
				−0·5	
			Cervical sympathetic	+2	+2 (2)
0·5*	20 hr.	Absent	Cervical sympathetic	+2·1 (4)	+1·6 (5)
1	42 hr.	Absent	Cervical sympathetic	+2·8 (3)	+2·6 (2)

* Injection into anterior chamber instead of vitreous body. In Exps. 2 and 3 the control eye received boiled toxin; in the others it was untreated.

Paralysis of the oculomotor nerve. On the other hand, stimulation of the oculomotor nerves showed a clear-cut difference in the magnitude, and sometimes in the promptness, of the pupillary response on the two sides. For example, in the second animal listed in Table 2, the usual constriction of the pupil was obtained six times on the control side (injected with boiled toxin) and averaged 3 mm. The contractions of the sphincter started at once and were maximal at the end of the 5 sec. period of stimulation. In contrast with this, there was no response in the intoxicated eye (0·1 μg. active toxin) at the end of

stimulation, but 1 min. later it was observed that the pupil had shrunk by 0·75 mm. The next stimulus was even less effective and two others subsequently had no effect at all; the average of the four measurements was −0·3 mm.

Delayed responses were observed in another experiment, in an eye inoculated with 0·05 μg. of toxin, and were timed more accurately. The first oculomotor stimulus constricted the pupil by 0·75 mm. after a delay of 25 sec.; the second, administered 6 min. later, was more effective (1·25 mm.) and with a shorter delay (10 sec.). The sluggish reaction to light, together with the delayed and diminished response to direct stimulation of the oculomotor nerve, illustrate the type of subtotal paralysis produced by insufficient doses of toxin. When the dose was raised to 0·5 μg., as in Exp. 4, listed in Table 2, the response to oculomotor stimulation on the intoxicated side was practically zero. On the control side, which was uninjected, the response to the first two stimuli averaged −2·8 mm.; subsequently there was some evidence of fatigue on this side (final average −1·3 mm.) which may possibly indicate a slight extension of the intoxication to the opposite side.

An analogous observation indicating a crossed effect of the toxin was made by van Ermengem (1897). In three rabbits, doses of toxin which eventually killed the animals in 18–24 hr. were injected into the anterior chamber on one side but produced paresis of the iris (to light) which was equal on the two sides.

The sensory fibres in the cornea. The integrity of corneal sensation was tested by applying mechanical or weak faradic stimuli to the cornea. In normal rabbits such stimuli elicit reflex retraction of the eyeball, with passive protraction of the nictitating membrane. This test was not available when the toxin was injected into the vitreous body because of the accompanying paralysis of the extrinsic muscles of the eye described below; but it was observed that the animals attempted to withdraw the whole head when the cornea was touched. However, when the toxin was injected into the anterior chamber of the eye, through the cornea, it was possible to apply this test and it was found, in all of three rabbits examined, that corneal sensation was unimpaired at a time when the paralysis of the third nerve was fully developed.

Unimpaired contractility of the sphincter pupillae. An indication of the functional state of the sphincter muscle in the intoxicated eyes could be obtained from a study of its response to acetylcholine injected into the anterior chamber. This experiment was performed on the second to the fourth day of the intoxication, on five rabbits, two of which were not reacting to light at all and the other three hardly at all (responses of 0·5–1 mm. at most). In all of six experiments on these five animals, the injection of 0·2–2·5 μg. of acetylcholine-HCl into the anterior chamber produced a vigorous myotic contraction of the sphincter pupillae. After the same amount of acetylcholine was injected into the control eye, the final size of the pupil was equal on the two sides. One of these experiments may be quoted in detail to illustrate this point.

6 October 1947. Rabbit, 1·03 kg. Paracentesis of the anterior chamber of the left eye performed under nembutal anaesthesia (30 mg. intravenously). Puncture through the cornea 3 mm. anterior to the sclerocorneal junction. About 0·15 ml. aqueous humour withdrawn into a syringe. Without withdrawing the syringe needle from the eye, the syringe was changed for another containing the toxin. 1 mg. of toxin in 0·04 ml. sterile phosphate diluent injected. Right control eye uninjected.

9 October 1947. No reaction to light in the left eye (see Table 1). 2.36 p.m., 30 mg. of nembutal intravenously. 2.49 p.m., left pupil: 5·5 mm. 2 μg. of acetylcholine in 0·2 c.c. of sterile saline injected into the left anterior chamber. Subsequent measurements: after 60 sec., 2·5 mm.; after 2 and 4 min., 2 mm. 2.54 p.m., right pupil (control), 6·5 mm. 2·55 p.m., 2 μg. of acetylcholine injected into right anterior chamber. Subsequent measurements: after 45 and 75 sec., 2 mm.; after 2 min., 2 mm.

After each acetylcholine injection in this and some of the other experiments, the syringe needle was not withdrawn from the eye for 45–60 sec., and the first measurements of the acetylcholine effects were taken with the needle still in the anterior chamber. The reason for this is as follows. It is known that the fall in intraocular pressure which is produced by paracentesis is followed by myosis due to congestion of the iris. It was therefore essential to be certain that the response to acetylcholine occurred *before* any fluid leaked out of the eye; by leaving the needle in for a minute it was possible to show that the response of the sphincter pupillae did occur before the leak. The measurements showed that the acetylcholine effect was, 30–60 sec. after injection, only 0·5 mm. short of its eventual maximum.

These experiments show that there is no decrease in sensitivity to acetylcholine in botulism of the eye. The muscle fibres of the sphincter pupillae appear to be unaffected and the toxin does not block the action of acetylcholine in the manner of atropine.

The vitreous body and the retina. After injections into the vitreous body there was an opacity within the eye. Ophthalmoscopic examination showed that the opacity was inside the vitreous body. There was also a small unabsorbed haematoma and a patch of choroido-retinal atrophy opposite the site of injection, in the eyes injected with toxin whether active or boiled. These reactions may have been caused by the trauma of injection.

Paralysis of the extrinsic muscles of the eye

Ophthalmoplegic symptoms were first observed after inoculations of the vitreous, but were later reproduced by injecting active toxin subconjunctivally. In the first case, the paralysis appears to be caused by a leakage of toxin out of the eye. When the syringe needle was withdrawn from the vitreous cavity of the eye a leakage of fluid out of the eyeball was observed in every instance. This fluid was trapped subconjunctivally, where, within a few minutes, it formed a small bleb, followed sometimes by the appearance of conjunctival oedema in the upper fornix. In two experiments, in which indian ink was mixed with the toxin and the needle was inserted into the vitreous body to a depth of 1–1·5 cm., the indian ink did not appear in the subconjunctival bleb at any time. Therefore it seems that the bulk of the injection does not leak out of the eye at once, for the indian ink at least is trapped in the vitreous. However, judging by the paralysis of the extrinsic muscles of the eye, the molecules of toxin are able to diffuse out along this path into the tissues of the orbit.

The changes which indicated a physiological action of the toxin inside the orbit were: (*a*) paresis of the m. levator palpebrae superioris with a resulting droop of the upper eyelid and a narrowing of the palpebral fissure; (*b*) paralysis of the m. retractor bulbi. This is a striated muscle which is supplied by the oculomotor nerve (Krause, 1884); as it retracts the eyeball, the nictitating membrane is drawn forward passively across the eye. In the intoxicated orbits the eye was slightly proptosed, and active retraction of the eyeball and reflex nictitation in response to corneal stimulation were abolished. In severe cases the absence of blinking led to the appearance over the cornea of white threads of stringy mucus. The cornea itself had a dry appearance, which may indicate some impairment of the secretion of tears.

Third-nerve stimulation. When the third nerve is stimulated in normal rabbits there is, besides the usual lateral and rotatory movement of the eye, visible retraction of the eyeball and nictitation. In these experiments the skin over the cranium and upper eyelid was removed prior to decerebration and, when viewed from above, it was seen that each time the eyeball was retracted a pad of orbital fat was forced out, as a bulge upwards, into the space between the frontal bone medially and its supraorbital process laterally.

These responses to third nerve stimulation were always present on the control sides, whether uninjected or injected with boiled toxin, but with adequate doses of active toxin they were all abolished. In a few experiments it was possible to produce this type of 'local' botulism without the appearance of generalized symptoms, by subconjunctival injections of 0·02–0·12 μg. of toxin. When larger doses of toxin were used it was necessary to perform the experiments within 24–48 hr., when the symptoms were still localized to the site of injection.

Analysis of the botulinic effect. In preliminary experiments it was noticed that the 'paralysed' extrinsic muscles of the eye would still retract the eyeball when acetylcholine (0·3–0·4 mg.) was injected into the homolateral common carotid artery. Further investigations were restricted to one of the extrinsic ocular muscles, namely the m. rectus superior. For these experiments 0·2–1·5 μg. of toxin was injected subconjunctivally either into this muscle or, more probably, around it in five rabbits. On the next day there were symptoms of paralysis in the upper group of extrinsic ocular muscles. Corneal stimulation elicited only slight downward rotation of the eyeball. In one of these animals it was noticed that the cornea had a dry appearance.

When tension records were taken from the m. rectus superior, it was found, on stimulating the oculomotor nerve, that the paralysis was incomplete in two of these animals. It was evident, in one of these, that the dose of toxin had been too small (0·2 μg. injected 20 hr. previously) and, in the other, that the toxin had not been allowed to act for a sufficiently long time (24 hr. after 0·6 μg.).

In the other three experiments, which were performed 27–43 hr. after the injection of 0·3–1·5 μg. of toxin on one side, a comparison of the myograms from the two superior recti showed that the paralysis was complete on the intoxicated side. Thus, faradic stimulation of the oculomotor nerve on the control side elicited repeatable tetanic contractions of the m. rectus superior on that side (Fig. 1 A and 2 A). In contrast, faradic stimulation of identical intensity applied to the nerve on the intoxicated side was repeatedly ineffective (Fig. 1 B and C; Fig. 2 C). A small contraction was obtained, in Fig. 1 D, when the electrodes were applied directly to the muscle.

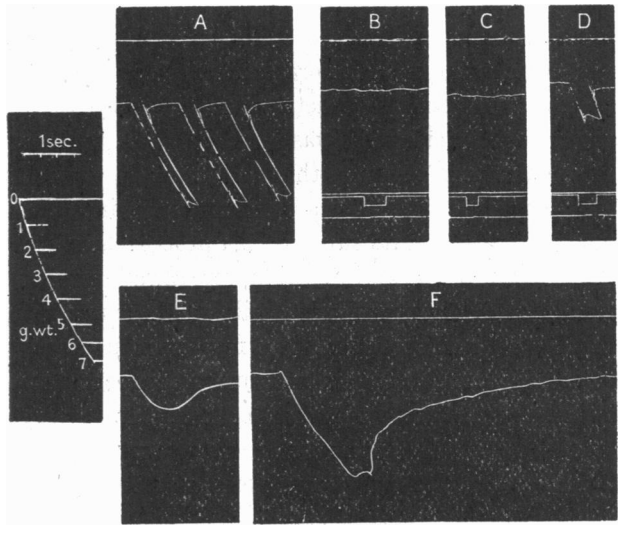

Fig. 1. Rabbit, 1·8 kg. 28 hr. after a local injection of 0·3 μg. of botulinum toxin round the left m. rectus superior. Decerebrated under nembutal (63 mg.); 2·5 mg. of atropine. Isometric myograms of the two recti superiores: A and E from the normal side; B, C, D and F from the intoxicated side. A, Three contractions of the normal muscle on electrical stimulation of the right oculomotor nerve (secondary coil at 7 cm.). B and C, An identical stimulus is applied twice to the left oculomotor nerve. The botulinic effect is fully developed and there is no response from the intoxicated muscle, which is however, contracted by direct electrical stimulation (at D). E and F, Effect of homolateral intracarotid injections of acetylcholine (300 μg.; pH_4). E, Normal muscle. F, Intoxicated muscle. Calibration in g.; the line of zero tension is shown at the top of each tracing. Time in 1 sec.

In all three of these experiments the paralysed muscles responded to acetylcholine. The animals were under the influence of atropine throughout the experiments, in order to prevent fatalities due to the 'muscarinic' action of acetylcholine on the heart, but as shown by Duke-Elder & Duke-Elder (1930) atropine does not interfere with the 'nicotinic' action of acetylcholine on the extrinsic ocular muscles.

In the first experiment a small contraction (1·3 g.) was recorded from the paralysed rectus superior muscle after the injection of 0·55 mg. of acetylcholine into the marginal ear vein. In the next experiment 1 mg. of acetylcholine (pH 4) intravenously was ineffective, but 0·3 mg. injected into the homolateral common carotid artery produced a contraction (5 g.) of the 'paralysed' muscle (Fig. 1 F) which was larger than the response to the same dose of acetylcholine

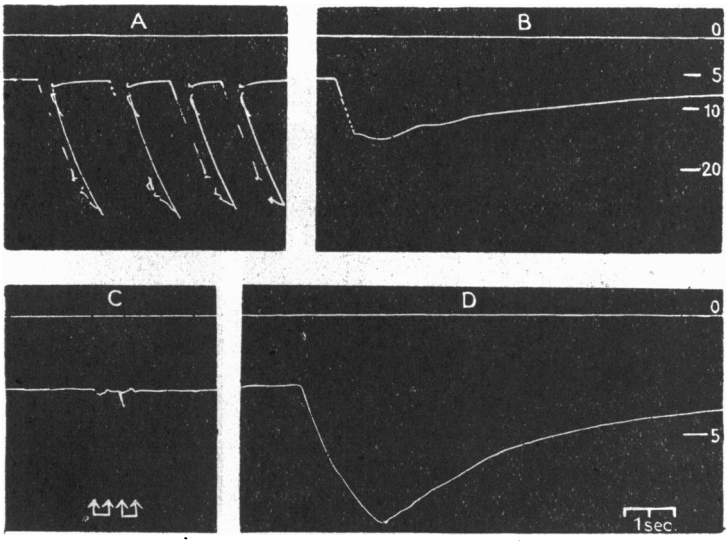

Fig. 2. Rabbit, 2·7 kg. 48 hr. after a local injection of 1·5 μg. of botulinum toxin round the m. rectus superior on one side only. Decerebrated under nembutal (78 mg.); 2 mg. atropine. *Control side.* A, Four responses to oculomotor nerve stimulation (secondary coil at 7 cm.). B, Effect of 1 mg. neutral acetylcholine injected into homolateral common carotid artery. Calibration in g. for A and B. *Intoxicated side*: (sensitivity of the lever increased in order to detect smaller responses). C, Ineffectiveness of oculomotor nerve stimulation repeated twice (secondary coil at 7 cm.). D, Response to 1 mg. neutral acetylcholine injected into homolateral common carotid artery. Calibration in g. for C and D. Line of zero tension shown at the top of each tracing. Time in 1 sec.

on the sound side (Fig. 1 E). In the last experiment (Fig. 2 D) 1 mg. of neutral acetylcholine, also intra-arterially, again produced contractions of the paralysed muscle, but this time the contractions were slightly smaller (6–7 g.) than the response (8 g.) to the same dose of acetylcholine on the opposite side.

Confirmatory experiments on the tibialis anticus muscle (cats)

Since this paper was submitted for publication an earlier paper by Guyton & MacDonald (1947) has come to the author's notice. The results described in this section are in agreement with their observations on the gastrocnemius in rabbits and guinea-pigs, and with those of Burgen, Dickens & Zatman (1948) on the rat's phrenic-diaphragm preparation.

Cats are able to tolerate larger doses of botulinum toxin, and one of the animals in this series survived for 8 days after it had received 50 μg. into one of its tibialis anticus muscles. The majority of these experiments, however, were performed on the third day after the intra-muscular injection. In every case it was found that, in doses of 10, 40, and 50 μg. the toxin had produced a total paralysis of the tibialis anticus (see Fig. 3B), whereas the muscle on the control side (injected with the same amount of boiled toxin in three of these experiments) responded normally to peroneal nerve stimulation (Fig. 3A). In

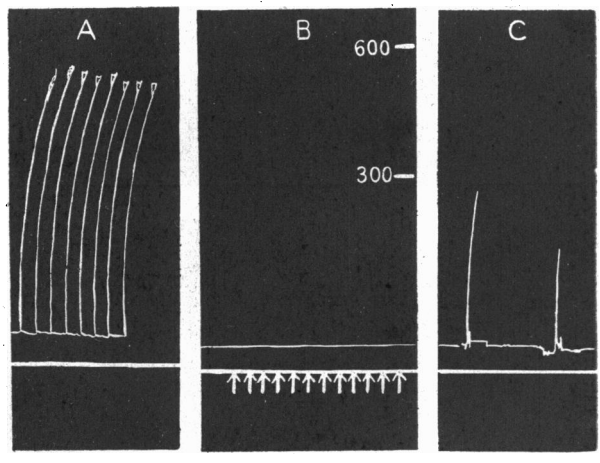

Fig. 3. Cat, 2·7 kg. Injected 3 days previously with 50 μg. of botulinum toxin into the right, and with the same amount of boiled toxin into the left, tibialis anticus. Anaesthetized with 105 mg. of nembutal intraperitoneally (and subsequent maintenance doses totalling 49·5 mg. over the 4 hr. of the experiments). A, Left tibialis (*control*). Twitches elicited by supramaximal stimulation of the left peroneal nerve once every 12·5 sec. B and C, From right tibialis (*intoxicated side*). B, Twelve stimuli of identical intensity and frequency were applied to the right peroneal nerve at the arrows; showing the muscle to be totally paralysed. C, Effect of close arterial injection of 10 μg. of acetylcholine, repeated twice. Bottom line indicates zero tension in all the records. Calibration in g. shown in B.

another experiment 10 μg. produced complete paralysis within 46 hr.; it is probable that this dose of toxin is effective within a much shorter time interval, but no experiments were performed to determine this. With 5 μg. of toxin paralysis was total after 45 hr. in one experiment; in a second cat, 45 hr. after the same dose, there were still measureable responses from the intoxicated muscle on motor-nerve stimulation, but the tension developed was only 25–45 g. as compared with 750 g. on the opposite side. It was noticed, in some of these experiments, that although there was no response from the intoxicated tibialis anticus on stimulating the peroneal nerve, there was, however, synchronously with each neon discharge during the period of stimulation, an extension of the toes on the same side. This appears to be due to excitation of the

m. extensor digitorum brevis, which is supplied by the same nerve lower down.

In four of these experiments, in which total paralysis had been obtained, a twitch could be elicited from the 'paralysed' muscle by close arterial injections of acetylcholine. One of these experiments is illustrated in Fig. 3, where the effect of two successive injections of 10 μg. of acetylcholine is shown at C.

These and the experiments in the preceding section show quite clearly that in somatic botulism, the voluntary muscle fibres, although effectively paralysed, can still react to acetylcholine. There is thus no similarity between this type of paralysis and that produced by curare.

Systemic effects (rabbits)

Intravenous injections. An approximate idea of the m.l.d. by this route can be gained from Table 3. All but one of the animals developed the classical symptoms of botulism. In addition to paresis and flaccidity of the muscles in the limbs, the neck and the abdominal wall, there was considerable salivation and the appearance of moisture round the nostrils. When the animals were moribund, the heart rate tended to be rather irregular and slow. Sluggishness of the pupil was seen in only one animal. At autopsy the lungs presented a consolidated, haemorrhagic appearance. Histological sections of these lungs showed that the consolidation was due to oedema and congestion; there were also areas of scattered haemorrhages into the substance of the lungs. The alveolar exudate contained no fibrin, polymorphs or red cells, which distinguishes these botulinic lesions from the pathological changes found in pneumonic consolidation.

Vitreous body injections. In view of the leakage of toxin out of the eye which has been mentioned, it is not surprising that some of the animals injected by this route developed symptoms of general botulism. But in order to produce the same effect approximately ten times as much toxin is required as by the intravenous route. The lung changes described above were found in one rabbit, (3·4 μg. toxin).

Conjunctival instillation. It is known that rabbits are relatively resistant to botulinum toxin by ingestion, but for reasons which will be apparent in the next paragraph, it was necessary to know whether the toxin is absorbed from the conjunctival sac, and the nasolachrymal duct. Very large amounts of toxin (4·54, 8 and 44·4 μg/kg. respectively) were therefore instilled into one or both conjunctival sacs in three rabbits. In all three there were no observable symptoms of botulism, either local or general, and the animals were still alive and well 16 days later. This confirms an old observation of van Ermengem (1897) in which, however, the amounts of toxin used were not specified.

Injections into the anterior chamber. After the injections a certain amount of aqueous humour was seen leaking out into the conjunctival sac, but as we have

just seen, the toxin is not absorbed through the conjunctiva. Therefore, if any systemic effect is produced by this route, it must indicate an absorption of toxin from the anterior chamber of the eye. With appropriate doses of toxin (see Table 3) this was found to be the case and the animals died with general

TABLE 3. Occurrence of systemic effects in rabbits (see text)

Route of injection	Dose of toxin (μg./kg.)	Time when general symptoms were first observed	Subsequent course	Findings at autopsy
Intravenous	0·035	—	Alive and well 16 days later	—
	0·045	70 hr.	Died between 80 and 92 hr.	Mild haemorrhagic lung lesions (confirmed histologically)
	0·05	45 hr.	Killed at 4 days (moribund)	Autopsy not performed
	0·08	45 hr.	Killed at 49 hr. (moribund)	Characteristic lung lesions
	0·25	19 hr.	Killed at 24 hr (moribund)	Lung lesions: oedema (confirmed histologically)
Anterior chamber	0·25	4 days	Killed at 6 days (moribund)	Lung lesions
	0·26	—	—	—
	0·29	—	Killed at 19 hr.	Autopsy not performed
	0·29	3rd day	Died between 3rd and 5th day	,, ,, ,,
	1	—	Alive and well 11 days later without general symptoms	—
	1·5	—	Alive and well 14 days later	—
	1·66	50 hr.	Died between 50 and 65½ hr.	Characteristic lung lesions (confirmed histologically)
	2·35*	—	Died between 24 and 65 hr.	,, ,,
	2·77	—	Alive and without general symptoms 9 days later	—
	3·37	—	Died in 18 hr.	No autopsy
Vitreous body	0·007 to 0·11 (6 rabbits)	—	—	—
	0·15	68 hr.	Killed at 68 hr.	Lungs appear normal at autopsy
	0·31	—	Killed at 70 hr.	,, ,,
	0·88	—	Killed at 42 hr.	No autopsy
	2·43	—	Died at 20 hr.	,,
	3·4	24 hr.	Died between 29 and 44 hr.	Characteristic lung lesions
Conjunctival instillation	4·54	—	Alive and well 16 days later. No symptoms then or subsequently	—
	8	—	,, ,,	—
	44·4	—	,, ,,	—

* Half this dose was injected into the anterior chamber proper. The other half accidentally into the substance of the cornea itself.

symptoms of paralysis and with characteristic lesions in the lungs. The presence of oedema was confirmed histologically in two of these animals. It would seem therefore that despite its very large molecular weight (1,130,000) the toxin can permeate out of the eye into the general circulation.

DISCUSSION

The results show that *Cl. botulinum* provides us with a neurotoxin which acts specifically on the nerve fibres of the cholinergic group. In one and the same organ, the adrenergic and sensory nerve fibres are relatively unaffected by it, although they have been exposed to exactly the same concentrations of toxin, within the eye, which are adequate to paralyse the cholinergic fibres. There is, therefore, no reason to believe that the toxin interrupts the conduction of nervous impulses, unless we are to believe that the basis of nervous conduction in cholinergic fibres is intrinsically different from that of other nerve fibres in the body, for which there is no evidence. And indeed, in several preliminary experiments in which 5–20 μg. of toxin have been injected within the sheath of the sciatic nerve well above its bifurcation, it has been found, that stimulation of that nerve 2–3 days later still elicits as usual vigorous contractions of the tibialis anticus (in cats). It is therefore, more probable that the specific action on the fibres of the cholinergic group is located at the nerve endings and that the toxin interferes with the process of humoral transmission. The suggestion that this interference is of the nature of a 'block', such as is seen after curarization or with atropine, receives no support from these experiments; on the contrary, it is evident that the muscle fibres, both plain and striped, respond vigorously to acetylcholine. It is more probable, therefore, that humoral secretion is at fault.

This neurotoxic effect shows a certain resemblance to the paralytic action of tetanus toxin previously described (Ambache, *et al.*, 1948). Both organisms, *Cl. tetani* and *Cl. botulinum*, are fairly close members of an anaerobic group of bacteria. It is perhaps worth stressing here that 'tetanic' symptoms may occur in botulism (convulsions and salivation) and botulinic symptoms in tetanus (ophthalmoplegia, and paralysis of the lingual and facial nerves). Despite the great difference in the molecular weight of these two bacterial products, it may be that their active parts, or the toxic derivatives produced from them within the body, are more closely related than was suspected.

One may perhaps make one further inference from these experiments. The molecular weight of botulinum toxin, which has been isolated in crystalline form as a single protein, has been found to be of the order of 900,000 to 1,130,000 (Kegeles, 1946; Putnam, Lamanna & Sharp, 1946). It is difficult to see how a molecule of this size could (*a*) be absorbed from the gut, (*b*) pass out of the blood stream through an intact capillary endothelium to reach the cholinergic nerve endings, and (*c*) escape out of the eye after anterior chamber injections. It appeared possible that the toxin was capable of producing a necessary change in membrane permeability by possessing a lecithinase activity as shown by Owen, Langohr & Blakely (1947) who used a crude toxin preparation. This might, for example, have accounted for the severe oedema of the lungs which

was found at autopsy. However, the toxin preparation which was used for these experiments was tested (Rees, 1948) by the lecithovitellin reaction and did not display any lecithinase activity. Nevertheless, it is possible that the toxin can, in some other way, induce a change in capillary permeability permitting its passage through the capillary membrane. Alternatively, or in addition, the original bacterial product may be a 'pro-toxin' which, when it enters the living organism, is broken down to smaller units of diffusible size which are still neurotoxic. In this connexion it was shown by Schübel (1923) that the toxin, although a protein, can withstand both peptic and tryptic digestion without loss of activity. It is also known (Krause, 1934) that both the anterior chamber and the vitreous body contain a protease, which might break down such a protoxin before it can escape out of the eye.

SUMMARY

1. The local effects produced by the intraocular injection of *Cl. botulinum* type A toxin have been studied in rabbits.

2. In appropriate doses the toxin produces a total paralysis of the cholinergic nerve fibres to the sphincter pupillae, which fails to react to light or to oculomotor nerve stimulation. The muscle fibres in the sphincter still respond at this stage to acetylcholine in small doses.

3. The adrenergic fibres within the eye are relatively unaffected by the toxin, and stimulation of the cervical sympathetic nerve dilates the pupil as usual.

4. The function of the sensory nerve fibres in the cornea appears to be intact and, if the toxin is injected into the anterior chamber of the eye, reflex blinking and retraction of the eyeball can still be elicited by mechanical or electrical stimulation of the cornea.

5. There is evidence that the toxin can escape into the general circulation from the anterior chamber and produce lesions in the lungs. The implications of this are discussed.

6. When injected subconjunctivally, or when it leaks out from the vitreous body, the toxin produces additional local effects on the extrinsic muscles of the eye. These include the paralysis of the m. retractor bulbi, which results in proptosis and a loss of nictitation.

7. The m. rectus superior was chosen for an analysis of the toxic effect on voluntary muscles. The 'paralysed' muscle does not respond to oculomotor nerve stimulation but is still contracted by intracarotid injections of acetylcholine.

8. These results were confirmed on the tibialis anticus m. of the cat. After local injections of toxin the muscle was completely paralysed when its motor nerve was stimulated, but responded to close arterial injections of acetylcholine.

9. These and other findings suggest that botulinum toxin exerts its paralytic action by means of a selective peripheral effect on the cholinergic nerve-endings.

I wish to express my gratitude to Dr G. L. Brown, for instructing me in the preparation of tibialis anticus, and to Prof. C. Lovatt Evans and Sir Stewart Duke-Elder for their advice and interest. My thanks are also due to Miss Jean Barrett for her help throughout this investigation, the expense of which was borne by a grant from the Medical Research Council.

REFERENCES

Ambache, N., Morgan, R. S. & Payling Wright, G. (1948). *J. Physiol.* **107**, 45.
Brown, G. L. (1938). *J. Physiol.* **92**, 22*P*.
Burgen, A. S. V., Dickens, F. & Zatman, L. J. (1948). *J. Physiol.* **107**, 41 *P*.
Dickson, E. C. & Shevky, R. (1923). *J. exp. Med.* **37**, 711.
Duke-Elder, W. S. & Duke-Elder, P. M. (1930). *Proc. Roy. Soc. B.* **107**, 332.
Edmunds, C. W. & Long, P. H. (1923). *J. Amer. med. Ass.* **81**, 542.
van Ermengem, E. (1897). *Arch. int. Pharmacodyn.* **3**, 213, 499.
Guyton, A. C. & MacDonald, M. A. (1947). *Arch. Neurol. Psychiat., Chicago*, **57**, 578.
Kegeles, G. J. (1946). *J. Amer. chem. Soc.* **68**, 1670.
Krause, A. C. (1934). *The Biochemistry of the eye.* Baltimore: Johns Hopkins Press.
Krause, W. (1884). *Die Anatomie des Kaninchens.* Leipzig: Engelmann.
Owen, C. R., Langohr, J. L. & Blakely, E. (1947). *J. Path. Bact.* **59**, 261.
Putman, F. W., Lamanna, C & Sharp, D. G. (1946). *J. biol. Chem.* **165**, 735.
Rees, R. (1948). Unpublished observations.
Schübel, K. (1923). *Arch. exp. Path. Pharmak.* **96**, 193.