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A FURTHER SURVEY OF THE ACTION OF *CLOSTRIDIUM BOTULINUM* TOXIN UPON DIFFERENT TYPES OF AUTONOMIC NERVE FIBRE

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The work done hitherto on botulinum toxin suggests that it acts by producing some functional derangement of cholinergic nerve fibres, probably at their secretory endings. It was shown in a previous investigation (Ambache, 1949) that intraocular administration of this toxin to rabbits, in amounts more than sufficient to paralyse the cholinergic fibres to the iris, did not seem to have any adverse effect upon the adrenergic fibres in the same eye. From these and other experiments it was concluded: (*a*) that the toxin does not interfere with the process of nervous conduction—a conclusion substantiated by the electrophysiological section of Guyton & MacDonald's (1947) paper; (*b*) that the toxin exerts its action specifically upon fibres of the cholinergic variety; and (*c*) that it probably produces some defect in acetylcholine secretion—a deduction which receives support from the results of Burgen, Dickens & Zatman (1949) on the failure of poisoned phrenic nerve-diaphragm preparations to release acetylcholine into the surrounding bath fluid. However, although the possibilities have been narrowed down, the nature of this paralysis still remains somewhat obscure. Burgen *et al.* have suggested that it might be due essentially to an injury of the non-myelinated terminal portion of the motor nerve fibres. This appears rather unlikely for the following reason: in the iris, the adrenergic fibres are non-myelinated throughout their length, whereas the cholinergic fibres are myelinated, except probably at their endings; this is the only known instance of myelination of post-ganglionic fibres in the parasympathetic system (Gaskell, 1920). Yet, as we have seen, the toxin distinguishes between the two sets of fibres, and acts preferentially upon those that are myelinated up to their endings. This phenomenon of the sparing of adrenergic fibres in the eye has now been confirmed in another species, the cat, and it is also shown in the present paper that these fibres, and their endings, are resistant to 200–300 times an amount of toxin which completely paralyses the cholinergic endings in the same iris.

In order to discover more about this toxin, it was felt that a survey of its action upon several different kinds of cholinergic fibres was desirable. The recent experimental work on it has been concerned largely with its effect on motor nerves, and its other known actions have not been subjected to a close analysis. For instance, it was shown in 1923, by Dickson & Shevky, that the toxin paralyses the following cholinergic nerves in the autonomic system: the oculomotor, the chorda tympani, the vagus, and the pelvic nerves to the bladder. But the site of the lesion in these nerves has not been accurately located, for interference with them could be due to a paralysis of either the pre- or the post-ganglionic endings, or of both. Unfortunately, in most parasympathetic nerves the post-ganglionic elements are short and inaccessible, being embedded in the effector organ. Their inaccessibility renders them unsuitable for an experimental analysis. The one exception is found in the orbit where the post-ganglionic fibres in the short ciliary nerves are of convenient length for electrical stimulation. Another convenient preparation of long post-ganglionic fibres is found in the sudomotor nerves. These nerves are of particular interest because, although they belong to the sympathetic system, their constituent fibres are classified as cholinergic.

From experiments on these two types of fibre, reported in this paper, it is clear that botulinum toxin does paralyse post-ganglionic fibres that are cholinergic, irrespective of their origin and of myelination. The paralyses in the autonomic nerves enumerated above could be accounted for entirely on this score, and the question then arises whether the toxin has, in fact, any effect upon pre-ganglionic nerve endings. Such an action is to be expected, since these fibres are also grouped as cholinergic. A study of the ciliary ganglion is reported in this paper, which shows that its preganglionic fibres are also poisoned by the toxin. A similar investigation on the susceptibility of the superior cervical ganglion is in progress, and will be reported later.

METHODS

The toxin preparation of type A has been the same throughout; the details of its toxicity (LD_{50}: 0·1 μg./kg. mouse), and of its cold storage in the dark as a 0·5 % solution in a 50 % glycerinated phosphate buffer (three batches of pH 6·84, 6·84 and 6·9 respectively) have been given in a previous paper (Ambache, 1949). The stock solutions tended to deposit a brownish sediment; they were therefore thoroughly stirred until this sediment was dispersed, before making dilutions from them in sterile saline.

Only two experiments were carried out with a type B botulinum toxin, made available through the kindness of Drs D. W. Henderson and J. Keppie, of the Microbiological Research Station, Porton, as a powder prepared by acid precipitation and freeze-drying; its toxicity (LD_{50}: 0·06 μg./kg. of mouse) was approximately 1·7 times as great as that of the A toxin. It was ground slowly, and suspended in the usual glycerol-buffer mixture (pH 6·84). The optimum pH, from the point of view of solubility and maximum stability, of pure type B toxin is said to be on the acid side of pH 4·5 (Lamanna & Glassman, 1947), which makes this toxin rather unsuitable for some kinds of experiment, e.g. those on 'local' botulism, because of the possibility of side-effects produced by such acid solutions.

Preliminary injections of toxin into various tissues were carried out under ether or intraperitoneal nembutal (40 mg./kg.) anaesthesia. Controls were performed by injecting into the corresponding tissue on the opposite side of the body an identical amount of toxin boiled previously for 5 min.; it may be necessary to prolong the boiling when very large doses of toxin are used.

Intra-ocular injections. Two procedures were used: (*a*) Before injecting toxin (active or boiled) into the anterior chamber, an equal volume of aqueous humour was withdrawn. The needle was left in the anterior chamber, but the syringe containing the aqueous humour was disconnected, and rapidly changed for one containing toxin. During the change-over a drop of aqueous humour was lost, so that the volume withdrawn was always larger than the volume of toxin injected. When the toxin was in the anterior chamber, it was thoroughly mixed with the remaining aqueous humour by a to-and-fro movement of the plunger. With this method there was usually a rush of aqueous humour on withdrawing the needle from the eye. Because of this leak through the puncture in the cornea, the effective amount of toxin was almost certainly not the whole of the dose administered, but some unknown fraction of it. (*b*) For this reason, the procedure was altered slightly for the last five experiments, in which it was essential to make quite sure that the very large doses of toxin which were used in these particular experiments (100–750 μg.) remained in their entirety in the anterior chamber. This was achieved by making the needle track through the cornea very oblique, and consequently valvular. A sharp needle is essential in order to leave a track with clean edges; it is best to use a new one for each injection. The needle (short-bevelled no. 27 s.w.g.) is driven into the cornea almost parallel with the corneal surface; once in the cornea, the needle is driven forward between the corneal lamellae for 2–4 mm., after which the point of the needle is dipped inwards into the anterior chamber. In these experiments the toxin solution was made up fresh from the powder, and dissolved in saline or in diluted glycerol-phosphate buffer (final concentration: 0·9% NaCl, 10% glycerol, 0·03% gelatin, 0·04% Na_2HPO_4), in order to avoid introducing unduly large amounts of glycerol into the eye. It was injected in a volume of 0·05 – 0·1 ml. without removing any aqueous humour, as it was felt that the valve-like needle-track would seal itself more efficiently at a higher intra-ocular pressure. Also, slow withdrawal gave better results, presumably by allowing time for the track to seal itself in the wake of the needle. The method was tested out with a dye on both a rabbit's and on a cat's eye, and proved effective for the retention of injected dye. It will be referred to in the text as the 'sealed' type of injection—the ordinary injections being 'unsealed'. A sample of the toxin solution used in two of these experiments was examined bacteriologically; both samples were found to be sterile (no growth in a broth culture after 72 hr.).

Pupillary measurements refer to the horizontal diameter of the pupil, as measured with calipers, except for those experiments illustrated by photographs in the text.

Intoxicated animals appear to be more susceptible to nembutal. When large doses of toxin have been administered, it is useful to reduce the anaesthetic dose of nembutal to 26 mg./kg. in the final experiment; even then, it may be necessary to apply artificial respiration.

Experiments on sudomotor nerves. Young kittens were anaesthetized and given a preliminary injection of toxin into the central hairless pads of the feet; one forelimb and one hindlimb pad was injected. The contralateral pads were in each case injected, as controls, with the same amounts of boiled toxin. With one exception, each injection was subdivided and given at four points on the pad (two in the middle section, and one in each lateral section of the pad).

Subsequently the cats were anaesthetized with ether or nembutal, 18–72 hr. after the administration of toxin. Both sciatic nerves were ligated and cut high in the thigh. The brachial plexus was also exposed on each side, and picked up in a loop of thread for stimulation.

Retrobulbar injections. The nictitating membrane was retracted slightly to the nasal side, and the tip of a blunt forceps was spread open in the conjunctival fornix on that side of the eye, pushing the eyeball gently laterally. A no. 15 syringe needle was then driven in through the fornix, tangentially to the eyeball at about '9 o'clock' (medially), or even at '8 o'clock' (slightly inferomedially), and then backwards with a slight movement curving round the eyeball, until the needle had been driven in for a distance of 1–1·5 cm. from its tip. Preliminary injections on a

cadaver, with the orbit dissected, showed that, if the syringe needle was driven in for the specified distance in this way, there was no injury to the eyeball; also, the tip of the needle was still well away from the optic and ciliary nerves and ganglia, but was within the conical space formed by the recti muscles. Toxin was injected in a volume of 0·1–0·3 ml.

Exposure of the ciliary ganglion and short ciliary nerves. On the appearance of the botulinic effect, 19–72 hr. from the moment of injection, the cats were anaesthetized again with nembutal, and decerebrated. The nembutal was necessary to suppress head movements and elevation of the blood pressure, which may arise reflexly during the dissection of the orbit. A trephine hole was made on each side of the skull, and the calvarium was completely removed with a bone cutter, to give a wide exposure of the brain. After incision of the dura, the brain stem was transected at the level of the bony tentorium, with care to avoid injuring the third nerve on each side. These nerves were either picked up gently in a loop of thread *in situ*, or cut and tied as close to the brain stem as possible. In order to free a greater length of nerve for stimulation, the dura was sometimes snipped carefully with scissors where it overlies the oculomotor nerve (the nerve can be seen through dura for some distance on its way to the superior orbital fissure). This removes an edge in the dura across which the oculomotor nerve tends to break on manipulation. The clamps on the carotid were released as soon as possible after the decerebration, before proceeding with the rest of the dissection.

The skin and fascia were then incised along the supra-orbital margin, and, with a blunt blade inserted through this incision, the orbital periosteum was separated from the bony plate which forms the roof of the orbit. On the superior side of this bone, the overlying dura was also incised and completely stripped away. The whole of this triangular plate of bone was then removed as far back as its apex at the optic foramen. Near the apex this must be done with care, because of the presence of four arteries which enter the orbit on the lateral side of the optic nerve (see Davis & Story, 1943, fig. 5). If these vessels are avoided the rest of the dissection is bloodless. A fine-pointed 'coldlite' illuminator was placed just above the orbit. Its use, and the application of warm saline swabs at intervals, prevented any drying of tissues in the orbit, an early index of which is a rapid blackening of the eyeball, which seems to be due to an increase in the transparency of the sclera, allowing the choroid to be seen through it.

Proceeding with the dissection, the ethmoidal and frontal vessels and nerves, which are seen immediately under the orbital periosteum, were cut between ligatures. The middle 1 cm. of the superior and lateral rectus muscles, and of the corresponding segments of retractor bulbi, was removed between ligatures, and these two muscle groups were pulled widely apart at their origin. This dissection exposes about 1 cm. of the optic nerve at the back of the eyeball, with the ciliary artery winding round it. The accessory ciliary ganglion is also visible, with two lateral short ciliary nerves arising from it, on the dorsolateral side of the optic nerve. Both short ciliary nerves were picked up on a fine thread for electrical stimulation with 100 μ. electrodes of pure silver 1·5 mm. apart, and insulated up to 2 mm. from the tips.

When necessary, the dissection was carried farther back to the ciliary ganglion proper, and its two efferent roots were included in a loop of thread. The disposition of the branches of the main and accessory ciliary ganglia in the cat is shown in a diagram by Whitteridge (1937). The position of the ciliary ganglion proper, as shown in that figure, is somewhat misleading. It is true that, at the end of a dissection, particularly after enucleation of the eyeball as in Whitteridge's experiments, the ciliary ganglion may be found pulled out laterally to the optic nerve, but in present experience it was found to lie either inferior or inferomedial to the optic nerve; it is necessary to curve round, and slightly underneath, the optic nerve in order to find it. This point was confirmed in a dissection of a cat's head preserved in formalin.

The cat was the animal of choice for these experiments on the ciliary ganglion. Guinea-pigs are too small, and rabbits were found to be unsuitable because of the existence in them of a large, bag-like, venous sinus (Davis, 1929, figs. 15, 16), enveloping the whole eyeball and the posterior two-thirds of the recti muscles. It is difficult to expose the apex of the orbit without incising this sinus, and the bleeding from it obscures further dissection.

Modified orbital dissection when using dibenamine. The dissection of the short ciliary nerves right back to the ciliary ganglion, as just described, was necessary for the following reason. In the cat, sympathetic fibres enter the larger, lateral, short ciliary nerve, usually at the level of the accessory ciliary ganglion (Christensen, 1936, p. 230). It was found that stimulation of the lateral short ciliary nerve beyond the accessory ciliary ganglion, or even near this ganglion on its proximal side, usually resulted in a dilatation of the pupil, i.e. the parasympathetic effect was masked by the simultaneous excitation of sympathetic fibres. To avoid this it was necessary to apply the electrodes well back, as near the main ciliary ganglion as possible, and with care to avoid spread of current. This necessitated the resection of the superior and lateral recti, and equivalent segments of the retractor bulbi. It was felt that this part of the dissection was undesirable because of possible interference with the venous return from the eye, which may anastomose with the veins of the extrinsic ocular muscles, and of the inevitable fall in temperature of the eyeball when its coverings are removed, both of which factors were perhaps responsible for the rather weak pupillary constrictions (about 2 mm.) sometimes obtained in normal eyes on stimulation of the short ciliary nerves in winter.

The experiment was therefore much simplified by blocking the adrenergic nerve supply to the iris pharmacologically with dibenamine (15–20 mg./kg. administered intravenously at least 30 min. earlier; Nickerson & Goodman, 1947). The effectiveness of this block was tested before the dissection was begun, by applying, at intervals, a maximal stimulus to the cervical sympathetic nerve pre-ganglionically until the dilator response was completely paralysed, or very nearly. After this, the dissection in the orbit was started, with the following modifications: the superior and lateral recti muscles and retractor bulbi were not resected; they were separated carefully as far back as possible, and a loop of thread was placed loosely round each of these two muscle groups, allowing retraction, at intervals, for the purpose of stimulation. On retraction, the optic nerve and its overlying ciliary nerves (long and short) became visible, and the electrodes were applied to the lateral short ciliary nerve *in situ*, a few millimetres behind the posterior pole of the eyeball.

Injection into the nictitating membrane (cats). The free cartilaginous end of the nictitating membrane was grasped at its pigmented border with iris forceps, and the movable part of the membrane was drawn forward. The needle was inserted into the nictitating membrane pointing backwards at a point 5 mm. or more from the free edge, on the nasal surface of the membrane; it was then directed on for 3–4 mm. under the epithelium towards the root of the membrane. The volume of toxin injected varied between 0·1 and 0·2 ml., and produced a visible swelling at the root of the membrane.

In the subsequent experiment (19–72 hr. later) the animals were anaesthetized with nembutal, and the contractions of the two nictitating membranes were recorded with an isometric lever. The threads from the nictitating membranes were disposed symmetrically over pulleys equidistant from the midline of the animal, and were tied to the same lever. When records were taken from one side, the pulley of the opposite side was raised until its thread was quite slack. It has been the aim to make the initial tension of the nictitating membrane the same for both sides, since it is known that the tension increment on nerve stimulation varies with the initial tension (Hampel, 1934), with a maximum at an initial tension of 10·75 g. The initial tensions varied from experiment to experiment in the present series, but were always very nearly the same for the two sides in any one experiment. The nictitating membranes were made to contract by maximal electrical stimulation of the cervical sympathetic nerves, both pre- and post-ganglionically.

A square wave generator (Attree, 1950; oscillator circuit of fig. 2, and pulse-generator of fig. 3) was used for electrical stimulation of the various nerves in these experiments. The characteristics of the stimulus, when quoted, are given in the following order: voltage, pulse width, frequency, duration of application.

RESULTS

Susceptibility of cholinergic postganglionic fibres

(1) *Parasympathetic: short ciliary nerves (myelinated).* In previous experiments on rabbits, referred to in the introduction, in which, after injections of toxin into the anterior chamber, there was neither a reaction to light, nor a response to oculomotor (pre-ganglionic) stimulation, it was inferred, largely by analogy with the phenomena observed in voluntary muscle, that the lesion was in the post-ganglionic nerve-endings within the iris. In view of the deliberate placing of toxin in the anterior chamber, this inference seemed reasonable. Although it would have been preferable to stimulate the short ciliary instead of the oculomotor nerves, i.e. post- instead of pre-ganglionic fibres, this was not feasible because, as already mentioned, the exposure of the short ciliary nerves in rabbits is attended by haemorrhage. But it has now been possible to test this point in the present experiments on cats. The species susceptibility of the cat to botulinum toxin is far lower than that of rabbits, so that higher doses of toxin have been used in the present series. All the injections referred to in this section were 'unsealed' (see Methods), so that the threshold dose of toxin is probably smaller than that found by this procedure.

Type A toxin was injected into the anterior chamber of one eye in eight cats. The doses of toxin were 5, 10, 10, 10, 12, 15, 25 and 500 μg. respectively, and the control eye usually received an equal amount of boiled toxin. Type B toxin (10 μg., pH 6·89) was used in only one experiment, with identical results.

The first of these cats was observed for several weeks, but was not used for a subsequent experiment, because the ensuing paralysis was subtotal. The pupil in the eye which had received 5 μg. of active toxin was much wider than the control, but there was still a residual reaction to light. In one other animal (10 μg.) the paralysis was subtotal at the time of experiment 19 hr. later. But in the remaining seven cats there was, sooner or later, both a marked difference in pupillary diameter between the two eyes, and a complete absence of reaction to light on the intoxicated side, indicating a total paralysis in the third nerve. The animals were then anaesthetized and decerebrated (1–11 days later), and the effect of nervous stimulation was observed.

In four of these experiments, stimulation (5–10 V., 1–3 msec., 20 cyc./sec., 5 sec.) of the intracranial part of the oculomotor nerve, i.e. pre-ganglionically, elicited a miotic response on the control side; the response was obtained at intervals on repeating the stimulus. On the intoxicated side there was not the slightest movement of the iris when the same stimulus was applied repeatedly.

In two of these experiments (both after 10 μg. type A), the ciliary ganglion was exposed on each side, and both medial and lateral short ciliary nerves were picked up in the same loop of thread for stimulation (2–5 V., 1–2 msec., 10–20 cyc./sec.) just distally to the ciliary ganglion. In both experiments the

results were clear-cut. Stimulation of the short ciliary nerves on the control side, as near the ciliary ganglion as possible, repeatedly constricted the pupil. The same stimulus applied to the short ciliary nerves on the intoxicated side was completely ineffective. The effect was tried of raising the voltage applied to the short ciliary nerves on the intoxicated side to 10 and 20 V.; this made no difference to the result, and raising the voltage further produced dilatation of the pupil, presumably through spread of current to the long ciliary nerves.

Use of dibenamine. As mentioned in Methods this type of experiment was greatly simplified by the use of dibenamine; and by blocking the adrenergic nerve endings in the eye, anomalous effects, due to spread of current from the short to the long ciliary fibres, could be avoided. The protocol of one experiment will illustrate this and several other points.

6.xii.49. Cat, 3·1 kg.; ether. 10 μg. type B toxin injected into left anterior chamber (unsealed). Right eye uninjected.

8.xii.49. Left pupil widely dilated but still reacts very slightly (1–2 mm.) to light.

12.xii.49. Horizontal diameters of the pupils in diffuse light: right 5 mm., left 13 mm.; left not reacting to light. 3.03 p.m., 110 mg. nembutal intraperitoneally. 3.21 p.m., right 2·5 mm., left 13 mm.

Subsequent maximal stimulation (5 V., $\frac{1}{2}$ msec., 50 cyc./sec., 5 sec.) of the cervical sympathetic nerve dilated the pupil by 8 mm. on the right (average of three readings) and by 2 mm. (13→15 mm.) on the left (average of two).

3.55–4.05 p.m., slow intravenous infusion of 40 mg. dibenamine HCl in 20 ml. of warm saline.

4.34 p.m. stimulation of cervical sympathetic dilates right pupil by 1·5 mm. (average of three); left pupil, by 0·5 mm. (twice).

Exposure of the optic nerve and stimulation, *in situ*, of the overlying short ciliary nerves (pulse width raised to 1 msec., and voltage 5 or 10 V.).

Right side. Constrictions at:

5.05 p.m., 7·5→5 mm.
5.12 p.m., 7·5→5 mm.
5.15 p.m., 7·5→6 mm.
5.16 and 5.22 p.m., 7 →6 mm.

Left side. Repeated stimulation completely ineffective; no flicker of movement in the iris.

(2) *Sympathetic: sudomotor fibres (non-myelinated).* As has been noted by earlier workers (see Burn, 1922), the secretion of sweat is easier to elicit in young animals, probably because the epithelium on the hairless pads of the feet tends to thicken with age. Accordingly, four kittens weighing between 0·85 and 1·7 kg. were injected subcutaneously with toxin into the central hairless pad of one fore- and one (usually the crossed) hindlimb; the control pads on the heterolateral (fore or hind) limbs were injected with the same amount of boiled toxin. The doses of toxin were 10, 20, 20, and 100 μg. After intervals of 21, 18$\frac{1}{2}$, 72 and 19 hr. respectively, the animals were used for experiment. In two of these experiments the kittens were anaesthetized with ether; at a certain stage in the anaesthesia sweat appeared on the control but not on the intoxicated pads,

showing the pattern of the 'denervation'. In a third animal there was no sweating at all during ether induction.

In all four of these experiments the sciatic nerves were exposed, and in three the brachial plexus was exposed as well. Stimulation of these nerves was begun on the control side, and the characteristics of the stimulus which was adequate to excite sweating were discovered. This stimulus was then repeated at intervals, producing the appearance of sweat on the pad and toes in all the control limbs. The appearance of sweat was timed with a stopwatch, and delays of 5, 10, 10 and 20 sec. were recorded in the four experiments. The same stimulus was then applied for the same duration, or longer, to the nerve on the intoxicated side. In every instance sweat appeared after the usual interval *on the toes*, which had not been injected with toxin. But on the pads there was either no sweat at all (in two of the forelimbs) or only a very few beads of sweat on the lower margin of the pad, and none in the centre of the pad (in four of the hindlimbs), contrasting markedly with the profuse sweating which had been observed on the control side. Raising the voltage of the stimulus further did not increase the amount of sweat on the intoxicated pads.

Action of drugs. The glands in the intoxicated pads could be made to sweat, sometimes copiously, by the administration of drugs. Sweating was elicited by acetylcholine (50–100 μg.) injected directly into the intoxicated pad, in two out of three experiments; in the third experiment, the responses to acetylcholine were very poor even on the control side.

In one experiment, sweating followed the injection of 0·1–0·2 μg. of 2268F into an intoxicated pad. 2 μg. elicited sweating in a control pad within 15 sec.

When these effects had subsided, the administration, in three of these experiments, of pilocarpine (0·25 and 0·5 mg. intravenously or subcutaneously) produced generalized copious sweating on all toes and pads in all the limbs within 0·75–2 min.

Thus, in every case, the administration of these various drugs produced sweating in what had been previously a sweatless area in the intoxicated pads.

Pre-ganglionic fibres

Ciliary ganglion. The symptoms produced by retrobulbular injection of active toxin, are twofold: those related to an impairment of function in the ciliary ganglion, and those related to other structures. To take the latter first: there was paralysis of the extrinsic ocular muscles in all of ten cats receiving 20–75 μg. of toxin; such animals would follow moving or distracting objects with the sound eye, whilst the other eye remained immobile. There was also a slight narrowing of the palpebral fissure.

The effect on the ciliary ganglion was manifest within 24–48 hr. (usually within 24). The pupil on the side which had received active toxin was widely dilated, and did not react to light. The difference between the two pupils is quite

as striking as that produced by injections of active toxin into the anterior chamber, although, as we shall see, it is due to a different type of lesion. It is shown in Pl. 1 where, for the sake of photographic convenience, advantage was taken of the fact that during deep nembutal anaesthesia the pupils, in the cat, become slit-like even in diffuse light, if the eyelids are open. Thus in Pl. 1 A and C, before the injection of toxin, nembutal produced a slit-like pupil on both sides. After these photographs were taken, toxin was injected retrobulbarly on one side, and boiled toxin on the other. Next day (Pl. 1 D), and 3 days later (Pl. 1 B), the animals were again anaesthetized with the same amount of nembutal, to show the disparity between the pupils. On the side with boiled toxin the pupil narrowed down to a slit as usual, but on the side with active toxin it remained widely dilated. Although, in the conscious animal, there was no reaction to light in the eye on the intoxicated side, yet careful illumination of that eye alone, by shining a light on it down a long paper tube, elicited a consensual reflex in the opposite, normal, iris. There was thus no evidence from these observations of any ill effects of the toxin upon the optic nerve fibres behind the eye; nor is there any impairment of the sympathetic fibres in the orbit, as we shall see below.

Analysis of the effect on the ciliary ganglion. In six of the above cats, preganglionic stimulation of the oculomotor nerve at its emergence from the brain stem was completely ineffective on the intoxicated side (e.g. Pl. 2 F); identical stimulation of the oculomotor nerve on the control side produced the usual constriction of the pupil (e.g. Pl. 2 E).

In four of these experiments, *post-ganglionic* stimulation of the short ciliary nerves on the intoxicated side, elicited a distinct constriction of the pupil, which varied in magnitude in the different experiments. The effect was photographed in the last experiment of this series, in which the use of dibenamine permitted the simplified version of the dissection; it is shown in Pl. 2 H. In one of these experiments (20 μg., 68 hr.), the pupil tended to remain constricted for some time after stimulation of the short ciliary nerves at 100 cyc./sec., and it was convenient, in this experiment and in one other (50 μg., 48 hr.), to dilate the pupil, in between short ciliary stimulation, by stimulation of the long ciliary nerves *in situ*. The constriction response was repeatable.

It has not been possible to obtain a maximal constriction of the pupil on stimulating the short ciliary nerves (frequency of stimulation ranging between 20 and 100 cyc./sec., and pulse width of $\frac{1}{2}$–3 msec.) either in intoxicated, or in normal, orbits. In three of the four experiments quoted above, this may have been due to simultaneous stimulation (see Methods) of the antagonistic adrenergic fibres both in the long ciliary nerves, and in the short ciliaries beyond the accessory ciliary ganglion—though Luco & Salvestrini (1942), stimulating beyond the accessory ciliary ganglion, i.e. a mixture of pupillo-constrictor and dilator fibres, have recorded constrictor effects.

In the fourth experiment (Pl. 2) this reason could not apply because the sympathetic fibres were blocked with dibenamine. The reason may lie in the condition of the eye, especially its temperature, after the orbital dissection.

The possibility was entertained of a spread of toxin from the retrobulbar injection site to the iris, for instance by diffusion through the sclera to the perichoroidal space, and thence to the iris. If this were happening, part of the paralysis described in this section could have been post-ganglionic.

Other forms of spread were considered, but seemed to be excluded. Thus the entry of toxin into the eye via the blood stream, after absorption into the general circulation, would appear to be insignificant, since the contralateral, control, eye showed no signs of intoxication. Again, lymphatic connexions between the inside of the eyeball and the outside are non-existent (Duke-Elder, 1938).

Three experiments were performed to test the passage of toxin through the sclera. Toxin placed subconjunctivally near the limbus lies on a layer which is continuous with the retrobulbar sclera; if the toxin could permeate or diffuse through the sclera, it would soon gain entry into the anterior chamber, and as little as 2·5 μg. (see below) would be enough to paralyse the sphincter. But this has not been the case, even after subconjunctival injection a few mm. from the limbus superolaterally, of several multiples of that dose, e.g. 50, 100 and 150 μg. of toxin. After 4, 3 and 1 days respectively, the condition of these three cats was the same. The toxin had produced a slight ptosis, and a paralysis of the extrinsic ocular muscles, as a result of which the cat would follow moving objects only with its unpoisoned eye. Tilting of the head produced rolling movements in the sound eye only. But there was no sign of paralysis in the iris, which reacted to light equally on both sides, and showed no tendency to dilate as it does after intracameral injections; in fact the pupil on the intoxicated side was slightly smaller than the control in the last two experiments. Moreover, this showed that, though the toxin had obviously spread back as far as the extrinsic ocular muscles, significant amounts had not reached the ciliary ganglion, which lies within the apex of the cone formed by these muscles.

It is also worth mentioning that in the cats receiving 50–500 μg. of toxin in one nictitating membrane (see below) the pupils were usually equal (smaller in one case), and both irises reacted to light 19–72 hr. later.

In conclusion, the experiments described in this section provide the first instance of a preganglionic paralysis produced by botulinum toxin. It is clear that the preganglionic fibres in question fall into line with other cholinergic fibres as regards susceptibility to this toxin.

The sparing of adrenergic postganglionic fibres

Previous experiments on rabbits' eyes (Ambache, 1949) have shown that botulinum toxin appears to act selectively on the cholinergic nerve supply to the iris. Doses of toxin which produced a complete paralysis of this set of nerve fibres seemed to have little or no effect on the pupillo-dilator fibres, which are adrenergic. This point has now been confirmed, and further elaborated, on cats; much larger doses of toxin have been used, some 200–300 times more than the threshold dose required to paralyse the cholinergic fibres, and the period of exposure to the toxin was prolonged to 2–3 days.

It was also thought desirable to investigate this problem of the apparent insusceptibility of adrenergic fibres to the toxin, on another muscle with adrenergic innervation. A bilateral structure is preferable for experiments of

this type, which require a control on the same animal, and the nictitating membrane of the cat presents itself as the most suitable preparation.

Pupillo-dilator fibres. (*a*) *Toxin in the anterior chamber* (*'unsealed' injections*). This section is based on the experiments already partly described on p. 6. The state of the adrenergic fibres was investigated only in those experiments in which the cholinergic fibres were completely paralysed.

Stimulation of the long ciliary nerves in two experiments (10 μg. type A toxin) and of the cervical sympathetic nerve preganglionically in three others (15 and 500 μg. type A; and 10 μg. type B toxin, already quoted in protocol on p. 7) produced repeated dilatation of the pupil. The dilator responses were measured only in the latter three experiments. They were of course smaller in magnitude in the intoxicated eye than in the control, but this is perhaps due only to the fact that the pupil is already considerably dilated on the intoxicated side, before sympathetic stimulation. But the pupil in the intoxicated eye appeared to dilate to its fullest extent as shown by the measurements below:

Type A toxin: (1) 15 μg. (*unsealed*); *11th day.*

Responses to identical cervical sympathetic stimulation:

Control 0·5→11 mm., 0·5→12 mm.
Intoxicated 9·5→13 mm., 10·5→14·5 mm.

(2) 500 μg. (*unsealed*); *3rd day.*

Responses to identical cervical sympathetic stimulation:

Control 1→ 9 mm., 1→10 mm., 1→10 mm.
Intoxicated 9→14 mm., 9→14 mm., 9→14 mm.

(*b*) *'Sealed' method of injection.* The sealed method of intra-cameral injection was introduced to overcome an element of uncertainty, present in the foregoing experiments, about the exact dose of toxin remaining in the anterior chamber after injection. This was a serious drawback in quantitative studies, for instance when it was desired to expose the iris to large, known, amounts of toxin.

The first experiment was performed to obtain some idea of the threshold amount of toxin which, when injected by this method into the anterior chamber will paralyse the cholinergic pupillo-constrictor fibres. A 'sealed' injection of 2·5 μg. into the left eye, and of 4·5 μg. into the right eye of the same cat, produced the usual mydriasis and loss of reaction to light; the paralysis was almost total on both sides (reactions of about 1 mm.) within 16–24 hr., and total within 3 days. Subsequently, e.g. 8 days later, the left iris would occasionally respond (*c.* 1 mm.) to light. From this experiment the threshold dose of toxin would appear to lie between 2·5 and 4·5 μg., but nearer to 2·5 μg.

In four other experiments 100, 500, 500 and 750 μg. respectively of toxin were injected into the anterior chamber. The toxin, in a volume of 0·05–0·1 ml., was mixed several times with the aqueous humour (the volume of which, in a cat, is about 1 ml.) before withdrawing the needle from the eye. On withdrawal, the eye was carefully observed, but no leakage comparable to that which occurred

in the previous experiments was seen. Once or twice a single small drop of fluid was expressed from the needle track, during the instant it took to close up after withdrawal. This loss represented a relatively insignificant fraction, estimated at 5% of the dose administered, as the toxin was now distributed in the whole 1 ml. of the aqueous humour. The anterior chamber did not become shallow, and the eye remained hard, and could be squeezed gently without loss of fluid through the puncture.

In these eyes there was no reaction to light in the conscious animal. Illumination of the intoxicated eye elicited a consensual light reflex in the sound eye in one experiment after 500 μg. During deep nembutal anaesthesia, the paralysed iris did not contract down to a slit (see Pl. 3A) as it is wont to do in normal eyes. It must be mentioned also that massive doses (100 μg. and above) of the toxin gave rise to severe reactions in the injected eyes. Within 24 hr. there was an exudate, fibrinous in appearance, and thought to be non-infective in origin, in the anterior chamber, and signs of keratitis in the whole cornea. One of the cats receiving 500 μg. was showing symptoms of general botulism on the second day.

Nevertheless, and despite these pathological changes induced by massive doses of the toxin, stimulation of the cervical sympathetic nerve on the 3rd day (2nd day in experiment of Pl. 3), still dilated the pupil of the intoxicated eye in all 4 experiments. The extent of this dilatation was: in experiment no. 1 (100 μg.) 2·2 mm., average of seven observations; no. 2 (500 μg.) 2·5 mm., average of seven; no. 3 (500 μg.) 3 mm. (see Pl. 3B); no. 4 (750 μg.) 1·7 mm., average of three observations made just after death of the animal. Assuming the threshold dose required to produce complete paralysis in the cholinergic endings of the short ciliary nerves to be *c.* 2·5 μg. (from the first experiment), then these experiments show that exposure of the iris for 3 days to about 200–300 times that amount (500 and 750 μg.) of toxin, failed to extinguish the function of the adrenergic nerve-endings in the same iris.

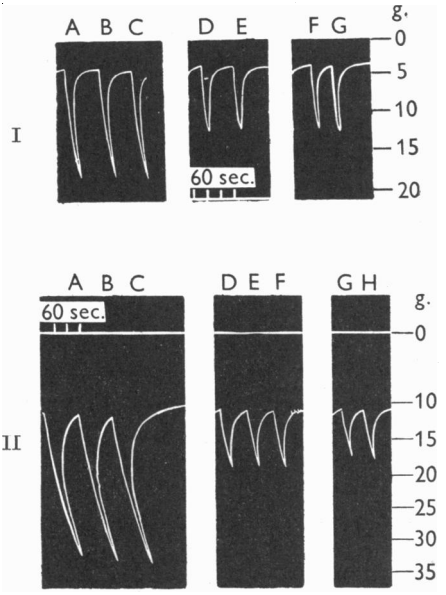
(*c*) *Retrobulbar toxin.* The sparing of the adrenergic fibres by a retrobulbar injection of a paralytic dose of toxin (50 μg.) is illustrated in Pl. 2C.

Nerve supply to the retractor muscle in the nictitating membrane

Five cats were injected with 50, 100, 100, 250 and 500 μg., respectively, of toxin into the nictitating membrane on one side; in experiment no. 3 the cat had also received 100 μg. of toxin subconjunctivally. The heterolateral membrane was injected with boiled toxin in the first two, and uninjected in the other three experiments. The response of the membrane to nerve stimulation was examined 68, 72, 50, 19 and 46 hr. later.

A comparison of the effect of maximal pre-ganglionic stimulation of the cervical sympathetic nerves on the two sides showed that the toxin had produced a reduction in the response of the nictitating membrane in all five

cats. This reduction amounted to 38, 49, 55, 52 and 62% respectively. The response of the intoxicated nictitating membrane was usually the same, whether the stimulus was applied pre- or post-ganglionically (Text-fig. 1), though



Text-fig. 1. Effects of botulinum toxin on the nictitating membrane. I. Cat, 3·2 kg., injected with 50 μg. active toxin into the left, and 50 μg. of boiled toxin into the right, nictitating membrane, under ether. Acute experiment 68 hr. later, under nembutal. Alternate recording from the two membranes (contractions downwards) at the same initial tension. *A*, *B*, *C*, responses to maximal pre-ganglionic sympathetic stimulation on the control side. *D*, *E*, responses (on the intoxicated side) to maximal *pre-ganglionic*, and *F*, *G*, to maximal *post-ganglionic* sympathetic stimulation. The responses to pre- and post-ganglionic stimulation are equal on the intoxicated side, indicating absence of ganglion block, but are only about 60% of the response obtained on the control side. The difference could be attributed to paralysis of cholinergic fibres in the post-ganglionic supply of the nictitating membrane.

II. Cat, 4·9 kg. Right nictitating membrane injected with 500 μg. of active toxin; left side uninjected. Acute experiment 48 hr. later under nembutal; alternate recording from the two nictitating membranes at the same initial tension. *A*, *B*, *C*, contractions of the left, normal, nictitating membrane produced by maximal (*A* and *C*) and twice-maximal (*B*) preganglionic stimulation. *D*, *E*, *F*, contractions of the right (intoxicated) nictitating membrane produced by maximal preganglionic stimulation. *G*, *H*, contractions of the right nictitating membrane produced by maximal (*G*) and supramaximal (*H*) postganglionic stimulation. The response to preganglionic stimulation is reduced by 62% on the toxin side, but is still not extinguished despite the very large dose of toxin administered.

occasionally it was slightly smaller on postganglionic stimulation, presumably because of injury to the postganglionic trunk in the process of dissecting it away from the vagus.

Before interpreting these results, it is necessary to comment on the size of the doses used in the last two experiments. Previous experience has shown that 5 μg. of this toxin are enough to produce complete paralysis of the entire tibialis anticus muscle in cats in 45 hr. In contrast, after injections of 50–100 times as much toxin into the nictitating membrane, a very much smaller structure, there is no extinction of its response to nervous stimulation. This is not due to lack of diffusion of toxin, as observation showed that the toxin had spread to other structures in the orbit. For instance, there was in all five experiments a paralysis of the extrinsic muscles of the eye, which remained immobile, even when the animal followed distracting objects with the other eye.

Thus, the persistence of a certain fraction of the nictitating response in the face of such large doses of toxin, may be taken to indicate a considerable degree of resistance of the adrenergic fibres to this toxin. The observed reduction in the response may be due, at least in part, to paralysis of the cholinergic fibres which are known to be present in the nictitating membrane. It is known that this smooth muscle has a dual pharmacology; the responses to adrenaline and to acetylcholine are identical, and sum along the same curve (Morison & Acheson, quoted by Cannon & Rosenblueth, 1937, p. 186). From this, one might suspect the existence of a dual innervation, and indeed Bacq & Fredericq (1935) found evidence thereof in three out of five cats examined. The present results suggest that it may occur with greater frequency. At the same time, the present experiments cannot exclude the possibility that a fraction of the reduction in the nictitating response might be due to lesion of adrenergic fibres. It is also not known what would happen if the toxin were allowed to act for longer periods than 3 days.

DISCUSSION

The first section of this paper establishes the susceptibility to botulinum toxin of cholinergic postganglionic nerve-endings in both divisions of the autonomic system. Qualifying for inclusion in this section is the finding that, after local injections of toxin into the wall of the intestine, the motor response to stimulant doses of nicotine is lost or sometimes inverted, indicating a paralysed state of the cholinergic post-ganglionic neurons imbedded in the gut. The details of these experiments are being published separately (Ambache, 1951), because of the light they throw on the presence in the intestine, of local inhibitory ganglion cells, which appear to be the cell-bodies of short adrenergic neurons.

The last section of the paper establishes the resistance of adrenergic fibres, in two different muscles, to many times the threshold dose of toxin which will paralyse the cholinergic fibres in the same situation. Whether other types of non-cholinergic fibres and endings are equally resistant is a matter for conjecture at the moment, although it is known, clinically, that sensation is unim-

paired in botulism. If they should turn out to be so, then this toxin may prove to be useful as a weapon of physiological investigation, for instance for mapping out cholinergic neurons in the central nervous system. Even now, it is already helpful in distinguishing between cholinergic and adrenergic fibres in the peripheral nervous system, and in producing a differential 'denervation' of the former in a tissue with a mixed innervation.

The middle section provides the first instance of a pre-ganglionic paralysis produced by this toxin. Although a release of acetylcholine at the preganglionic endings in the ciliary ganglion has never been demonstrated, it is known (MacIntosh, 1941) that this ganglion has a fairly high content (12 μg./g.) of acetylcholine. Taken together with this fact, the susceptibility of this ganglion to botulinum toxin is suggestive of the presence of a cholinergic transmission mechanism within it.

Analogous observations have been made in preliminary experiments on the superior cervical ganglion, after local application of toxin. A preganglionic paralysis is produced in the pupillo-dilator pathway, resulting in a pupillary disparity in the conscious animal, with the smaller pupil on the toxin side.

SUMMARY

1. The susceptibility to botulinum neurotoxin of a number of different nerves of the autonomic system has been examined.

2. Postganglionic fibres of the cholinergic variety are susceptible, whether they occur in the parasympathetic, or in the sympathetic, system. The short ciliary nerves were chosen as an example of the former, and the sudomotor fibres as an example of the latter.

3. Paralysis of the ciliary ganglion has been produced by retrobulbar injections of toxin. Analysis of the effect has located the lesion in the preganglionic fibres.

4. Adrenergic postganglionic fibres to the dilator pupillae are resistant to 200–300 times the dose of toxin required to paralyse the cholinergic fibres in the iris.

5. Despite the fact that the postganglionic innervation of the nictitating membrane is mixed, some evidence was obtained of the resistance of a proportion of the nerve fibres, probably the adrenergic ones, to large amounts of toxin.

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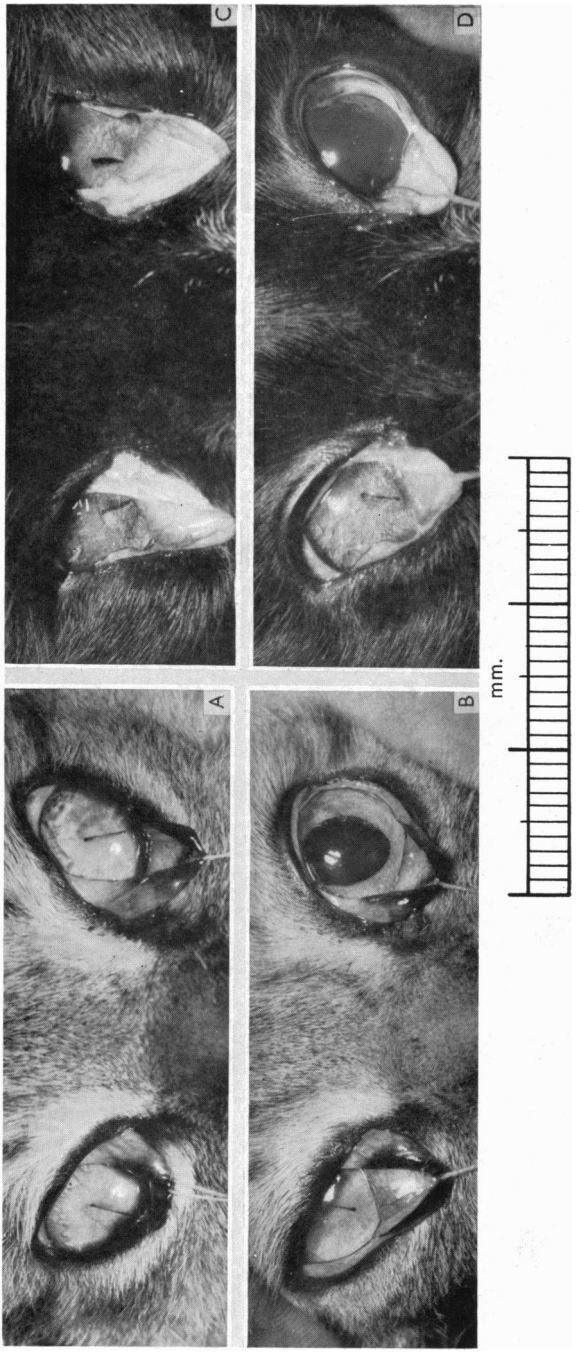
EXPLANATION OF PLATES

Plate 1

The type of pupillary paralysis produced by retrobulbar injection of botulinum toxin, in two cats. A and C show the initial equality of the pupils, before the retrobulbar injections; both cats anaesthetized with nembutal. The pupils are slit-like, which is the normal appearance, in diffuse light, of a cat's pupil during deep nembutal anaesthesia. After these photographs were taken, 50 μg. of active toxin were injected retrobulbarly on the left, and 50 μg. of boiled toxin on the right. This eventually abolished the reaction to light on the left in both cats, in the conscious state. B. Grey cat, photographed 72 hr. later, again under nembutal (same dose as before). The right pupil has narrowed down to a slit as usual, but the left remains dilated. D. Black cat photographed 22 hr. after the injection of toxin, and showing the same phenomenon, but more markedly. Calibration in mm. The nictitating membranes are retracted by means of threads sewn through the free border of the membrane, in order to uncover the iris for photography. This and all subsequent photographs taken with short flashes of 0·2–0·25 msec. duration.

Plate 2

Cat, 3·5 kg.; under nembutal. Preganglionic paralysis in the ciliary ganglion produced by a retrobulbar injection of botulinum toxin (50 μg.) on the left side 2 days earlier; the same amount of boiled toxin was injected retrobulbarly on the right. A. Shows the difference between the pupils on the two sides. On the right the pupil is slit-like as usual. On the left the pupil, which did not react to light in the conscious cat, remains widely dilated. B. Dilatation of the pupil on the right (control) side by maximal stimulation of the right cervical sympathetic nerve for 5 sec. (50 cyc./sec.). C. Identical stimulation of the left cervical sympathetic nerve produces further dilatation of the pupil on the intoxicated side, to its maximum; showing that the long ciliary fibres in the orbit are resistant to the toxin. Between



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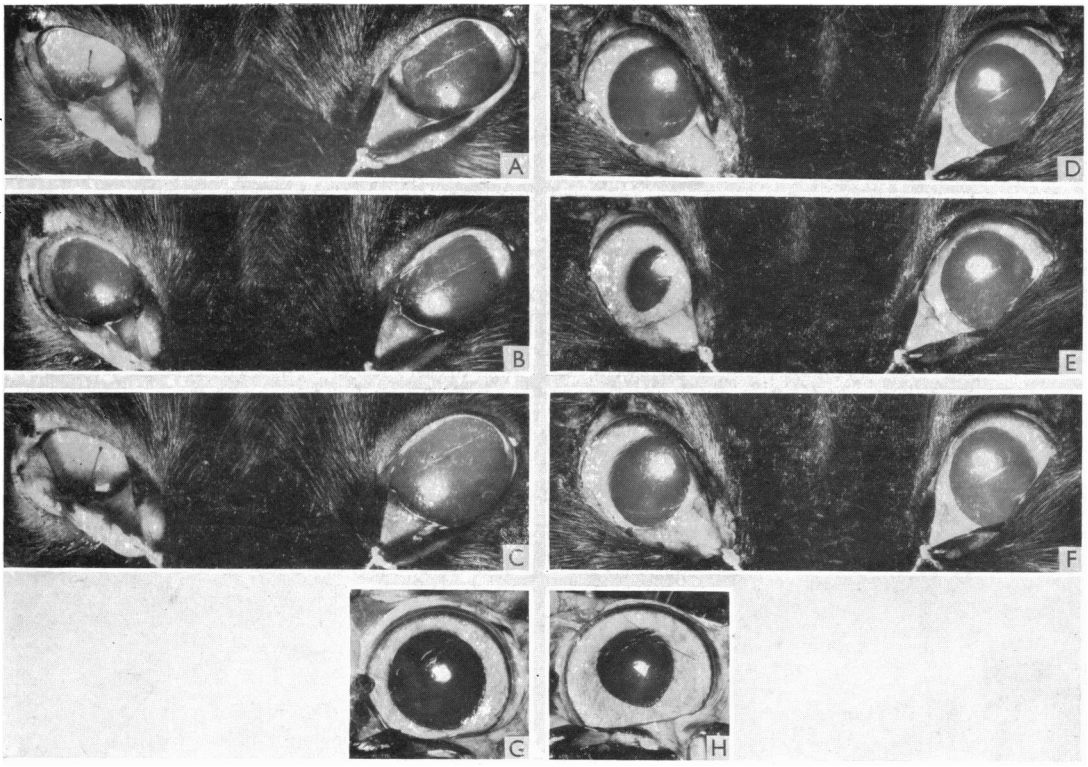
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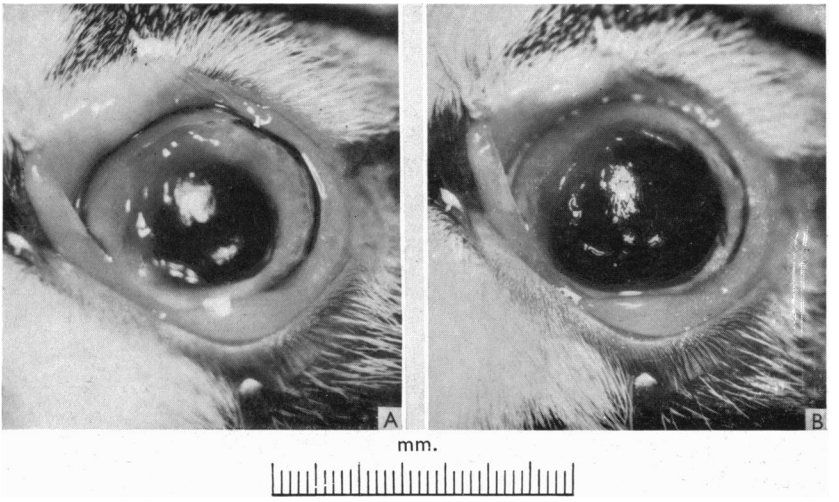
A
B
mm.

C and D the cat was decerebrated (which abolished the difference between the pupils, and a slit was made through the outer canthus on each side, for better exposure of the iris. Slow intravenous infusion of 70 mg. of dibenamine HCl ¾hr. before D. D. Shows the equality of the pupils after decerebration. E. Miotic response photographed at the end of the fifth second of stimulation (30 cyc./sec.) of the right (control) oculomotor nerve, preganglionically. F. Taken at the end of the fifth second of identical stimulation of the left oculomotor nerve. No response whatsoever, on the paralysed side, to preganglionic stimulation. Between F and G, dissection of the left orbit. G. The left eye at the end of the dissection. H. Effect of stimulation of the left short ciliary nerves *in situ* for 2 sec. at 100 cyc./sec. Note the appearance of a response on the intoxicated side to post-ganglionic stimulation.

PLATE 3

Experiment illustrating the sparing of the adrenergic fibres in the iris, even by very large doses of botulinum toxin. Cat, 3·2 kg.; under nembutal, 52 hr. after an intracameral injection by the 'sealed' method, of 500 μg. of active toxin into the right eye. The cat was exhibiting symptoms of general botulism. Because of the milky opacity of the cornea produced by such large doses of toxin, the photographs of the iris were taken with infra-red light. A. The right pupil at rest. It is dilated (10 mm.) as usual, even though the cat is under nembutal, owing to the paralysis of the sphincter nerves. B. The integrity of the dilator muscle and of its nerve supply is shown by the further dilatation (to 13 mm.) of the pupil, which is obtained on maximal preganglionic stimulation of the cervical sympathetic nerve. Photograph taken at the fifth second of stimulation. Evidence of the keratitis is provided by the broken reflexions from the whole surface of the cornea.