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THE ACTION OF TETANUS TOXIN ON THE RABBIT'S IRIS

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As a result of his investigation of the peripheral action of tetanus toxin, Harvey (1939) suggested that the condition of 'local tetanus' is caused by a disturbance of the normal processes of cholinergic transmission at the neuromuscular junction of skeletal muscle. The effect of this toxin on smooth muscles innervated by cholinergic fibres does not appear to have been studied hitherto. We have begun such an investigation by observing the effects on the muscles of the iris that result from the injection of a small quantity of tetanus toxin into the anterior chamber of the eye. This has proved a satisfactory method of localizing the effect of the toxin, and has shown that this substance exerts a paralysing action on the cholinergic fibres to the sphincter pupillae.

MATERIALS AND METHODS

Dried preparations of ammonium sulphate-precipitated toxin were made from cultures of the CN655 strain of *Cl. tetani* by the method described by Ipsen (1941*a*). The powdered toxin was kept in a vacuum desiccator, and fresh solutions of it in sterile 0·9% NaCl were made shortly before each inoculation. No solution was more than ½ hr. old at the time that it was injected. The toxicity of the dried preparation was repeatedly tested by intravenous injection into mice and showed little deterioration over the period of the experiments. The LD_{50} of the dried toxin, calculated from Ipsen's (1941*b*) Table 83, was 0·075 μg. The powder contained a small amount of $(NH_4)_2SO_4$ as an impurity.

Before inoculation, the rabbits were lightly anaesthetized with 'veterinary nembutal' (0·75–1·5 c.c. intravenously), and, to ensure local insensibility, 2 drops of 5% cocaine-HCl were instilled into the conjunctival sac. When the cornea became anaesthetic, the eyeball was immobilized as much as possible by pressing on it from below with one finger placed on the medial half of the lower eyelid—the pressure being applied dorso-laterally towards the roof of the orbit. The carefully sharpened needle (size 26) of a tuberculin syringe was then introduced into the anterior chamber through the cornea just in front of the sclero-corneal junction at the inner or outer canthus (see Fig. 2, 2), and 0·05 c.c. of the solution was injected. The amount of toxin varied from 0·25 to 200 μg., but in most of the experiments a standard dose of 25 μg. was adopted.

In control experiments, the same volume of one of the following solutions was injected into the opposite eye: (1) toxin solution of the same concentration, in which the toxin had been neutralized by the previous addition of an amount of antitoxin (Wellcome tetanus antitoxin globulins:

1000 i.u./c.c.) which was estimated to represent about fifty times the immunological equivalent of the toxin; (2) toxin solution of the same concentration but previously mixed with a potent *Cl. botulinum* Type A antitoxin (containing less than 1/100 i.u. of tetanus antitoxin)—it was estimated that in the final mixture any tetanus antitoxin present would neutralize less than $\frac{1}{1000}$th of the toxin present; (3) the usual toxin solution boiled for 5 min.; (4) 0·9% NaCl solution.

In the experiments upon the sympathetically denervated iris, the superior cervical ganglion was removed aseptically on both sides in two rabbits. The structures removed were examined histologically and identified as sympathetic ganglion. An interval of 9 and 10 days respectively was allowed for the degeneration of the post-ganglionic fibres to the iris, before proceeding to inoculate the eye with toxin.

The inoculated eyes were observed at intervals, and the pupillary diameter was measured (to the nearest mm.) on both sides with a ruler placed just in front of the eye. This measurement was carried out for the two sides under identical conditions in diffuse daylight, and was recorded without correction for the error introduced by the refractive power of the cornea. The reaction to a sudden illumination with a 60 W. lamp at 7 cm. distance from the eye, was also measured on both sides. In addition, a number of photographic records of pupils was obtained by a method essentially the same as that described by Thomson (1947), to whom we are indebted for the loan of the necessary apparatus. This consisted of a Cine-Kodak special camera fitted with a device for synchronizing electrically the flash from a photo-flood lamp with the opening of the camera shutter; this flash was produced by the opening of an electromagnetic shutter fitted to the front of a light-tight box containing the photo-flood lamp (750 W.), and lasted 0·01 sec. By this means, photographs were taken of the iris before it had had time to react to the light. The rabbit was placed in a rabbit-box and its head held immobile at a standard distance from the lens. For the purpose of subsequent measurement, a millimetre scale was also photographed at the same distance. The pupils were photographed in each experiment (i) in diffuse light; (ii) after a period of dark-adaptation, which, owing to the difficulty of keeping the animal still for a longer period, was standardized at 3 min.; and (iii) after a 30-sec. period of intense illumination with two 100 W. lamps with reflectors at a standard distance (25 or 50 cm.). For the photographic studies, albino rabbits were used.

In the experiments on electrical stimulation of the nerves supplying the iris, the rabbits were anaesthetized with nembutal and the cervical sympathetic nerves were exposed on both sides. The skull was trephined and its bony vault removed with bone forceps. Haemorrhage was controlled by diathermic coagulation of bleeding points. The cerebral hemispheres were scooped out, exposing the optic nerves which were cut anteriorly to the chiasma. The intracranial portion of each oculomotor nerve was identified on the roof of the cavernous sinus.

In these last experiments the diameter of the pupil was measured on both sides with calipers. Measurements were taken before and after anaesthesia, and before and after faradic stimulation of equal intensity and duration of the cervical sympathetic and oculomotor nerves on both the intoxicated and the control sides.

RESULTS

Nature of pupillary changes produced by the toxin

Mydriatic effect. Within 24–48 hr. of the inoculation, a change was observed in the size of the pupil on the side injected with active toxin. In diffuse daylight the pupil was visibly larger than before the inoculation and more open than that on the control side. Ruler measurements made on the two sides are listed in Table 1. Similar differences in size were obtained from measurements of the photographs of the two eyes after they had become dark adapted.

This mydriatic effect was once produced with as small a dose of toxin as 0·25 μg.; we failed, however, to obtain it in two other animals with this dose

and later in the same animals with 2·5 μg. It was produced with unfailing regularity in ten rabbits by a dose of 25 μg. Other rabbits inoculated with 50 μg. or more also showed the same pupillary changes. In none of these animals did any sign of tetanus appear either in the muscles of the orbit or elsewhere; the effect of the toxin appeared to be entirely localized to the eye. In a few instances the inoculation gave rise to a small patch of turbidity in the aqueous humour of the anterior chamber. This cleared up after 5 or 6 days and was not the cause of the paralysis observed, since the paralysis developed in the absence of any turbidity, and in those in which turbidity did appear, the mydriatic effect persisted long after the opacity had disappeared.

TABLE 1. Changes in pupillary diameter in twelve rabbits, produced by intra-ocular tetanus toxin (ruler measurements)

Dose of toxin (μg.)	Control eye	Time of measurement (days after inoculation)	Vertical diameter of the pupil in diffuse light (mm.)	
			Control side	Intoxicated side
0·25	Uninjected	7	5	10
25	Uninjected	3	7	11
		7	8	12
25	Uninjected	3	5	9
		6	6	10
		13	7	10
		17	6	9
		20	7	7
25	Same amount of toxin boiled for 1 min.	1	4	10
		5	5	10
		30	6	10
25	25 μg. tetanus toxin + excess of tetanus antitoxin	10	5	11
25	25 μg. tetanus toxin + *Cl. botulinum* antitoxin	5	10	10
		11	10	9
		18	6	10
25	25 μg. tetanus toxin + *Cl. botulinum* antitoxin	1	10	10
		5	12	12
		12	12	12
		15	10	12
25*	Uninjected	8	8	10
50*	Uninjected	1	8	8
		3	7	10
		8	6	10
		15	7	9
50	0·1 c.c. of sterile 0·9 % saline	1	8	10
		5	8	10
50	Uninjected	3	5	11
		6	7	11
		14	7	11
		17	6	10
50	Uninjected	1	4	8
		3	6	11
		8	5	11

* Both superior cervical ganglia excised 9 and 14 days previously.

The pupillary changes were usually obvious within 48 hr. of inoculation: in several instances they began within 24 hr. The mydriasis lasted for 2½–5 weeks.

Reaction of the iris to light. For 24–48 hr. after the mydriatic effect had developed, weak reactions to light were sometimes obtained, but by the fourth day this response had disappeared (Pl. 1, fig. 1). At this more advanced stage, ruler measurements taken before and during bright illumination (60 W. lamp at 7 cm.) showed a complete absence of the ipselateral light reflex on the inoculated side, whereas it was present in every case in the control eye. The more accurate photographic measurements, which confirm this absence of reaction to light, are listed in Table 2. In these experiments, the eye was

TABLE 2. Absence of reaction to light in tetanus intoxicated eyes (photographic measurements)

		Horizontal pupillary diameter (mm.)			
		Control side		Intoxicated side	
Dose of toxin (μg.)	Time of measurement (days after inoculation)	Dark adapted (3 min.)	Light adapted (30 sec.)	Dark adapted (3 min.)	Light adapted (30 sec.)
25	4	6·5	3	9	9
25	6	9	6	10	10
25*	10	5	3·5	11	11
50	3	7	4	10	9·5

* This rabbit received the same dose of toxin, neutralized by an excess of tetanus antitoxin, in the control eye.

illuminated with a 200 W. lamp at 50 cm. distance for a period of 30 sec. before the photographs were taken. More intense illumination with a 750 W. photo-flood lamp at 13 cm. for 5 sec. was equally ineffective. The absence of light reflex did not appear to be due to any failure of light perception on the part of the retina, because when the inoculated eye was approached with some object or subjected to intense illumination, powerful nictitation of the three eyelids was observed.

Specificity of the reaction

The following control experiments were performed to demonstrate that these pupillary effects were produced specifically by the tetanus toxin:

(i) *Experiments with tetanus antitoxin.* Three rabbits were inoculated in the left eye with 25 μg. of toxin, and in the right eye with the same amount of toxin which had been neutralized just previously by a gross excess of tetanus antitoxin. In all three animals, the typical syndrome developed in the left eye, but the right was unaffected, the pupil showing no change in diameter and the iris reacting to light in the usual manner.

(ii) *Effect of a non-specific serum* (Cl. botulinum *antitoxin*). That the protective action of tetanus antitoxin is not due to a non-specific effect of the serum was shown in two experiments in which it was replaced in the mixture with the toxin, by a batch of *Cl. botulinum* Type A antitoxin which was practically

free from tetanus antitoxin. This antitoxin was chosen because the paralytic symptoms produced by tetanus toxin bear a certain resemblance to those seen in botulism, and it was, therefore, of interest to see whether they could be prevented by *Cl. botulinum* antitoxin. It was found that this antitoxin was devoid of any protective action since the tetanus toxin produced a mydriasis and loss of reaction to light as severe in its presence as without.

(iii) *Other controls.* In two other experiments, the control eye was injected with 0·05 c.c. of toxin solution (25 μg.) which had been boiled for 5 min., and with the same volume of sterile 0·9% NaCl solution, respectively. In neither of these controls did the responses typical of the intoxication appear.

Effect of the toxin on the iris after sympathetic denervation

To exclude the possibility that the dilator effect of the toxin was due to the overaction of the sympathetic nerve fibres and endings in the radial muscle of the iris, experiments were performed on two rabbits in which the superior cervical ganglion on both sides had been excised 9 and 10 days previously. Injection of the standard dose of toxin was followed by the usual mydriatic effect in these eyes, and this change was also associated with the usual loss of response to light.

Effect of acetylcholine

To test the contractility of the sphincter pupillae in the intoxicated eyes, four of the inoculated animals were anaesthetized with nembutal, and 0·05 c.c. of a sterile solution of acetylcholine-HCl dissolved in 0·9% saline was injected into the anterior chamber on the affected side. In the first experiment the dose of acetylcholine-HCl was 20 μg., but in the others it was 1 μg. In each instance there was a powerful constriction of the pupil (Pl. 1, fig. 2), within 10–15 sec. of the injection. In the last experiment (on a rabbit at the 30th day of the intoxication) the duration of this acetylcholine effect was compared with the effect on the normal side. It was prolonged on the intoxicated side and a slight myosis was still present 24 hr. after the injection.

Effect of eserine

A myosis could also be produced in the intoxicated eyes by instilling eserine into the conjunctival sac (Pl. 1, fig. 3). A 1% solution of eserine sulphate dissolved in 0·9% saline was used at first and myotic effects were obtained with 1–2 drops (0·06–0·12 c.c.) in four rabbits. The first noticeable change in the pupil (a reduction in diameter of 1 mm.) occurred after 5–6 min. and the change was complete within 10 min., the final diameter of the pupil being 2–3 mm.

To compare the time course of this myosis with that of the normal eye, experiments were made in which the eserine was instilled simultaneously into the conjunctival sacs on both sides, and measurements made of the two pupils

at intervals subsequently. With fresh 1% eserine, the myotic effect was so powerful that there was little difference between the control and intoxicated eyes, although the onset of the myosis appeared slightly earlier in the former. In later experiments weaker solutions of eserine (0·5, 0·1 and 0·01%) were used, and, with these, the myotic effect of eserine was often weaker on the intoxicated side, and was also slower in its development and sometimes in onset.

Effect of stimulating the oculomotor and cervical sympathetic nerves

On the induction of anaesthesia before the exposure of the nerves for stimulation, it was observed that the pupil of the intoxicated eye underwent contraction, while that of the control eye dilated, the diameters of the two becoming approximately equal. It was from this intermediate equilibrium position (see Langworthy & Ortega, 1943) that the effects of the subsequent brief faradic stimulation (5–10 sec.) of the oculomotor and cervical sympathetic nerves were determined. The immediate changes in pupil diameter which resulted from this excitation are shown in Table 3. After these measurements

TABLE 3. Changes in the diameters of pupils (averages of three to six determinations) after stimulation (of equal strength and duration) of the oculomotor and cervical sympathetic nerves on the control and intoxicated sides

Dose of toxin (μg.)	Days since inoculation	Nerve stimulated	Changes in pupil diameter (mm.) Control side	Intoxicated side
100	6	Cervical sympathetic	+2·0*	+2·0*
		3rd	−4·3	−0·2
100	19	Cervical sympathetic	+3·7	+2·5
		3rd	−1·6	nil
50	12	Cervical sympathetic	+2·3	+2·0
		3rd	−3·8	−0·5

* Single observations only.

had been made, eserine was introduced into the intoxicated eye, either by the instillation of 2–5 drops of a 1:1000 solution of eserine sulphate, or by the intraocular injection of 100 μg. of the drug. On repeating, after a few minutes, the stimulation of the oculomotor nerve to the intoxicated eye, small constrictions of the pupil occurred even when the paralysis had formerly been total; the pupils of the three animals showed contractions of 1·5, 1·0 and 1·0 mm. respectively.

These experiments show that tetanus toxin has little or no effect on the adrenergic nerve fibres which supply the dilator muscle of the iris. On the other hand, the cholinergic nerve fibres to the sphincter pupillae from the oculomotor nerve, appear to be almost completely paralysed by the toxin, though an improvement takes place in the presence of eserine.

DISCUSSION

The injection of small quantities of an active preparation of tetanus toxin into the anterior chamber of a rabbit's eye results in a slowly developing and persistent mydriasis. This effect might result either from an over-action of the dilator muscle of the iris (or of the adrenergic nerve fibres to it), or from a paralysis of the sphincter pupillae (or of its cholinergic nerve supply). It seems unlikely that the former explanation is correct because the pupillary response to light is lost simultaneously, and also because the mydriasis occurs in its typical form in animals in which the previous removal of the superior cervical ganglion has ensured the degeneration of the post-ganglionic sympathetic nerve fibres. Indeed, sympathetic denervation does not materially affect the development of this mydriasis, either by the interruption of inflowing nervous impulses, or by the sensitization of the dilator muscle fibres to humoral influences. It appears more likely that the pupillary dilatation and the absence of reaction to light, result from a paralysis of the more powerful sphincter pupillae, which in the albino rabbit is the muscle principally concerned with the response of the iris to light (Langworthy & Ortega, 1943). However, since this muscle still contracts promptly and powerfully when a small quantity of acetylcholine is injected into the aqueous humour, it is evident that the injurious effect of the toxin is not falling primarily on the muscle fibres themselves. Coupled with this fact, the absence of a normal response to oculomotor nerve stimulation, and its partial restoration in the presence of eserine would suggest that the paralysis results from a disturbance of the humoral transmission at the cholinergic nerve-endings in the sphincter. The paralysis from tetanus toxin thus differs radically from that produced by atropine, for, unlike atropine, the toxin does not appear to produce a block between the transmitter and the muscle fibres.

Stimulation of the cervical sympathetic nerves shows that the function of the adrenergic fibres is little affected by the toxin. From this fact, together with the augmentation by eserine of the myosis produced by oculomotor nerve stimulation, it may also be inferred that the toxin does not interfere so much with the conduction of nervous impulses as with humoral transmission.

The paralytic effect of tetanus toxin in this nerve-muscle system may be compared with that described by Harvey (1939) in voluntary muscles in local tetanus. He observed that despite the persistence of local spasticity the tension response to a maximal motor-nerve volley became progressively reduced as the intoxication developed, and after 2–3 weeks it sometimes disappeared entirely, although direct stimulation of the muscle fibres either electrically or by the intra-arterial injection of acetylcholine produced a contraction that was at least as great as normal. In his discussion, Harvey suggested that the toxin may bring about its paralytic effects by causing an

abnormal leakage from, and eventually a depletion of, the acetylcholine depots at the cholinergic nerve endings, and compared this state of the nerve-endings with that found during nerve degeneration. This associated paralysis which is present in experimentally produced local tetanus is in line with the occurrence of paralytic forms of tetanus which have been observed clinically in man (see Courtois-Suffit & Giroux, 1918), particularly in patients with cephalic tetanus, who may exhibit ophthalmoplegia and paralysis of the lingual and of the whole or part of the facial nerves.

There are minor clinical features of tetanus, usually thrown into the background by the more striking skeletal muscular disturbances, which suggest that the activity of the other parasympathetic nerves in the body may be similarly deranged in this intoxication. Retention of urine is of common occurrence, and manometric determinations of intravesical pressure made by Eastman & Nesbit (1942) on a patient with tetanus, have shown that the bladder may be completely atonic. Tachycardia, which cannot be accounted for by concurrent pyrexia, has been described in a series of patients suffering from tetanus by Dean (1917). Clinically, also, constipation is often a troublesome feature of this disease, and we have observed a complete absence of peristaltic movements and retention of faeces in the intestines of tetanus-intoxicated mice when the viscera have been examined immediately after death. There is no suggestion from these clinical data, or from our own experiments, that the onset of paralysis in these parasympathetic nerves is preceded, as in the voluntary motor nerves, by a phase of spasticity. Thus, we have not seen any sign of myosis at any time before the onset of the pupillomotor paralysis. The comparison between the effects of tetanus toxin and those of nerve degeneration is again applicable here, for when a parasympathetic nerve to smooth muscle is cut, there is no phenomenon in the muscle comparable to the fibrillation seen after motor-nerve section. The difference may be due to the absence of end-plates in the muscle systems concerned.

SUMMARY

1. The injection of tetanus toxin into the anterior chamber of the rabbit's eye results in a dilatation of the pupil and a loss of reaction to light. This effect starts within 1–2 days of the injection and lasts 2½–5 weeks.

2. The reaction is specific and is prevented by tetanus antitoxin, but not by the antitoxin to *Cl. botulinum* Type A.

3. The effect is produced in the sympathetically denervated iris.

4. Under anaesthesia the pupillary dilatation on the intoxicated side is reduced. Stimulation of the cervical sympathetic nerve then produces dilatation of the pupil on the intoxicated as well as on the control side. On the other hand, stimulation of the oculomotor nerve has little or no effect on the pupil of the toxin-injected eye, except after eserine when real myotic responses were observed.

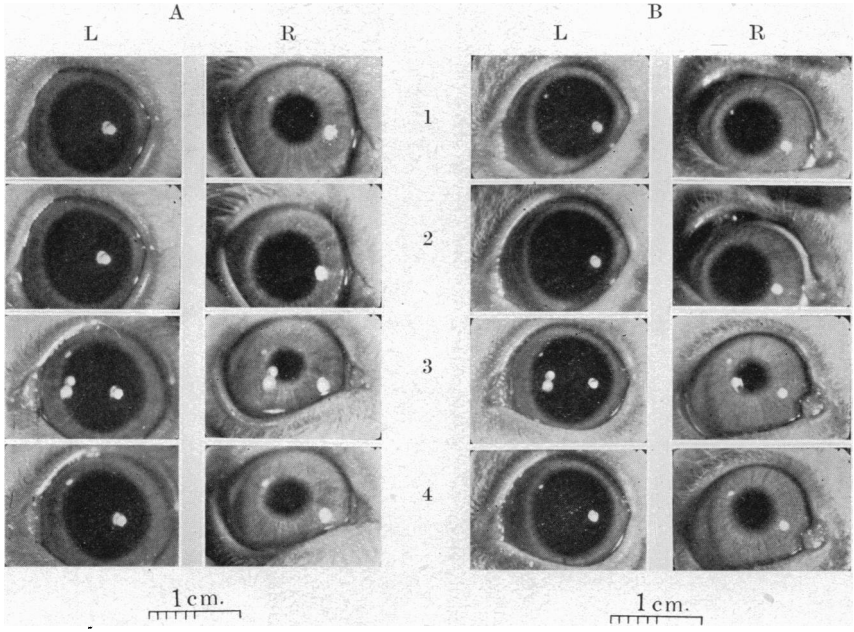

Fig. 1.

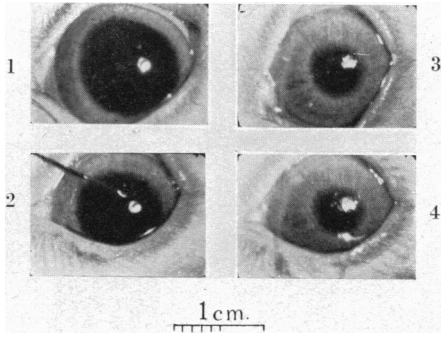

Fig. 2.

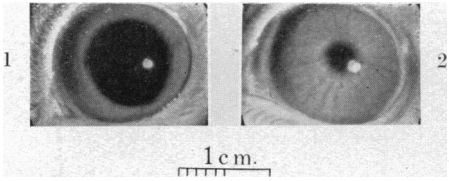

Fig. 3

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5. The mydriasis was not due to a paralysis of the muscle fibres of the sphincter pupillae, because this muscle can still respond vigorously to acetylcholine. The results suggest that tetanus toxin does not affect the smooth muscle or the adrenergic nerve fibres in the iris, but that it paralyses specifically the cholinergic nerve-endings of the oculomotor nerve.

We are grateful to the Director of the Wellcome Physiological Research Laboratories for providing us with the strain of *Cl. tetani* used for preparing the toxin, and for the gift of botulinus antitoxin. Most of these experiments were performed in the Department of Pathology, Guy's Hospital Medical School, but a few were continued by one of us (N.A.) at the Department of Physiology, University College, London; some of the expenses of this research were covered by a grant to him from the Ella Sachs Plotz Foundation.

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EXPLANATION OF PLATE 1

Fig. 1. Successive photographs of the pupil showing the mydriasis and loss of reaction to light which is produced by tetanus toxin. Two rabbits were injected with toxin in the left eye (50 μg. in A, 25 μg. in B) the right eye serving as a control. Photographs were taken of both eyes on the 3rd (in A) and the 4th (in B) days of the intoxication. 1. The pupils in diffuse light. 2. After 3 min. dark adaptation. 3. Effect of 30 sec. intense illumination (200 W. at 50 cm. distance). 4. Illumination just ended. Calibration in mm. The white spots are due to reflexion of lamps.

Fig. 2. Successive photographs of a rabbit's eye which was injected with 50 μg. of tetanus toxin 13 days previously, showing the response of the iris to an intraocular injection of acetylcholine. 1. Initial size of the pupil (10·5 mm. horizontal diameter). 2. The syringe needle is introduced into the eye and its point is seen in the anterior chamber; 1 μg. of acetylcholine HCl was injected at this moment and the needle was withdrawn. 3. 30 sec. later; the diameter of the pupil is now 6 mm. 4. 90 sec. after the injection (pupil 5 mm.).

Fig. 3. Myotic effect of eserine in an intoxicated eye (25 μg. of toxin injected 10 days previously. 1. Initial size of the pupil (10 mm.). 2. After eserine, 1 drop of 0·5% eserine sulphate was instilled into the conjunctival sac 20 min. before this photograph was taken. Pupil 4 mm.