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THE ACTION OF BOTULINUM TOXIN ON THE NEURO-MUSCULAR JUNCTION

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It has been known since the work of Edmunds and others (Edmunds & Long, 1923; Edmunds & Keiper, 1924; Dickson & Shevky, 1923*a*, *b*; Schübel, 1923; Bishop & Bronfenbrenner, 1936) that nearly all the phenomena of botulism could be explained primarily in terms of a peripheral action blocking somatic motor nerves and the parasympathetics. Guyton & MacDonald (1947) have recently confirmed and extended many of these observations in the intact animal. Modification of the factors involved in production of paralysis by the toxin can be more readily analysed in an isolated preparation.

We have found that if suitable concentrations of toxin are employed a rapid and reproducible neuro-muscular block can be produced in the isolated rat phrenic nerve-diaphragm preparation of Bülbring (1946). This preparation is adaptable and has proved very suitable for the analysis of the action of the toxin.

Torda & Wolff (1947) have claimed that choline acetylase is strongly inhibited by the toxin and that this might explain how the toxin produces neuromuscular block. The action of the toxin on this system and on the closely related aromatic amine acetylase has been investigated further.

METHODS

Toxin. Type A toxin was obtained from *Clostridium botulinum* (strain 4587) grown in a medium consisting of 25% tryptic digest of casein, 10% yeast extract, and 1% glucose. The ingredients were prepared as in CCY medium (Gladstone & Fildes, 1940). The toxin was purified by acid precipitation with HCl at pH 3·9, then rendered soluble by elution with 0·2 M-Na_2HPO_4 to pH 6·0. The solution was freeze-dried and stored as a powder *in vacuo* over phosphorus pentoxide. As required, the toxin was dissolved in a buffer consisting of 1% Na_2HPO_4 and 0·2% gelatin at pH 6·6 to give a concentration of 2×10^5 or 2×10^6 mouse LD_{50}/ml. (20 g. mice, intraperitoneally). The toxin contained $1{\cdot}3 \times 10^7$ LD_{50}/mg. N. (Crystalline type A toxin contains $2{\cdot}4 \times 10^8$ LD_{50}/mg. (Lamanna, McElroy & Eklund, 1946), i.e. this material was *c.* 5% pure toxin.) In the text the amount of toxin used is expressed in terms of mouse LD_{50}/ml. of bath fluid which is for convenience called a unit.

Immunized rats. Rats were immunized by four fortnightly, intramuscular injections of alum-precipitated botulinum toxoid. Representative groups were subsequently shown to survive a challenge dose of toxin containing approximately 1×10^6 rat LD_{50}. Nerve-diaphragm preparations and antitoxic serum were obtained from this group of rats.

Rat diaphragm preparation. The preparation was as described by Bülbring; the bath fluid was Tyrode solution containing 0·2% glucose and aerated with 95% O_2, 5% CO_2. The bath volume was 100 ml. The phrenic nerve was stimulated through submersible electrodes (Brown, 1948) by slightly supramaximal rectangular pulses of 0·25 m.sec. duration delivered at a rate of 6/min. from an electronic stimulator. Either a torsion wire isometric lever or a spring-loaded lever was used to record the contraction. The preparation was always stimulated for ½–1 hr. before starting an experiment to allow stretching of the muscle to become complete and for the preparation to settle down to a steady contraction. In control experiments the diaphragm continued to contract for at least 3–4 hr. without appreciable decline.

Acetylcholine output. Two half diaphragms without their costal attachments were suspended in a 15 ml. bath in Tyrode solution containing 1 part/million eserine sulphate. After tetanizing at 50 cyc./sec. for 20 min. the fluid was pipetted off and assayed for acetylcholine by its depressor effect on the blood pressure of the chloralosed cat.

Acetylation of sulphanilamide. The enzyme was an acetone-dried powder of pigeon liver (Lipmann, 1945) ground with ice-cold KCl and phosphate buffer (pH 7·4). The supernatant was added to the rest of the system containing 0·02 M-$MgCl_2$, 0·02 M-sodium acetate, 0·05 M-NaF, 0·5 mg. ATP-7′-P and 0·12 mg. sulphanilamide, in a total volume of 3 ml. After incubation at 37° C. for 1 hr. the reaction was arrested with trichloroacetic acid and the free sulphanilamide estimated by Bratton & Marshall's (1939) method.

Choline acetylase. (*a*) The procedure of Torda & Wolff was followed except that in order to avoid loss of acetylcholine the solution was made acid with N/3 HCl before boiling to destroy the enzyme, and subsequently neutralized with N/3 NaOH. The yield of acetylcholine from minced mouse brain by this method is poor.

(*b*) The acetone dried rat brain powder system, as described by Feldberg & Vogt (1948), was used but without added activator.

The acetylcholine was assayed on the frog rectus abdominis muscle.

Choline esterase. Choline esterase activity of 1 ml. guinea-pig serum was estimated at 37° C. by Mendel & Rudney's (1943) method using acetylcholine, acetyl-β-methylcholine, and benzoylcholine as substrates.

RESULTS

Action of botulinum toxin on the rat diaphragm

After the addition of 1000–5000 units of botulinum toxin (Type A) to the bath fluid there is a latent period of 25–40 min. during which no effects of the toxin are visible on the tension record of the diaphragm to single motor nerve twitches. The tension then declines, at first gradually, then more rapidly and later again more slowly, so that the tension record shows a sigmoid form ending within 70–100 min. in complete paralysis of the diaphragm to nerve stimulation (Fig. 1). If the amount of toxin added to the bath is increased to 20,000–40,000 units the latency is only slightly decreased to 15–30 min. and the rate of paralysis somewhat accelerated. With 200–500 units the latency is increased in duration and the rate of paralysis is slowed; with smaller amounts still, no change is detected in preparations run for about 2 hr. 2000 units of toxin can be relied upon to produce paralysis regularly in a convenient time and this dose has been used for most of the experiments.

Toxin heated in a water-bath at 100° C. for 15 min. became inactivated, and lost its power to produce paralysis.

If the Tyrode containing the toxin was replaced by fresh Tyrode after the toxin had been in contact with the diaphragm for 5 min. or more, both the duration of the latent period and the rate of paralysis were unchanged. When washing was carried out during the first 5 min., however, the duration of the latent period was prolonged and the rate of paralysis slowed—an effect equivalent to reducing the amount of toxin added initially. It is evident that the toxin is 'fixed' by the preparation early in the latent period and that slowness of diffusion into the muscle is not responsible for the delay in the onset of paralysis.

The rat diaphragm was found to contract well over a wide range of temperature from 12 to 38° C. On cooling from 38° C. the tension at first increased

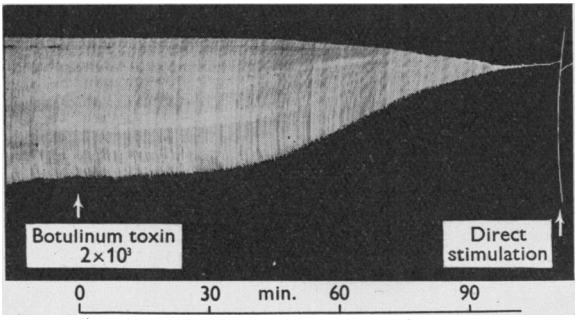

Fig. 1. Rat diaphragm preparation. Temp. 37·8° C. Botulinum toxin 2×10^3 mouse LD_{50}/ml. added. Normal response to direct electrical stimulation.

somewhat, reached a maximum between 25 and 30° C. and then began to decline slightly; below 20° C. the speed of contraction was considerably decreased. In all experiments in which the preparation had been in contact with toxin at 17–18° C. for 2½ hr. no signs of paralysis of the diaphragm to nerve stimulation were observed (Fig. 2). If an intermediate temperature was employed, e.g. 28° C., both the latent period and the progress of paralysis were considerably prolonged. Corresponding results have been obtained in frogs (*Rana temporaria*). At laboratory temperature (16–18° C.) an injection into the dorsal lymph sac of 15,000 mouse LD_{50} of toxin/15 g. frog took 5–8 days to kill, whereas at 30° C. the same dose of toxin killed frogs in under 16 hr. It is thus evident that the Q_{10} of toxin action is high, resembling that of a chemical rather than a physical process. In the biological range the Q_{10} of diffusion is $< 1{\cdot}1$ whereas that of chemical reaction is usually 1·5–3.

Effect of Type B botulinum toxin. The rat phrenic nerve-diaphragm preparation is only slowly paralysed by 25,000 mouse LD_{50}/ml. of Type B botulinum

toxin. These amounts are about 500 times greater than the amount of Type A toxin required to produce a similar rate of paralysis.

This great difference in the toxicity of Type A and Type B toxins was also seen in the whole rat, where the LD_{50} (intraperitoneal injection in 200 g. rats) of the two toxins were found to be approximately as follows:

$$\text{Rat } LD_{50} \text{ Type A toxin} = 25 \text{ mouse } LD_{50},$$

$$\text{Rat } LD_{50} \text{ Type B toxin} = 10{,}000 \text{ mouse } LD_{50}.$$

When, however, the phrenic nerve-diaphragm preparation was obtained from young guinea-pigs (150–200 g.) typical neuro-muscular block followed the addition of 2000 units/ml. of either Type A or Type B toxin. There were no marked differences in the latent period or rate of paralysis between Types A and B toxins on this preparation. The guinea-pig is known to be susceptible to Type B toxin.

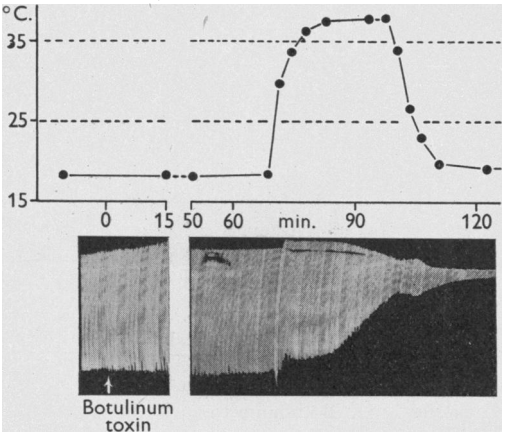

Fig. 2. Rat diaphragm preparation. Initially at 18·2° C. The graph shows the changes in temperature of the Tyrode solution during the course of the experiment.

Analysis of the paralysing action of botulinum toxin. Comparison with curare

The paralysis of the diaphragm to nerve stimulation is the result of neuro-muscular block; the toxin does not affect the muscle fibres or the nerve trunk. As shown in the experiment of Fig. 1 the muscle responds normally to direct electrical stimulation when the paralysis to nerve stimulation is complete. To test the effect of toxin on the phrenic nerve trunk, a length of nerve together with the stimulating electrodes were immersed in a small glass cup of 1 ml. capacity not in communication with the main bath. The addition of 5000 units of toxin to the cup produced no change in the contraction of the muscle over the next 80 min., whereupon 2000 units of toxin were added to the main bath and paralysis ensued as usual 30 min. later.

Post-tetanic potentiation. Brown & v. Euler (1938) showed that in normal and partially curarized muscle, a short tetanus of the motor nerve increased the tension developed in the succeeding single twitches. Fig. 3 shows this phenomenon in the diaphragm where the effects of nerve stimulation at 6/min. are increased after a 5 sec. tetanus at 50 cyc./sec. After the addition of the toxin, the post-tetanic potentiation is tested at 10 min. intervals. The effect is unchanged during the latent period and during the paralysis the post-tetanic potentiation remains proportional to the twitch tension. As Brown & v. Euler (1938) showed that the post-tetanic potentiation was essentially a muscular phenomenon, and was present on direct stimulation of the fully curarized or denervated muscle, it is not surprising that it should be unchanged in botulinum poisoning and this fact offers further evidence of the lack of direct action on the muscle.

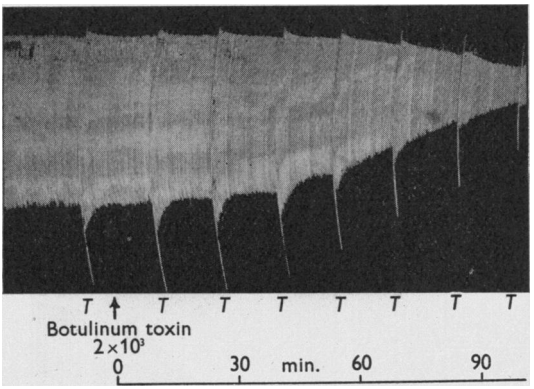

Fig. 3. Rat diaphragm preparation. Stimulated at 6/min. At points marked *T* rate of stimulation increased to 50 cyc./sec. for 5 sec. Botulinum toxin 2×10^3 units was added as indicated.

Tetani. When the phrenic nerve is tetanized at 50 cyc./sec. there is a rapid build-up of muscle tension in the first 5–10 sec. and the tetanus is then well maintained. The ratio of tetanus to single twitch tension is usually 2–3 : 1. After partial paralysis with toxin the response to tetanus remains perfectly normal in shape, but diminished in proportion to the decrease in twitch tension. Fig. 4 A, B, C shows 30 sec. tetani at different stages of botulinum intoxication. Fig. 4 D is from the same muscle before the addition of toxin, but when the twitch tension had been about 40% reduced by D-tubocurarine chloride 1 μg./ml. After an initial strong contraction which was about 50% greater than the twitch tension there was a rapid falling off of the tension.

Summation of two stimuli. When the summation curves of two successive stimuli applied to the phrenic nerve are examined (Fig. 5) it will be seen that the curve obtained with a muscle partly paralysed by toxin is very similar to the normal curve, showing only a small supernormal phase which is over in

about 4 m.sec. In the partially curarized muscle, however, there is seen a typical large and prolonged supernormal phase due to the 'decurarizing' effect of the first stimulus (Eccles, Katz & Kuffler, 1941).

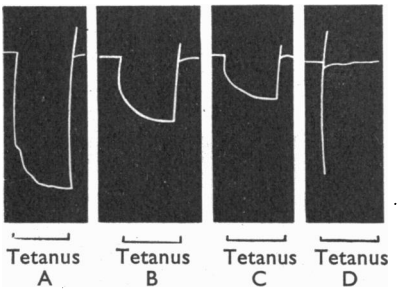

Fig. 4 A, B, C and D. Rat diaphragm preparation. Phrenic nerve stimulated at 50 cyc./sec. for 30 sec. A, B and C, stimulation at intervals as the course of the botulinum paralysis progressed. D, shows the response of the same preparation before addition of the toxin but when the twitch tension had been reduced to about 60% by 1 μg./ml. D-tubocurarine chloride.

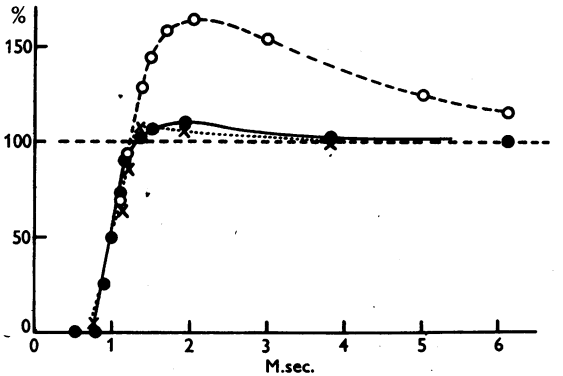

Fig. 5. Summation of two stimuli. Two stimuli delivered to the phrenic nerve from a Lucas pendulum and air-cored coils. Abscissa: interval between stimuli in m.sec. (approx.); ordinate: per cent increase in tension. ●—●, control preparation; ○—○, partly curarized preparation; × ···· ×, preparation poisoned with toxin.

Effects of drugs on the paralysis: eserine, prostigmine and *tetraethylpyrophosphate* (TEPP) were tested on the partially paralysed diaphragm. In low dosage (eserine 0·1–1 μg./ml.; prostigmine 0·01–0·1 μg./ml.; TEPP 0·01–0·1 μg./ml.) they produced a small, temporary increase of tension (Fig. 6*a*) which occasionally amounted to a maximum increase of 50% over the previous tension. This is similar to the degree of increase seen in the normal muscle. Larger amounts of prostigmine (1–5 μg./ml.) and TEPP (1 μg./ml.) produced paralysis in the poisoned diaphragm as in normal muscle. With curarized muscle, on the other hand, the small doses of all three drugs especially TEPP and prostigmine

produced striking recoveries in the force of contraction of several hundred per cent, and the larger amounts of TEPP and prostigmine had no paralytic effect.

Potassium chloride added to the bath in concentrations of 2–15 mg./ml. produced in normal and poisoned preparations a small transient increase in tension followed by depression. In some poisoned preparations the initial transient augmentation was absent. This contrasts strikingly with the powerful anticurare action of KCl (Wilson & Wright, 1936).

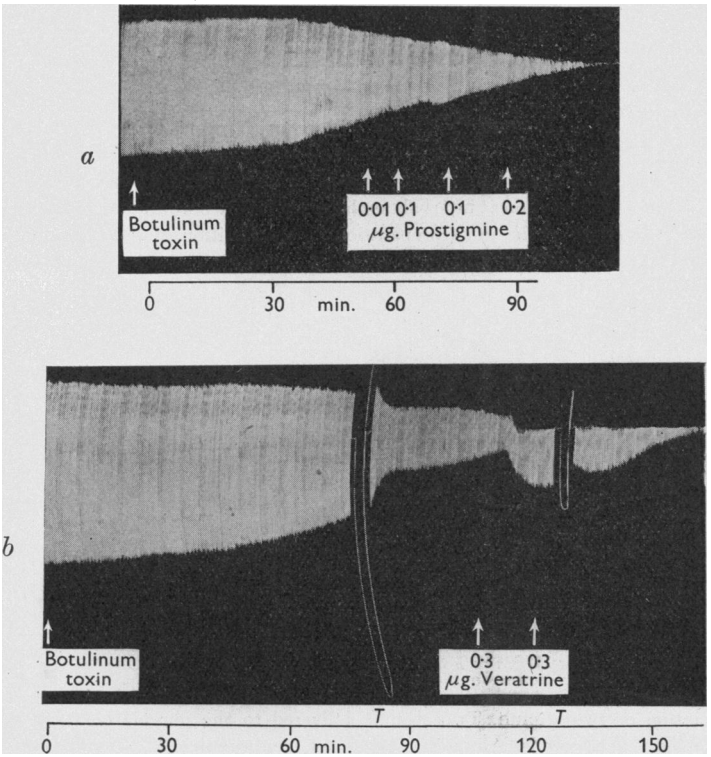

Fig. 6. Effects of drugs. Rat diaphragm preparation. Botulinum toxin 2×10^3 units. (*a*) Prostigmine 0·01, 0·1, 0·1, 0·2 μg./ml.; (*b*) veratrine 0·3–0·6 μg./ml. At *T*, *T* a maximal motor nerve tetanus at 100 cyc./sec., for 20 sec. was given.

Veratrine (mixed alkaloids) in suitable doses (0·1–1 μg./ml.) produced a striking increase in tension in normal muscle and also in that paralysed by toxin (Fig. 6 *b*). The twitch tension became comparable to that produced by a maximal motor nerve tetanus. Veratrine did not prevent ultimate paralysis.

Adrenaline (1–20 μg./ml.) occasionally produced a very small increase in tension in both the normal and poisoned preparation.

Close intravascular injections of acetylcholine. The neuro-muscular block produced by curare results from its action on the motor end-plates which become

insensitive to the acetylcholine released from the motor nerve endings. During the neuro-muscular block caused by botulinum toxin, however, the motor end-plates remain sensitive to acetylcholine, as shown by the following experiments.

Acetylcholine, even in large amounts, will not reliably produce contraction of the diaphragm when added to the bath fluid. Close intravascular injections are, however, readily performed on the isolated rat diaphragm. The arterial supply is complex and unsuitable for this purpose, but practically the whole of each dome of the diaphragm drains into one large vein which empties into the inferior vena cava as it passes through the tendinous arch of the diaphragm (Fig. 7). Retrograde injection through the veins yields essentially the same results as close arterial injection.

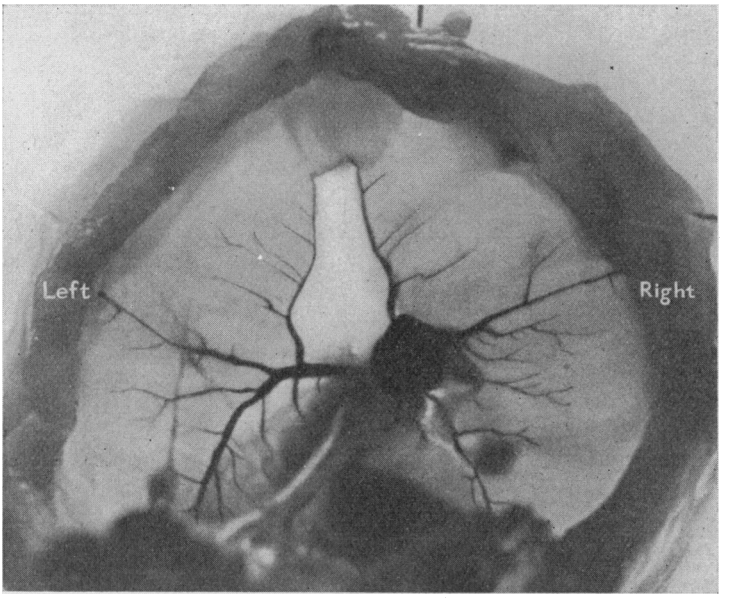

Fig. 7. Injected preparation of the venous drainage of the rat diaphragm. The large vein draining the left half of the diaphragm and the main right lateral vein can readily be seen.

The inferior vena cava is tied immediately above the entry of the hepatic veins and a needle cannula is inserted downwards into the thoracic inferior vena cava. The vein from the left side of the diaphragm is tied as it crosses the central tendon. A sector of the right diaphragm is cut on either side of the main lateral vein. The tendon end is then tied to a bar at the bottom of the bath and thread tied to the rib margin attached to the lever. Injections may be made or perfusion carried out through the cannula with only slight leakage of the injected fluid.

In Fig. 8 A the effect of injecting 10 μg. acetylcholine is seen. A rapid twitch followed by a slight contracture is produced. Control injection of saline produced only very small mechanical effect. Fig. 8 B shows the response to

acetylcholine of the same preparation when the tension due to motor-nerve stimulation had been reduced to about 10% by toxin and Fig. 8 C shows the acetylcholine response when the toxin had rendered motor-nerve stimulation completely ineffective. The response to 10 μg. acetylcholine is unaltered throughout the poisoning.

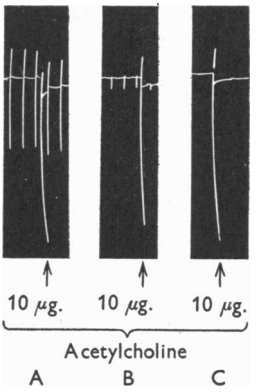

Fig. 8. Close intravenous injection of acetylcholine. A, normal preparation stimulated at 6/min. At the arrow 10 μg. acetylcholine were rapidly injected into the inferior vena cava. B, the same preparation after toxin when the response to nerve stimulation was reduced to 10–15% of the initial. C, the same preparation when it was unresponsive to nerve stimulation.

Liberation of acetylcholine on motor-nerve stimulation. Under favourable conditions the acetylcholine liberated in the diaphragm preparation by motor-nerve stimulation can be assayed. After the diaphragms had been contracting at 6/min. in Tyrode containing 1 part/million eserine sulphate the fluid was replaced twice. The nerve was then tetanized at 50 cyc./sec. for 20 min., and 30 sec. later the fluid was aspirated and assayed for acetylcholine. The nerve was then stimulated at 6/min. in fresh fluid for 15 min. and this procedure repeated. 5000 units of toxin were then added to the fluid bath. Seventy minutes later, when the preparation was almost completely paralysed, the

TABLE 1. Output of acetylcholine on stimulation of normal and poisoned rat diaphragm preparations

	Output of acetylcholine (μg./20 min.)	
	Before toxin	When paralysed by toxin
Exp. 1	0·125 0·120	<0·020
Exp. 2	0·105	<0·010

stimulation and assay were repeated as before (Table 1). It will be seen that when the diaphragm preparation is paralysed by toxin the output of acetylcholine on motor-nerve stimulation is greatly reduced.

The immune diaphragm. Diaphragms from immune rats were set up in the usual way and carefully washed free of blood. Amounts of 2000 units of toxin never produced paralysis of the preparation. In one experiment a further 30,000 units were added an hour later, and after a further 75 min. still no paralysis had occurred. In another experiment 20,000 units of toxin were added at the beginning, and after 1 hr. a slow paralysis developed at a rate one might expect from the addition of about 200–300 units of toxin to a normal diaphragm. It seems most unlikely that the very small amount of occluded serum could account for this 100-fold increase in resistance to toxin. When diaphragms obtained from young guinea-pigs immunized to Types A and B toxin respectively were tested with both toxins, resistance was demonstrable only against the toxin type used to produce immunity.

Effect of antibotulinum serum. Homologous antibotulinum serum was used. When 1 ml. of serum was added to normal preparations 10 min. before the usual 2000 units of toxin, no paralysis ensued after 1 hr. A further 20,000 units of toxin, however, produced paralysis in 20 min. To see if the poisoned preparation could be restored by antitoxin 2000 units of toxin were added to the bath and 10 min. later the excess of toxin was washed out. Fifteen minutes later the usual paralysis made its appearance, and when it was half complete 2 ml. of antiserum were added. The paralysis proceeded at the usual rate and no recovery was observed during the next hour. Other experiments using high potency horse antibotulinum serum (100 antitoxin units/ml., i.e. sufficient to neutralize 10^6 units of toxin/ml.) were likewise unsuccessful in reversing botulinum paralysis.

Action of botulinum toxin on enzyme systems

Acetylation of choline. Botulinum toxin exerted no inhibitory effect on the acetylation of choline by either fresh or acetone dried rat brain.

TABLE 2. Choline acetylase activity of (*a*) minced, (*b*) acetone dried brain

	Method *a* Acetylcholine synthesized (μg./g. wet brain/4 hr.)	
	Exp. 1	Exp. 2
Control	3·4 (2·9, 3·9)	5·5 (5·4, 5·6)
33 LD_{50} toxin/ml.	4·1 (3·4, 4·8)	7·7 (7·2, 8·3)
333 LD_{50} toxin/ml.	—	6·7 (6·6, 6·8)
	Method *b* Acetylcholine synthesized (μg./g. acetone powder/hr.)	
	Exp. 1	Exp. 2
Control	204 (184, 224)	243 (235, 251)
20 LD_{50} toxin/ml.	208 (200, 216)	217 (204, 230)
200 LD_{50} toxin/ml.	—	240 (225, 255)

These results are not concordant with those of Torda & Wolff, who, however, were using a culture filtrate of unspecified purity as a source of toxin, and it is possible that some impurities present in their preparation may have been responsible for the inhibition of acetylcholine synthesis found in their experiments.

Acetylation of sulphanilamide. In ten control experiments, 56·7 % (S.E. ± 0·7) of the added sulphanilamide was acetylated. Botulinum toxin produced no decrease in the acetylation of sulphanilamide, whereas 0·02 M-hydroxylamine produced its usual powerful inhibition (Table 3).

TABLE 3. Acetylation of sulphanilamide

	Acetylation % of control
Toxin 66 LD_{50}/ml.	96 (97, 97, 94)
Toxin 666 LD_{50}/ml.	96 (97, 95)
Toxin 6666 LD_{50}/ml.	96 (95, 97)
Hydroxylamine 0·02 M	8 (11, 5)

Choline esterase. Botulinum toxin even in very high concentrations has no effect on the 'true' or 'pseudo' choline esterase of guinea-pig serum. The absence of inhibitory effect is not due to a lag period, as when the toxin-enzyme mixture was incubated at 37° for 1 hr. before addition of substrate, there was still no appreciable effect on choline esterase activity (Table 4).

TABLE 4. Choline esterase activity with and without addition of botulinum toxin

	μl. CO_2/ml. serum/10 min.		
	Acetyl choline	Acetyl-β-methyl-choline	Benzoyl choline
Serum alone	293·8	23·2	124·5
Serum + 40,000 LD_{50} toxin/ml.	280·8	24·2	123·5
Serum + 40,000 LD_{50} toxin/ml.; activity tested 1 hr. later	—	25·5	—

Metabolism of rat brain slices. 100 units toxin/ml. had no significant effect *in vitro* on the Q_{O_2} of rat brain slices determined in the Warburg apparatus as compared with controls.

160 LD_{50}/g. wt. were injected intraperitoneally into a rat. After 4½ hr. the animal was moribund, its brain was removed and the Q_{O_2} determined on slices. Again there was no significant difference from the normal values.

DISCUSSION

Botulinum toxin produces paralysis of the diaphragm preparation after a characteristic delay which is related to the size of dose given. This delay is typical of tetanus, diphtheria, and other toxins, and Pappenheimer (1947) has shown for tetanus toxin that the duration of the latent period before signs appear in the intact animal is roughly inversely proportional to the logarithm of the dose of toxin. That this delay in the case of botulinum toxin is not due to diffusion of a large molecule has been clearly shown here, since replacing with fresh Tyrode during the latent period—a procedure which reverses the diffusion gradient into a muscle—does not prevent or alter the course of the subsequent

paralysis. During the latter part of this phase the toxin is fixed and presumably producing some change at the neuro-muscular junction which ultimately leads to block. This process is unlikely to be purely physical as the duration of the latent period and the rate of paralysis are much prolonged by lowering the temperature, the process having a high Q_{10} which is characteristic of a chemical reaction.

The irreversible paralysis produced by botulinum toxin has been shown to result from a neuro-muscular block leaving muscle and nerve trunk unaffected. The mechanism by which the toxin produces the block is entirely different from the block produced by curare. The normal type of tetanic response, the normal summation curves, and the lack of response to anticurare drugs suggest that the partially poisoned preparation contains some motor units that behave normally in all ways, and blocked motor units in which transmission is not restored by conditioning stimuli or other means.

The site of the block has been localized. The end-plate is normally sensitive to acetylcholine but the output of acetylcholine from the nerve endings is greatly diminished, whereas curare renders the end-plates insensitive to acetylcholine without decreasing its release from the motor-nerve endings (Dale, Feldberg & Vogt, 1936). The diminished acetylcholine output can be conceived as occurring in the following possible ways: (1) Transmission of the nerve impulse through the fine unmyelinated nerve fibres to the 'acetylcholine release region' in apposition to the end-plates may be blocked; in this case *no* acetylcholine will be released at the affected neuro-muscular junctions. (2) The synthesis of acetylcholine by the nerve endings is inhibited; since all or none inhibition of enzymes has not been described, in this case it would be expected that the power to synthesize acetylcholine would gradually decline, until the amount of acetylcholine fell below the threshold needed to activate the end-plate. (3) The release of acetylcholine may be decreased, perhaps by a permeability change in the nerve endings; a change of this nature is also unlikely to be all or none.

A diminished acetylcholine output due to increased choline esterase activity and hence greater breakdown can be excluded, since the output of acetylcholine was measured in the presence of eserine, a choline esterase inhibitor, and because of the lack of effect of toxin on choline esterase *in vitro*.

If mechanism (1) were correct, the effect of anticholinesterase drugs would be similar on a partly paralysed preparation to that on the normal preparation, i.e. the production of a brief tetanic contraction in response to a single nerve impulse in those muscle fibres whose neuro-muscular junctions were still unaffected by toxin; at the blocked neuromuscular junctions the anticholinesterase will be ineffective since acetylcholine is not being released at all and hence cannot be preserved by choline esterase inhibition. On the other hand, if explanation (2) were correct, the amount of acetylcholine released at the blocked

neuro-muscular junctions has fallen below threshold and might be raised above threshold by inhibition of choline esterase and conduction restored in at least a proportion of the blocked junctions. Together with the normal effect on the unpoisoned junctions, the resultant increase in force of contraction would be more marked than we have seen in botulinum poisoning. *In vitro*, contrary to the results of Torda & Wolff, we have found no inhibitory effect on choline acetylase in minced mouse brain, nor on the far more active system utilizing an acetone dried powder of rat brain. Since Lipmann & Kaplan (1946) have shown the liver enzyme system acetylating aromatic amines has the same coenzyme (coenzyme A) as choline acetylase, we tested the effect of toxin on this system without finding any inhibitory effect. Explanation (3) seems to us unlikely, but cannot confidently be excluded owing to lack of knowledge of the final mechanism of acetylcholine release.

While it is not yet possible to localize with certainty the site of action of botulinum toxin, the evidence presented could most readily be explained by an interaction of toxin with the fine unmyelinated nerve fibres entering the end-plate region, thus causing a block in the transmission of the nerve impulse.

Botulinum type A toxin has been crystallized and is now known to be a globular protein of mol. wt. 900,000–1,100,000 (Putnam, Lamanna & Sharp, 1946). A complete amino-acid analysis (Buehler, Schantz & Lamanna, 1947) of the toxin has revealed no abnormal amino-acids or suggestion of abnormal peptide links or any considerable amount of constituents other than amino-acids. These analytical results make the problem of how this toxin produces its effects very obscure. Its high molecular weight makes it most improbable that it could enter a cell at all readily, and yet the very minute amounts of toxin required are rapidly and selectively taken up by the neuro-muscular region. Since the ultimate mechanism of action of the toxin is at present so obscure, the present demonstration of specific toxic action on an isolated preparation should prove of value in facilitating further investigation.

The degree of insensitivity of the rat diaphragm preparation to Type B toxin as compared with Type A toxin correlated well with the insensitivity of the whole rat to Type B toxin, and would suggest that the differing sensitivity of other animals to toxin is related to the sensitivity of their motor-nerve terminals rather than to differing modes of distribution or disposal of the toxin. The results with guinea-pig diaphragm illustrate this point since this diaphragm, like the intact guinea-pig, does not show a marked discrimination for either toxin.

The resistance of the preparation obtained from rats actively immunized with toxoid is of great interest. These results cannot be explained as due to occluded serum antitoxin, since an amount of serum from the same animals equal to about three times the volume of the diaphragm failed to give the same protection. It may be that the antitoxic activity of the whole preparation is greater than that of an equivalent amount of serum, but the possibility also

exists that this resistance may be a tissue immunity induced by inoculation that renders the motor-nerve terminals highly resistant to the toxin. A good deal of further work will be needed before the exact mechanism involved can be decided. Lastly, the lack of reversal by antitoxin was not unexpected in view of the results of Zamecnik & Lipmann (1947) with *Cl. welchii* lecithinase. These authors found that once this enzyme was in contact with substrate, subsequent addition of antitoxin, except in massive amounts, exerted only a minor antagonistic effect.

The experimental findings discussed here are concordant with the symptoms and signs of clinical botulism. Guyton & MacDonald found that after local botulism of the gastrocnemius muscle of guinea-pigs, partial recovery of function occurred only very slowly over the following year. The lack of action of antiserum on the paralysed preparation is in agreement with the observation that antiserum is ineffective in reversing clinical botulism.

Note added in proof. We have now shown that results identical with those described above are given by an electrophoretically homogeneous toxin of $180 \times 10^6 LD_{50}$/mg. N. Since this paper was submitted similar conclusions have been reported from experiments on the intact animal by Ambache (1949).

SUMMARY

1. Botulinum toxin produces irreversible paralysis of the isolated rat phrenic nerve-diaphragm preparation after a latent period.

2. Boiled toxin does not produce paralysis.

3. Removal of the toxin during the latent period does not prevent the paralysis.

4. The latent period is greatly prolonged and the rate of paralysis slowed by lowering the temperature.

5. Prior addition of antibotulinum serum will prevent the development of paralysis by the toxin, but addition of antitoxin subsequent to toxin will not affect the paralysis.

6. Diaphragms from immune rats are highly resistant to botulinum toxin.

7. Some 500 times larger doses of Type B botulinum toxin than of Type A toxin are required to paralyse the rat phrenic nerve-diaphragm preparation; this correlates with the larger LD_{50} of Type B toxin in the rat. Both the whole guinea-pig and the guinea-pig phrenic nerve-diaphragm preparation are sensitive to both Type A and Type B toxins.

8. The paralysis produced by botulinum toxin on the rat phrenic nerve-diaphragm preparation is due to neuro-muscular block. Conduction in the nerve is unaffected and the muscle responds normally to direct stimulation.

9. The neuro-muscular block produced by toxin differs from that of curare in that motor-nerve tetani are well maintained, the summation curve for two stimuli is normal and that the paralysis is unaffected by eserine, prostigmine, TEPP, potassium or adrenaline.

10. The neuro-muscular block produced by the toxin differs from that of curare in that the motor end-plates remain sensitive to acetylcholine and in that the output of acetylcholine on motor-nerve stimulation is greatly reduced.

11. A method is described by which close intravascular injections of acetylcholine can be made into the diaphragm preparation by retrograde injections through a cannula tied into the vein.

12. The toxin does not affect the enzymes acetylating choline and sulphanilamide, nor choline esterase.

13. It is assumed that any effect of the toxin on the motor nerve must occur after it has lost its medullary sheath on entering the end-plate. By an irreversible fixation of the toxin to the fine nerve fibres, transmission of the impulse through them is abolished, consequently no release of acetylcholine takes place, resulting in the observed neuro-muscular block.

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