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COMPARISON OF THE ACTIVITY OF CL. BOTULINUM NEUROTOXIN IN TWO STRAINS OF MICE AND IN AXENIC VERSUS CONVENTIONAL RATS BY IN-VITRO TEST METHODS

FINAL REPORT

by

Marvin J. Bleiberg, Ph.D.

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Marvin J. Bleiberg

Woodard Research Corporation

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13. ABSTRACT

In-vitro tests of the paralytic effects of Botulinus toxin Type A were conducted in parallel on electrically-stimulated phrenic nerve-diaphragm preparations of axenic and conventionally bred Fischer strain (F-344) rats, and Charles River versus Fort Detrick strain mice. The data showed no consistent difference in regard to time of onset or degree of paralysis with respect to species. (U)

In-vitro tests of effect of toxin on post-ganglionic parasympathetic nerves were conducted using isolated eye preparations of Charles River and Fort Detrick strain mice. Preliminary data confirm that endings of the short ciliary nerves which innervate the pupillary sphincter are blocked by toxin as measured by blockade of miosis produced by cholinesterase inhibitors. The data are not conclusive regarding the sensitivity of the Charles River strain to botulinum toxin, but suggest on the basis of sensitivity to hemicholinium in addition to other data, that Fort Detrick mice have greater in-vitro cholinergic activity. (U)

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Isolated eye						
Miosis						
Mouse strains						
Mydriasis						
Neostromys albicaudatus						
Neuromuscular junction						
Post-ganglionic nerves						
Phrenic nerve-diaphragm						

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COMPARISON OF THE ACTIVITY OF CL. BOTULINUM NEUROTOXIN IN TWO STRAINS OF MICE AND IN AXENIC VERSUS CONVENTIONAL RATS BY IN-VITRO TEST METHODS

SUMMARY

The basis of the greater sensitivity to the paralytic effect of the neurotoxin of *Clostridium botulinum* Type A, as exhibited by mice of the Charles River CD-1 strain versus the Fort Detrick strain (Fish, 1972), and by axenic versus conventionally bred animals (LaManna and Ward, 1970) was investigated. *In-vitro* tests of time of onset and degree of paralysis were conducted in parallel on electrically-stimulated phrenic nerve-diaphragm preparations. Two preparations, one from an animal of each strain, were immersed together in the same solution in an organ bath. The muscle contractions were recorded on separate channels of a physiograph. By this means the action of the toxin was elicited while by-passing such factors as absorption, distribution, blood and tissue binding. The data showed no consistent detectable difference between axenic and conventional Fischer strain (F-344) rats in regard to time of onset or degree of paralysis. In a definitive mouse experiment no difference was noted between Charles River and Fort Detrick strains. There was an indication in an *in-vitro* experiment, that the African White-Tailed Rat (*Mystromys albicaudatus*) exhibits a greater sensitivity to toxin than the Charles River strain albino rat. *In-vivo*, Mystromys showed a greater variability in sensitivity, but similar LD_{50}.

Also investigated were the paralytic effects of toxin on the pupil of the eye in-vitro, adapting the methods of Beaver and Riker (1962). In this technique, the pupil of the eye is observed and measured with a microscope reticle. Preliminary data confirmed the data of Ambache (1949), that the cut central endings of the short ciliary nerves, which originate at the ciliary ganglion and innervate the pupillary sphincter, are effectively blocked by toxin. Mice of the Charles River strain were compared with Fort Detrick strain by the extent of mydriasis produced or blockade of miosis after exposure to cholinesterase inhibitors (parathion or ambenomium), electrical field stimulation, or a ganglionic-stimulating agent, dimethylphenylpiperazinium. The data are not conclusive regarding sensitivity of the Charles River strain to botulinum toxin, but do suggest on the basis of sensitivity to hemicholinium in addition to the other data, that Fort Detrick mice have greater in-vitro cholinergic activity as tested in this system.

FOREWORD

This project was authorized by Contract No. DAHC 19-72-C-0011, P-0001 by the U.S. Army Research - Durham. The direction and guidance provided by Dr. C. LaManna is gratefully acknowledged.

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on The Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

EFFECTS ON NEURO-MUSCULAR JUNCTION

PROBLEM

To compare the sensitivity *in-vitro* of neuromuscular junctions and neural tissues of Charles River versus Fort Detrick strains of mice, and of axenic versus conventional rats.

Fish (1972) reported that the lethal dose of botulinum toxin Type A in mice of the Charles River strain was one-half that of the Fort Detrick thus indicating a greater sensitivity of the Charles River mice to botulinum toxin. LaManna and Ward (1970) showed a similar sensitivity of axenically-bred mice as compared with conventionally-bred animals. The purpose of this study was to investigate whether there is an intrinsic difference between mouse strains in neural sensitivity to botulinum toxin. For this purpose *in-vitro* nerve-muscle

preparations were studied. The processes of absorption, distribution and tissue binding are therefore by-passed in this study.

MATERIALS AND METHODS

Isolated phrenic nerve-diaphragm tissues were prepared according to the methods of Bulbring (1946). These were mounted in a 100-ml tissue chamber surrounded by a thermostatically controlled water bath maintained at 37°C. The tissues were bathed in Krebs-Henseleit solution, while being continually gassed with 95% O_2-5% CO_2. In each experimental procedure, two isolated preparations were run in parallel in the same bath. Electrical field stimulation of the phrenic nerve was provided by the stimulator portion of a Heathkit Impscope through silver electrodes. Maximal isotonic or isometric contractions elicited were converted to electrical signals by transducer arrangements for recording on separate channels of a physiograph (Narco Biosystems, Houston). Either a pressure transducer or a displacement transducer was utilized. In many experiments the sensitivity of the preparations to D-tubocurarine was tested prior to adding toxin to the bathing solution. This assured electrical stimuli eliciting muscular contractions indirectly via the neuromuscular junction rather than by a direct effect on muscle fibers. In these experiments the time for complete paralysis to develop was determined and compared.

Crystalline Type A toxin was furnished through the courtesy of Dr. Edward A. Schantz. A stock solution of toxin containing $10^{6.4}$ mouse lethal dose units/ml in an acetate buffer at

pH 4 was kept in a refrigerator. The working solutions were prepared by dilution with phosphate buffered saline at pH 7.4 before addition to the tissue bath.

Mice of the Charles River CD-1 strain and rats (both axenic and conventional) of Fischer strain (F-344) were obtained from Charles River Breeding Laboratories, Inc. Axenic rats were shipped in groups of two in special containers to maintain the germ-free state. The axenic animals were used within several days of receipt. Fort Detrick mice had been previously received (Fish, 1972). In some instances the progeny obtained from breeding these mice in this laboratory were used in this study.

In several experiments nerve-muscle preparations of the African White-Tailed Rat (Mystromys albicaudatus) of a special colony maintained at this laboratory were compared with the Charles River strain rat (Sprague-Dawley). The African White-Tailed rat has been reported to exhibit resistance to radioactivity (Knott, Wright and Bleiberg, 1969) which is attributable by some to a sterile gastro-intestinal tract and relative state of dehydration (Hall, et al. 1967). It was therefore believed of interest to test effects of toxin on the neuromuscular junction of this strain.

RESULTS

Charles River versus Fort Detrick Mice

Toxin Added	Time	Results
$1 \times 10^{6.4}$ units	4 hrs	Incomplete blockade (approximately 80%); degree of blockade similar in each strain

The mouse preparation was delicate and somewhat tedious to prepare and use.

Axenic versus Conventional Rats

Toxin Added	Time	Results
$1 \times 10^{6.4}$ units	3 hrs	Essentially complete paralysis both rats; no remarkable difference
$2 \times 10^{6.4}$ units	1.5 hrs	Essentially complete paralysis both rats; no remarkable difference
$2 \times 10^{6.4}$ units	1.5 hrs	Essentially complete paralysis both rats; no remarkable difference

Mystromys versus Charles River Rats

Toxin Added	Time	Results
$1 \times 10^{6.4}$ units	5 hrs	Equal effect
$1 \times 10^{6.4}$ units fresh solution	1.5 hrs	Mystromys showed greater blockade

Mouse preparations were somewhat fragile and difficult to maintain. A good preparation was one which showed a definitive effect and, prior to the addition of toxin, showed sensitivity to D-tubocurarine (Figure 1). One experiment showed blockade of contractions four hours after adding $10^{6.4}$ mouse lethal dose units of toxin to the bath which was parallel in preparations from Charles River CD-1 and Fort Detrick mice. Therefore, further work was done with rats.

The preparations of the Fischer strain rat were more robust. These showed essentially complete paralysis between 1.5 to 3.0 hours after adding 1-2 ml of $10^{6.4}$ mouse lethal dose units of toxin to the bath (Figure 2). Addition of smaller amounts of toxin were without detectible effect within six hours, the duration of these experiments. No remarkable differences were noted between preparations from axenic and conventionally raised animals.

IN-VIVO STUDIES - COMPARISON OF MYSTROMYS ALBICAUDATUS WITH CHARLES RIVER RATS

A range-finding study was conducted to compare the toxicity of botulinum neurotoxin Type A in Mystromys with its toxicity for the Charles River rat.

Rats or Mystromys received interperitoneal injections of serial dilutions of toxin prepared from a stock solution containing $10^{6.4}$ mouse lethal dose units per ml. Injections of 1-ml volume were administered. The results are presented in Table 1.

SUMMARY

The data indicate that there is greater variability in toxicity of botulinum toxin in Mystromys as compared to the Charles River rat. The median lethal dose was approximately $10^{3.9}$ mouse lethal dose units (MLD) in both species.

An observation of interest was a severe atrophy of both hind limbs noted in one Mystromys 12 days after injection of 1:10,000 dilution ($10^{2.4}$ MLD). This animal survived the

TABLE 1

COMPARISON OF MYSTROMYS ALBICAUDATUS

WITH CHARLES RIVER STRAIN RATS (SPRAGUE DAWLEY)

MATERIAL TESTED: Botulinum toxin - $10^{6.4}$ units/ml in acetate buffer, pH - 4.0

ROUTE OF ADMINISTRATION: Intraperitoneal, two animals per dose level

DOSAGE LEVEL: 1 ml/animal

DILUENT: Phosphate buffered saline, containing gelatin, pH 7.4

	Mortality after Injection			
	Mystromys			
Dilution	Day 1	Day 2	Day 4	Day 12
Undiluted	2	2	2	2
1:10	2	2	2	2
1:100	1	1	1	1
1:1,000	1	1	1	1
1:10,000	0	0	0	1

	Mortality after Injection			
	Charles River			
Dilution	Day 1	Day 2	Day 4	Day 12
Undiluted	1	1	2	2
1:10	2	2	2	2
1:100	1	2	2	2
1:1,000	0	0	0	0
1:10,000	0	0	0	0

LD_{50}: $10^{3.9}$ mouse lethal dose units for each species

immediate acute effects of toxin but was found dead in the cage with the atrophy noted. Local hemorrhage and necrosis evidently contributed to the death.

EFFECTS ON EYE

PROBLEM

To compare the *in-vitro* sensitivities of post-ganglionic cholinergic nerves of Charles River versus Fort Detrick strains of mice to botulinum neurotoxin.

MATERIALS AND METHODS

The method of Beaver and Riker (1962) for the study of the isolated eye was adapted for the purposes of this investigation. The pupillary diameter serves as a measure of release of acetylcholine at the synapse of the short ciliary nerves with the sphincter of the pupil. This, in turn, depends on the activity of the short ciliary nerves. The central endings of the short ciliary nerves were, in turn, severed from the ciliary ganglion.

Ambache (1949) showed that Type A botulinum neurotoxin has no appreciable effect on either sensory or adrenergic nerve fibers within the eye but that it affects cholinergic fibers specifically. Ambache also states that the cholinergic paralysis is not due to existence of a block between transmitter and effector cell. He showed that the pupil of the rabbit eye does not respond to light by miosis after

periocular injection of toxin. The paralysis was attributable to blockade of the short ciliary nerves.

Mice were sacrificed by cervical fracture or injection of parathion. Eyes were enucleated using sharp scissors and placed in holes drilled in black Plexiglass plates for this purpose, and bathed in Krebs-Henseleit bicarbonate solution contained in Lucite dishes. The solution was gased with 95% O_2-5% CO_2 and maintained at 38°C by a thermostatically maintained water bath. The eyes were viewed through a microscope containing a calibrated reticle. Because the iris is not pigmented a dark background facilitated distinguishing the outline of the pupil. The size of the pupil was measured at each viewing. Observations of pupillary diameter were made at 10- to 30-minute intervals for up to six hours.

At least one-half hour was allowed after enucleation for equilibrium to be established before further procedures were carried out.

Several procedures were tried. The aim of these was to stimulate the cut endings of the short ciliary nerves to induce miosis and to compare treated eyes with control eyes. It was expected that eyes treated with botulinum toxin would not show miosis and that the extent of mydriasis would reflect the sensitivity to toxin.

The procedures examined were electrical field stimulation (square waves 240 pulses/minute, 50 volts); sacrifice of the

mice by systemic injection of parathion, a cholinesterase inhibitor, to induce miosis; addition of cholinesterase inhibitors in-vitro; and addition of a ganglionic stimulating agent, dimethyl phenylpiperazinium in-vitro. In later experiments one eye of each mouse served as a control and was not exposed to botulinum toxin. In two experiments hemicholinium-3, a compound which depletes nerve of acetylcholine by blocking its synthesis, was tested.

RESULTS

In most instances the effects in Fort Detrick and Charles River mice were parallel. The observation of Beaver and Riker (1966), that the pupils slowly dilate after enucleation of the eyes, was confirmed.

In several experiments, eyes which were placed in a solution containing toxin (1 ml of stock solution) immediately after removal dilated to a greater extent than parallel controls placed in Krebs solution only (Table 2). In experiments 1 and 2 eyes of Charles River mice showed a greater dilatation than those of Fort Detrick mice, but this was not marked. In all three experiments the mean of Charles River mice was greater than Fort Detrick mice. The dilatation was similar to that observed by Beaver and Riker (op cit), who state that enucleated eyes, placed in solutions containing atropine, dilate to a greater extent than those placed in Krebs solution only. They stated that if the control eyes were then placed in atropine no further dilatation occurred.

TABLE 2

COMPARISON OF ENUCLEATED EYES PLACED IN SOLUTIONS CONTAINING BOTULINUM TOXIN VERSUS CONTROL EYES

Experiment	Charles River Mice			Fort Detrick Mice		
	Control	Toxin	Diff.	Control	Toxin	Diff.
Immediately after addition of Ambenomium (0.5%) to bath						
1	1.6	2.4	0.8	2.0	2.3	0.3
	1.8	2.1	0.3	2.2	2.1	-0.1
2	1.9	2.1	0.2	2.1	2.1	0.0
	1.6	2.1	0.5	1.8	2.1	0.3
3	2.4	2.4	0.0	2.1	2.5	0.4
	2.4	2.4	0.0	2.3	2.5	0.2
Three hours after addition of Ambenomium (0.5% to bath						
2	0.7	1.8	1.1	0.6	1.4	0.8
	0.8	1.9	1.1	0.7	2.0	1.3
3	0.7	1.9	1.2	0.7	1.5	0.8
	1.1	1.9	0.8	1.2	2.1	0.9

* Eye diameters in reticle divisions (1 mm = 1.5 reticle division)

Three experiments were conducted in which ambenomium (0.5%) was added to the bathing solution containing the eyes and miosis developed (Table 2). Control eyes of each mouse strain showed a similar degree of miosis. Miosis was blocked in botulinum-treated eyes of each strain to a similar extent.

In one of four experiments in which mice were sacrificed with systemic injection of 10% parathion, there was an indication of a strain difference (Table 3). In this experiment (Experiment 4) miosis had not developed after enucleation of the eye, but slowly became evident even after addition of toxin to the bath. A greater degree of miosis was not [illegible] in the pupils of Fort Detrick mice, reflecting greater cholinergic activity.

TABLE 3

DIAMETERS OF ENUCLEATED EYES OF MICE AFTER SACRIFICE WITH PARATHION - EFFECT OF BOTULINUM TOXIN IN-VITRO*

(IN PRESENCE OF ELECTRICAL FIELD STIMULATION)

Experiment	Charles River Mice			Fort Detrick Mice		
	Before	After	Diff.	Before	After	Diff.
1	0.3	2.0	1.7	0.7	1.8	1.1
	0.5	1.9	1.4	0.5	2.0	1.5
2	0.2	2.0	1.8	0.2	2.0	1.8
	0.2	1.5	1.3	0.2	2.1	1.9
3	0.1	2.0	1.9	0.1	1.9	1.8
	0.2	1.5	1.3	0.2	1.5	1.3
4	0.8	0.8	0.0	1.0	0.4	-0.6
	1.1	0.7	-0.4	1.0	0.6	-0.4

* Eye diameters in reticle divisions (1 mm = 1.5 reticle division)

An experiment was conducted in which scopolamine was used to achieve maximal dilatation of the eye (Table 4).

TABLE 4

COMPARISON OF SENSITIVITY OF ENUCLEATED EYES OF MICE SACRIFICED WITH PARATHION TO HEMICHOLINIUM-3 (HC-3) FOLLOWED BY SCOPOLAMINE*

(IN PRESENCE OF ELECTRICAL FIELD STIMULATION)

Experiment	Charles River Mice			Fort Detrick Mice		
	Before	After	Difference	Before	After	Difference
1	0.5	1.9	1.4 (2.5)**	0.5	1.3	0.8 (2.0)
	0.5	1.8	1.3 (2.4)	0.7	1.8	1.1 (2.1)
2	0.9	1.8	1.1	1.0	1.5	0.5
	1.0	1.8	0.8	1.1	1.7	0.6

* Eye diameters in reticle divisions (1 mm = 1.5 reticle division

** Number within parenthesis is diameter after scopolamine.

In two experiments in which hemicholinium-3 was added in place of botulinum toxin, Charles River mice were more sensitive (Table 4).

Dimethylphenylpiperazinium and electrical stimulation did not reveal any difference between strains of mice when tested on botulinum-treated eyes, although blockade of effects was noted in some instances.

The isolated eye prepararation of Beaver and Riker presents several practical technical difficulties. When used with eyes of albino animals, the lack of pigment in the iris presents the problem of adequately viewing the outline of the pupil. However, the use of subdued light and a dark background against the eyes facilitated distinguishing the pupil. The dose-response curve to cholinergic agents showed a low sensitivity as compared to other isolated preparations such as the ileum and responses developed slowly. Moreover, as stated by Beaver and Riker, reversibility of drug effect was not readily achieved. They showed that the cornea of the eye presents a formidable barrier to the passage of drugs within the eye. Thus the present results are believed to reflect activity at cut ciliary nerve endings rather than post-ganglionic effects directly at intra-ocular receptors and required the use of high concentrations of drugs, especially of ions to test effects. Stimulation of the short ciliary nerves in the intact animal has been shown to produce contraction of the pupillary sphincter (Westheimer and Blair, 1973). Electrical field stimulation of the isolated eye preparation was effective in inducing miosis.

The data of these experiments must be regarded as preliminary because of the practical difficulties presented by this preparation.

Marvin J. Bleiberg, Ph.D.
Pharmacologist

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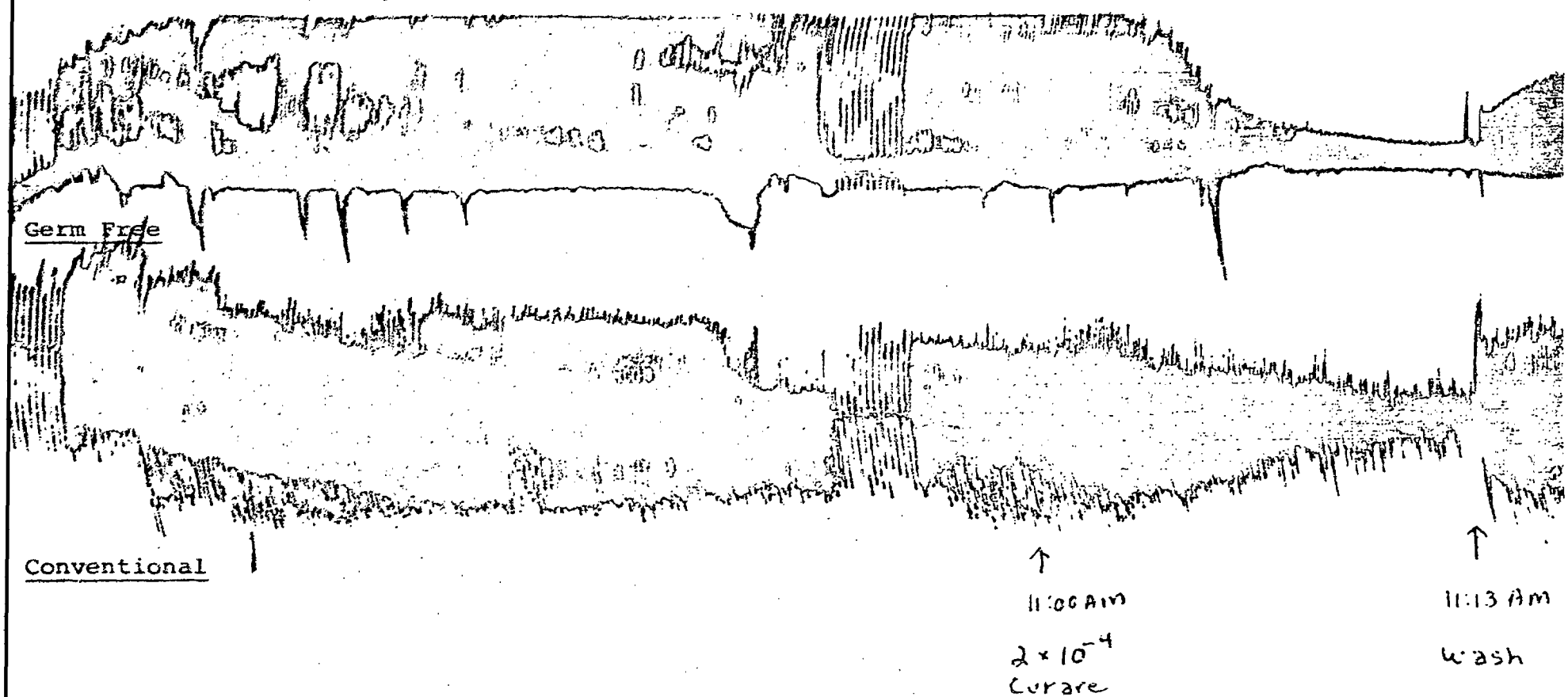
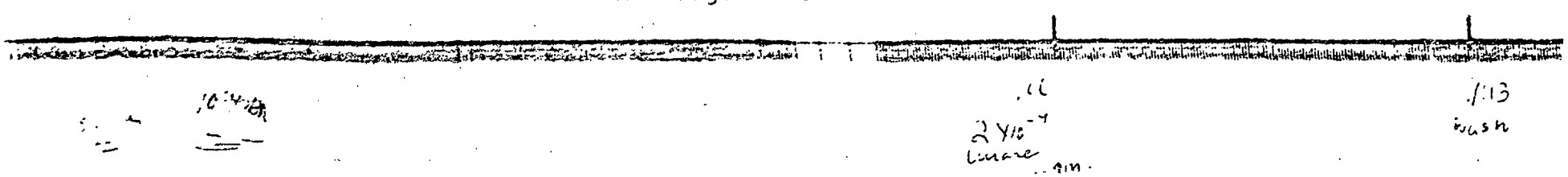


Figure 1. Effect of D-tubocurarine on Isolated Phrenic Nerve-Diaphragm Preparations of Axenic (Upper Tracing) and Conventional (Lower Tracing) Fischer Strain Rats Immersed in the Same Organ Bath



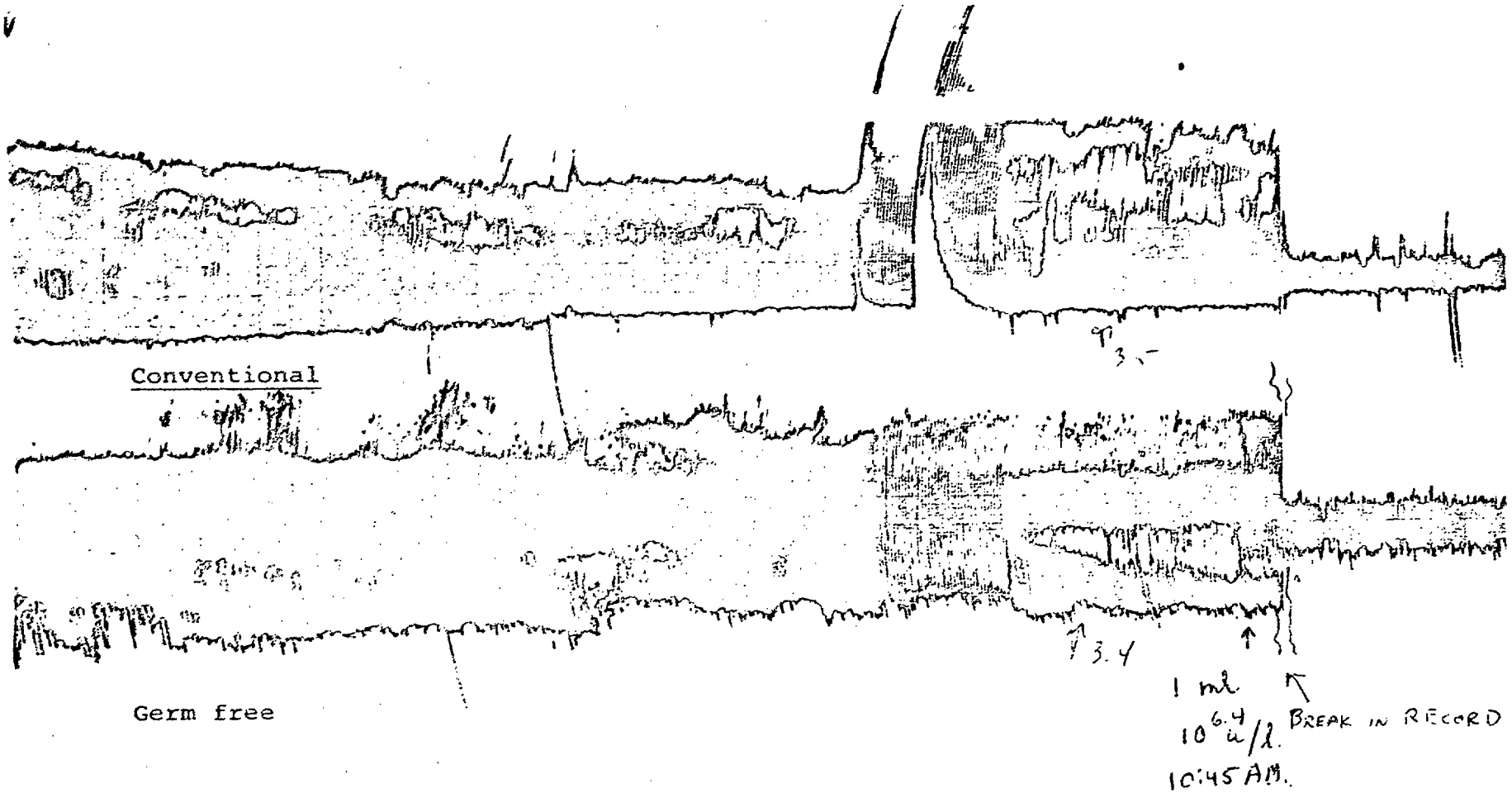
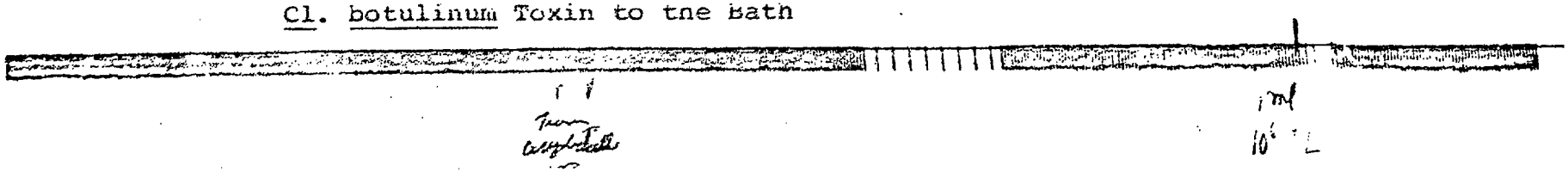



Figure 2. Contractions of Isolated Phrenic Nerve-Diaphragm Preparations of Conventional (Upper) and Axenic (Lower) Fischer Strain Rats Before and 4.5 Hours After Addition of 1 ml ($10^{6.4}$) MLD) of Cl. botulinum Toxin to the Bath