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INVESTIGATION OF THE PROLONGED RECOVERY TIME FOLLOWING BOTULINUM INTOXICATION - EFFICACY OF INTRAPERITONEAL VERSUS INTRAVENOUS INJECTION OF BOTULINUM ANTITOXIN

FINAL REPORT

by

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James F. Novotny, Ph.D.

August 10, 1973

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In order to study prolonged recovery times, mice were injected via the intramuscular route with sublethal concentrations of botulinum type A toxin. Proteolytic enzyme solutions were injected into the test site to determine if the removal of bound proteinacious toxin from neuromuscular junctions could influence paralytic recovery times. Parameters of observation were gait of animal, production of lesion at site of injection and activity of paralyzed and treated mice. Trypsin and protease at 1 mg concentrations appeared to markedly influence recovery whereas chymotrypsin, papain, ficin, pepsin and CaCl<sub>2</sub> did not significantly affect decrease of paralysis. (U)

The efficacy of intraperitoneal versus intravenous injection of botulinum antitoxin was studied by initiating a state of paralysis with resulting death of groups of mice and prophylactically administering specific antitoxin by either the intravenous or intraperitoneal routes. There appeared to be no effect of route of antitoxin administration on protective action of anticoxy thorage.

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Security Classification LINK A LINK C KEY WORDS ROLE ROLE ROLE Type A Cl. botulinum toxin
Botulinum paralysis
Botulinum therapy
Enzyme therapy
Botulinum prophylaxis
Swiss-Webster mice
Fort Detrick Inbrood mice Fort Detrick Inbreed mice

# INVESTIGATION OF THE PROLONGED RECOVERY TIME FOLLOWING BOTULINUM INTOXICATION - EFFICACY OF INTRAPERITONEAL VERSUS INTRAVENOUS INJECTION OF B. PULINUM ANTITOXIN

#### SUMMARY

Studies to provide information concerning botulinum intoxication were conducted in an attempt to determine what parameters influence the prolonged recovery time following intoxication and if the routes of antitoxin treatment could influence the rates of recovery and decrease mortality in test species.

In order to study prolonged recovery times, mice were injected via the intramuscular route with sublethal concentrations of botulinum type A toxin. Proteolytic enzyme solutions were injected into the test site to determine if the removal of bound proteinacious toxin from neuromuscular junctions could influence paralytic recovery times. Parameters of observation were gait of animal, production of lesion at site of injection and activity of paralyzed and treated mice. Trypsin and protease at 1 mg concentrations appeared to markedly influence recovery whereas chymotrypsin, papain, ficin, pepsin and CaCl<sub>2</sub> did not significantly affect decrease of paralysis.

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## INVESTIGATION OF THE PROLONGED RECOVERY TIME FOLLOWING BOTULINUM INTOXICATION

The course of botulinal intoxication following the binding of toxin to a specific site in the nerve fiber was studied by injecting mice with a sublethal paralyzing dose of botulinum type A toxin. Toxin which was not bound immediately following injection was neutralized intraperitoneal inoculation with specific antitoxin.

As reported in previous reports, testing criteria to determine the extent and severity of paralysis consisted of use of test mouse motion in an activity cage. As the mouse moves through beams of invisible light, activity is measured as counts per period of time.

The results of activity of mice following administration of toxin by the intramuscular, intraperitoneal and stomach intubation routes are presented in Table 1. Significant paralysis was observed following intramuscular injection of 1/2 LD<sub>50</sub> of toxin.

Following those initial experiments, tests were performed to determine if injection of various proteolytic enzymes could destroy botulinum toxin in vivo. In the previous experiments four mice were placed in the activity cage. Three mice per group were used in these experiments. One group of three received either two LD<sub>50</sub> of toxin in the left hind leg or PBS diluent. One hour later all animals received 0.1 m<sup>3</sup> of antitoxin and four hours later all animals received 0.1 ml of freshly prepared proteolytic enzyme containing 10 mg/ml in the

TABLE 1

ACTIVITY OF MICE FOLLOWING ADMINISTRATION

OF DIFFERENT AMOUNTS OF TOXIN - ACTIVITY

PRESENTED AS PERCENT OF CONTROL GROUP

Route of	Percent		Da	У	
Injection	LD <sub>50</sub> —	1	4	11	15
Intra- muscular	1/2 1/4 1/8	45.96 82.72 94.68	34.16 89.11 91.62	64.91 107.45 101.94	82.48 88.73 89.18
Intraperi- toneal	1/2 1/4 1/8	86.99 285.11 162.58	191.10 436.84 284.47	105.11 159.56 142.56	111.06 158.07 187.63
Stomach Intubation	1/2 1/4 1/8	29.84 67.91 90.97	12,20 119.75 136.03	106.79 94.93 173.28	137.62 129.00 158.79

same hind leg. Chymotrypsin, protease, trypsin and CaCl<sub>2</sub> (control) were used as saline solutions. The animals were observed for walk and stance, ability to walk down a vertical rod, and presence of enzyme-induced lesion or sore at the site of injection for two weeks. The results are presented in Tables 2, 3 and 4.

Tables 5 and 6 present activity cage results of mice treated with toxin to induce paralysis and then injected with proteolytic enzymes. This data demonstrated marked paralysis in all toxin-treated animals except those receiving protease and trypsin. Papain and ficin resulted in extensive tissue damage at the site of injection.

- 3 -

TABLE 2

VERTICAL ROD, GAIT AND STANCE, AND SORES AT THE

INJECTION SITE AS A RESULT OF ENZYME TREATMENT

	r r	oxin	Ani	mals		Di	luen	t An	imal	.s
Treatment	<b></b>	3	Day 7	<del></del>	16	<del></del>	3	Day 7	<del>-, , -</del>	16
	**************************************			11	10				11	<u>16</u>
Control										
Vert Gait Sore			+	++	+		+			
Vert		+	ш.	++	+					
Gait Sore	+	τ.	τ.	<b>T</b> T	<b>T</b>					
Vert Gait Sore	+		+		.÷					
Pepsin										
Vert Gait Sore	+ ++	++	+				•	-		
Vert Gait Sore	+	+	+ +·	+		+				
Vert Gait Sore	+	+	+	+	٠					
Trypsin										
Vert Gait Sore	+ +	4	+			++				
Vort Gait Sorc	+	. <b>+</b>	+	+	++	44				
Vert Gait Sore	+	* ++ , +	+++++++++++++++++++++++++++++++++++++++	±		ቀተ		. <del>ተ</del> ቀ		

- 4 -

TABLE 2 (Contd)

# VERTICAL ROD, GAIT AND STANCE, AND SORES AT THE INJECTION SITE AS A RESULT OF ENZYME TREATMENT

m			loxi		mals	}	D.	lluent		imal	.s
Treatment		<del></del>		Day			-	Ī	Day		
	•	<u>.</u>	_3	7	11	16		_3	_7	11	16
← Chymotrypsin											
Vert					+						
Gait		+					+				
Sore	•	+		+			+		+		
Vert.											
Gait							+				
Sore		+		+			•				
Vert					+						
Gait		+			·		+				
Sore		• +		+			•				
Papain											
Vert			++	+	4		4	+++	*		
Cait		++	+	+	·	•		+++			
Sore		+		+			++	++		•	
Vert	•						+			•	
Gait		+					+				
Sore		+		-			+				
Vert		4.	4.4		+	+				*	
Gait		++	+		•			•			
Sore		+.		+ ;	:	· .			+		
Ficin				•			٠, ٠				
•	-						•4			•	
Vert Gait			444	4.	+	+		+			
		44	44			-	+			•	
Sore	*	+		4	. +		. 4.		+		
Vert		+	+	4.	,+	+		++			
Gait		44						*			. •
Sore		+		+	+		+		+	+	٠.
Vert			+		÷	+		+++	4.	4	•
Gait		++	. +	++			+++	+++	4		•
Sore									+	+	

<sup>\*</sup> Loy badly crippled

- 5 -

TABLE 2 (Contd)

#### VERTICAL ROD, GAIT AND STANCE, AND SORES AT THE

#### INJECTION SITE AS A RESULT OF ENZYME TREATMENT

m	Ţ		Ani	mals			Di.			imal	s
Treatment	1	3	<u>7</u>	11	16		1	3	Day 7	11	16
Protease											
Vert Gait Sore	+			,			+				
Vert Gait Sore	+		+								
Vert Gait Sore	<b>+</b>						+		+	•	
CaCl <sub>2</sub> Vert Gait Sore	+	<b>+</b> +	. <u>±</u>				•				
Vert Gait Sore	+	+	土		· · · · · · · · · · · · · · · · · · ·						
Vert Gait Sore						; ; ;					
Uninjected Control	1		• .	٠	• (*)		•	•			
Vert Gait Sore										•	
Vert Gait Sore	· · · · · · · · · · · · · · · · · · ·	•			. *						
Vert Gait Sore							• • •	,			

TABLE 3

VERTICAL ROD, GAIT AND STANCE, AND SORES AT THE INJECTION SITE AS A RESULT OF ENZYME TREATMENT NEOSTIGMINE INDUCED HYPERSENSITIVITY

Treatment	Tox Vert	in Anima Gait	Sore	Dilu Vert	ent Animals Gait Sore
Control	+	+	+	-	<b>-</b>
Pepsin	+	+		Lunga	
Trypsin	•			-	
& Chymotrypsin	••	•••		***	
Papain	+				
Ficin	***	÷			
Protease	· •	· .		•	
CaCl <sub>2</sub>	4	***			
Uninjected Control	960				

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TABLE 4

#### VERTICAL ROD, GAIT AND STANCE, AND SORES AT THE

#### INJECTION SITE AS A RESULT OF ENZYME TREATMENT

#### RELATIVE TO ENZYME CONCENTRATION

			oxin		als		Di	Diluent Animals				
Treatment			D	ay		*****		I	Day			
		_1	4	_7	11	18	1	4	7	11	18	
Control									•			
Vert Gait Sore		+			<b>, *</b>							
Vert Gait Sore		<b>+</b> + 	· · ·	+	<u>±</u>							
Vert Gait Sore												
← Chymotryp	sin-1	nig	1		•	• •				_		
Vert Sait Sore Vert		**	4.44	*	+	* 1.00 *	+ + +	****** <b>+</b>	+	+	+	
Gait		+. +	+	· · · · · ·	+	+	+	+	+	; . , <b>+</b>	+	
Vert Gaita Sore		+++	4*4 4\$4 4	<b>,</b>	+		#++ <u>+</u> +	+	.,	+	+	
CaCl <sub>2</sub>	rati Sala Sala				.:	er e e Lid					21g	
Vert Gait Sore				n <b>d</b> in	+	<u>±</u>			1.			
Vert Gait Sore	,							· .				
Vert Gait Sore	<i>.</i>			* <b>±</b>	ik ik	İ						

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#### TABLE 4 (Contd)

#### VERTICAL ROD, GAIT AND STANCE, AND SORES AT THE

#### INJECTION SITE AS A RESULT OF ENZYME TREATMENT

#### RELATIVE TO ENZYME CONCENTRATION

	T	oxin		als		Di	Day   4   7   11   18   18   18   18   18   18			
Treatment	<del>- 1</del>	4	ay 7	77	18	<del></del>	<u>D</u>	ay ,	<del></del>	10
	·	. 4		11	10	1	-4-		77	T 0
Trypsin	-									
Vert Gait	+++	+	± ± +		土					
Sore	+	+	+	+	+	+	+	+	+	+
Vert Gait	+++	+		+	±	+++ +	+			
Sore	+	+	+	+	+	+	+	+	+	+
Vert Gait	+++	++		+		+	++			
Sire	+	+	+	+	+	+	+	+	+	+
0.33 mg						•				
Vert Gait Sore	+	-	+							
Vert Gait Sorc	. <b>+</b>			-		•				
Vert Gait	+++ +	•				+++				
Sore						+				
0.11 mg						•				
Vert Gait			<u>±</u>							
Sore						•				
Vert Gait Sore			土						+	•
Vert Gait Sore		++		<u>+</u>		·		-		

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TABLE 4 (Contd)

#### VERTICAL ROD, GAIT AND STANCE, AND SORES AT THE

#### INJECTION SITE AS A RESULT OF ENZYME TREATMENT

#### RELATIVE TO ENZYME CONCENTRATION

	T	oxin		als	<del></del>	Diluent Animals				
Treatment		D	ay					ay		
	1	4	7	11	18	1	4	7	11	18
Protease-1 mg										
Vert	+++	+				+++	++			
Gait	ł					+++				
Sore	+	+	+	+	+	+	+	+	+	+
Vert	+++	+++	++			+++	+++	+	土	
Gait	+++	+++	+			+++	++	+		
Sore	+	+	+	+	+	+	+	+	+	+
Vert	++	+		<u>+</u>		+	++		<u>+</u>	
Gait	+			_		+	+			
Sore	+	+	+	+	+	+	+	+	+	+
0.33 mg						•				
Vert	+					+				
Gait	•					·				
Sore						<b>+</b> '	+			
Vort		+								
Gait		•								
Sore										
Vert	++	++								
Gait		-tt-								
Sore	. <u>±</u>	. +								
2016										
0.11 mg			,							•
Vert	+++	++	4.4.	+	+	•				
Gait	+	• •	• •	•	•					
Sore	•									
Vert			+.		<u>±</u>				•	
Gait	•		1		-4-					
Sore										
Vert	4:4	+			<u>+</u>	•	. ·		<b></b> .	4.4
Gait		'			4.		•	. ±	· .	• •
Sore								· index		,
· ·								•		

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PROPHYLACTICALLY WITH PROTEOLYTIC ENZYMES

Treatment			Day	· · · · · · · · · · · · · · · · · · ·	
Treatment		3	7	11	16
Control	50.1	48.6	73.1	68.1	49.4
Popsin	37.2	41.2	46.1	39.1	53.0
Trypsin	88.1	80.4	81.9	86.2	115.8
Chymotrypsin	54.4	54.7	85.9	61.0	79.9
Papain	43.2	38.9	46.6	51.0	52.8
Ficin	74.6	57.0	68.8	86,2	71.5
Protease	66.8	121.8	110.7	121.4	94.2
CaCl <sub>2</sub>	43.2	33.9	51.5	53.1	57.5

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TABLE 6

PERCENT ACTIVITIES OF MICE TREATED WITH

DIFFERENT CONCENTRATIONS OF PROTEOLYTIC ENZYMES

FOLLOWING BOTULINAL PARALYSIS

,	Dosage	Pe	ercent De	ecrease d	of Contro	01
Treatment	Level			Day		
	mg	1	4	7	11	1.8
Control	•••	61.6	63.8	80.7	82.5	78.2
chymotrypsin	1	55.0	77.0	72.8	50.5	54.3
CaCl <sub>2</sub>	. 1	91.8	188.3	236.1	179.2	177.3
Protease	1	62.6	54.4	90.9	.84.1	90.4
Protease	0.33	109.4	89.9	222.4	210.9	184.4
Protease	0.11	52,9	73.7	73.8	56.4	79.2
Trypsin	1	49.2	51.1	75.3	52.4	62.3
Trypsin	0.33	144.5	245.9	591.7	380.7	292.3
Trypsin	0.11	50.7	81.3	47.6	76.3	87.3

### EFFICACY OF INTRAPERITONEAL VERSUS INTRAVENOUS INJECTION CF BOTULINUM ANTITOXIN

Studies conducted were designed to compare the difference in efficacy between intraperitoneal (IP) and intravenous (IV) injection of botulinum antitoxin following stomach intubation of type A botulinum toxin into Swiss-Webster mice. Mice were inoculated with 0.25 ml of toxin solution by the per os route by inserting a round end needle into the stomach of each mouse and expressing the toxin. Botulinum antitoxin (Wellcome Lot K 9710) was serially diluted in saline and 0.1 ml of each dilution was injected either by the IP or IV route into each toxin treated mouse. The results of initial studies are presented in Table 7. Marked differences in protection were not noted.

A second group of experiments was conducted in which greater dilutions of antitoxin were used in order to determine if the low death rates initially observed were due to high antitoxin concentrations. Again, a significant difference between the two routes was not observed. This data is presented in Table 8. In addition, species sex did not after the results which were obtained as shown in Table 9.

A chance observation within the laboratory indicated mice which are fasted become more susceptible to botulinal intoxication. Table 10 presents data obtained when mice were fasted for 24 hours and inoculated with 10-fold dilutions of botulinum type A toxin by stomach intubation. A total of three trials was conducted. Two of three trials provides expected doseresponse results.

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TABLE 7

INTRAPERITONEAL VERSUS INTRAVENOUS PROPHYLAXIS

OF BOTULINAL INTOXICATION IN MICE

noute of	Dilution			mber					
Antitoxin	of Antitoxin	- <u>P</u>	ost 23	Inje	<u>48</u>	n (H 56	ours 72	80	Dead/Total
Admn.	AUCTOXIU		43	31	40	30	12	00	
T. T. T.	10-4	1	4	1	1	0	0	0	7/20
The second second	10-3	0	4	0	0	0	0	0	1/20
1/2	10-2	1	1	0	0	0	0	O	2/20
	10-1	1	1	0	0 -	0	1	. 0	3/20
		,							
IF	$10^{-4}$	0	3	0	0	0	0	0	3/20
	1.0-3	2	1	U	O	U	O	O	3/20
	10-2	1	U	U	1	- 0	0	C	2/20
	© 10 <sup>-1</sup>	0	O	0	0	U	0	. 0	0/20

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TABLE 8

INTRAPERITONEAL VERSUS INTRAVENOUS PROPHYLAXIS

OF BOTULINAL INTOXICATION IN MICE

USING DILUTE CONCENTRATIONS OF ANTITOXIN

Route of	Dilution	Number of Deaths					Dead/				
Antitoxin	of	Post Injection (Hours)					Total				
Admn.	Antitoxin	4	20	28	44	<u>52</u>	68	76	92	106	Total
IV	10-5	0	1	1	0	0	0	Q	2	0	4/20
	10-4	0	0	O	0	0	0	0	0	0	0/20
	10-3	Q	1	0	0	0	0	0	0	0	1/20
	10 <sup>-2</sup>	0	1,	0	0	0	0	0	. 0	0	1/20
IP.	10-5	0	2	. 0	O.	0	0	. 0	<b>0</b> .	0	_/20
	10-4	0	0	0	0	0	0	. 0	0	0	0/20
	10-3	Ö	/ (0	0	0	0	.0	0	0	0	0/20
	10 <sup>-2</sup>	Ü	Ü	0	0	0	0	0	0	0	0/20
Control		O.	1.	0	1	0	0	0	0	. i	2/20
								• • •	,	•	

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TABLE 9 THE EFFECT OF SEX ON THE INTRAPERITONEAL VERSUS INTRAVENOUS PROPHYLAXIS OF BOTULINAL INTOXICATION IN MICE

Route of	Dilution	<del>,</del>				eaths		
Antitoxin Admn.	of Antitoxin	Sex	Post	1 In	<del>)。(</del> )	Hour:	3) 96	Dead/Total
	10 <sup>-5</sup>	2014	*******					1.40.0
IV	Δ	20M*	0	1	0	3	0	4/20
	10-4	20M	0	0	0	0	0	0/20
	10-3	10M	0	1	0	0	0	1/10
	_	10F**	0	0	0	0	0	0/10
	10-2	10M	0	1	0	0	1	2/10
		10F	0	0	0 -	1	. 0.	1/10
<b>TP</b>	10 <sup>-5</sup>	20M	0	Ü	0	,	0	1 /20
<u></u>	10-4	10M	0	1	. 0	1	U U	1/20
	TO.					_	-	2/10
	<u>.</u>	101	0	1	0	1	Û	2/10
	10-3	10M	0	. 0	0	Ü	1	1/10
		19F	1	. 0	0.	0	0.5	1/10
	10-2	10%	0	0	0	1	0	1/10
		10F	0	0	0	0		0/10
					•			
Control	•	3M	0	1	0	0	0	1/3
		12F	O	3	0	1	0	4/12
		•						

Males Females

MORTALITY OF MICE RECEIVING BOTULINUM TOXIN

FOLLOWING REMOVAL FROM FEED FOR 24 HOURS

Botulinum Toxin	Deat	tio	
Dilution	Trial 1	Trial 2	Trial 3
1:2	4/10	9/10	3/10
1:4	3/10	7/10	2/10
1:8	3/10	5/1/)	2/10
1:16	0/10	2/10	3/10
1:32	0/10	-	0/10
1:64	0/10		0/10

Following experiments to determine the potency of botulinum toxin by the stomach intubation route in fasted mice, tests were conducted in which mice intoxicated by this route were treated with antitoxin by the intraperitoneal route in order to determine if this treatment can indeed lessen the severity of symptoms or number of deaths. Three trials were conducted in which mice were inoculated by the stomach tube route and were injected by the intraperitoneal route at two hours with 0.1 ml of antitoxin (wellcome Batch K 9710). The mice were observed daily for five days and deaths and/or surviving animals were recorded. The results of these studies are presented in Table 11.

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MORTALITY OF MICE INJECTED WITH BOTULINUM TOXIN

AND TREATED INTRAPERITONEALLY WITH

BOTULINAL ANTITOXIN

Botulinum Toxin	Deat	h-Survivor Ra	tio
Dilution	Trial 1	Trial 2	Trial 3
10-1	2/20	-	_
10 <sup>-2</sup>	3/20	-	-
10-3	4/20	4/20	4/10
10-4	7/20	2/20	1/10
10-5	-	7/20	1/10
10-6	**	10/20	1/10
10-7	<b>-</b> ·	-	2/10
			•

Antisera therapy via this route appeared to be extremely variable and at the recommendation of the scientific technical representative, Dr. Carl LaManna, experiments in this area of study were terminated.

James F. Novotny, Ph.D. Microbiologist

Submitted: August 10, 1973

In conducting the research described in this report, the Investigator(s) 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.

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