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To determine the efficacy of 3,4-diaminopyridine (3,4-DAP) as a potential treatment for botulism, its effect on the survival times of mice injected with type A, B, E, or F botulinum toxin (BoTx) was examined. Mice were injected ip with 10, 20 or 40 LD<sub>50</sub> of BoTx. Three hr later, when the mice displayed symptoms of botulism, half of each group of mice was treated with 3,4-DAP, an agent which increases nerve-evoked transmitter release. At each dose of type A BoTx tested, 3,4-DAP definitely prolonged survival. In contrast, treatment with the drug did not significantly increase the survival time of mice injected with

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type B, E, or F BoTx. The differences in efficacy of 3,4-DAP against the four serotypes of BoTx may reflect differences in the molecular mechanism of action among the neurotoxins.

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EFFECT OF 3,4-DIAMINOPYRIDINE ON THE SURVIVAL OF MICE  
INJECTED WITH BOTULINUM NEUROTOXIN TYPE A, B, E, OR F

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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# ABSTRACT

Effect of 3,4-diaminopyridine on the survival of mice injected with botulinum neurotoxin types A, B, E, or F. Siegel, L.S., Johnson-Winegar, A.D., and Sellin, L.C. (1985). Toxicol. Appl. Pharmacol. , . To determine the efficacy of 3,4-diaminopyridine (3,4-DAP) as a potential treatment for botulism, its effect on the survival times of mice injected with type A, B, E, or F botulinum toxin (BoTx) was examined. Mice were injected ip with 10, 20 or 40 LD<sub>50</sub> of BoTx. Three hr later, when the mice displayed symptoms of botulism, half of each group of mice was treated with 3,4-DAP, an agent which increases nerve-evoked transmitter release. At each dose of type A BoTx tested, 3,4-DAP definitely prolonged survival. In contrast, treatment with the drug did not significantly increase the survival time of mice injected with type B, E, or F BoTx. The differences in efficacy of 3,4-DAP against the four serotypes of BoTx may reflect differences in the molecular mechanism of action among the neurotoxins.

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## INTRODUCTION

Botulism is caused solely by the protein neurotoxin produced by Clostridium botulinum. There are seven immunologically-distinct types of neurotoxin, designated A, B, C<sub>1</sub>, D, E, F, and G (Sugiyama, 1980). Types A, B, E, and F cause botulism in humans (Sugiyama, 1980), which usually occurs as a food-borne illness, caused by ingestion of preformed toxin from improperly preserved food (Sugiyama, 1980). Infant botulism, first described in 1976 (Pickett et al., 1976; Midura and Arnon, 1976), apparently results from the colonization of the intestine by C. botulinum followed by production of the toxin in vivo (Arnon et al., 1977). Occasionally, cases of human botulism result from infection of a wound by C. botulinum, with subsequent toxin production at the site (Merson and Dowell, 1973). The neurotoxin blocks the release of the neurotransmitter acetylcholine at cholinergic nerve terminals (Burgan et al., 1949) by a mechanism which remains to be determined. Respiratory failure, usually the cause of death is due to the inhibition of transmitter release from the phrenic nerve to the muscles of the diaphragm.

Numerous drugs have been tested on botulinum-paralyzed muscle preparations and in whole animals to determine their therapeutic benefits and/or to elucidate the mechanism of action of botulinum toxin (BoTx). Guanidine has been shown to increase the quantity of acetylcholine released from nerve endings (Otsuka and Endo, 1960). Since guanidine had been found to be effective in the management of myasthenia gravis (Minot et al., 1938) and Eaton-Lambert syndrome (Lambert, 1966), diseases similar to botulism in that neuromuscular transmission is compromised, guanidine was used to treat a case of human botulism (Cherington and Ryan, 1968). Since then, guanidine (at doses of 15-35 mg/kg/day) has been employed as an adjunct to standard

intensive care and antitoxin therapy in numerous cases of botulism. Case reports have noted beneficial effects for patients with type A botulism (Cherington and Ryan, 1970; Ryan and Cherington, 1971; Cherington and Schutz, 1977; Stahl et al., 1980) and with type B botulism (Jenzer et al., 1975; Oh et al., 1975). Treatment with guanidine resulted in improvement in ptosis and extraocular movement, as well as an increase in strength in the extremities. However, there was little or no improvement in respiratory function. In other cases of type A botulism (Faich et al., 1971; Kaplan et al., 1978) and type B botulism (Cherington and Ginsberg, 1971), guanidine treatment failed to produce any clinical improvement. In a double-blind crossover study involving 14 patients with type A botulism, guanidine (20 to 35 mg/kg/day) did not affect the rate of recovery or the clinical course of the disease (Kaplan et al., 1978). The drug produced diarrhea, nausea, gastric discomfort, and distal paresthesia when doses greater than 35 mg/kg/day were employed (Cherington and Ryan, 1968; Faich et al., 1971). Due to its side effects and its failure to improve respiratory function, guanidine remains a controversial therapeutic agent in the treatment of botulism.

4-Aminopyridine (4-AP) also increases the release of acetylcholine from nerve endings (Molgo et al., 1975; Yeh et al., 1976). It is 20 to 30 times more potent than guanidine in restoring neuromuscular transmissions in rat muscle preparations following paralysis by type A BoTx (Lundh et al., 1977a). In humans, 4-AP has been used clinically as an anticurare agent (Paskov et al., 1973), and to treat Eaton-Lambert syndrome (Lundh et al., 1977b) and myasthenia gravis (Lundh et al., 1979). During an outbreak of type E botulism in England, 4-AP (0.35 - 1.5 mg/kg) was administered to four patients (Ball et al., 1979). Transmitter release was restored

(electromyograph) and peripheral paralysis was almost completely reversed. However, these effects were transient and respiratory muscles were not affected. Increasing the dosage did not improve respiration, and doses of 1.5 mg/kg produced grand mal convulsions.

3, 4-Diaminopyridine (3,4-DAP), reported to be less convulsant than 4-AP in laboratory animals (Vohra and Pradhan, 1964), was six to seven times more potent than 4-AP in restoring neuromuscular transmission in rat muscles paralyzed with type A BoTx (Molgo et al., 1980). In addition, 3,4-DAP has been shown to be effective in prolonging the survival of mice (Lewis, 1981) and cynomolgus monkeys (Lewis and Wood, 1981) that had received type A BoTx. More recently, studies with isolated rat muscles demonstrated that 3,4-DAP was less effective in reversing paralysis by type B (Sellin et al., 1983b) or type F (Kauffman et al., 1985) than by type A BoTx. To further define the efficacy of 3,4-DAP as a potential treatment for botulism, we have examined its effect on the survival time of mice injected with four serotypes (A, B, E, or F) of BoTx.

## METHODS

### Botulinum Toxin Stock Solutions

Type A. The Hall strain of type A Clostridium botulinum was grown as previously described (Siegel and Metzger, 1979), and the toxin in the culture fluid was precipitated by adjusting the pH to 3.5. The precipitate was washed with distilled water, and the toxin was extracted from the precipitate with 0.2 M phosphate buffer at pH 6.0. After centrifugation, the supernatant fluid was divided into 1 ml aliquots and stored at -70°C. This preparation

contained  $2.8 \times 10^7$  mouse LD<sub>50</sub>/ml.

Type B. Type B toxin was produced using the Bean strain of C. botulinum (Siegel and Metzger, 1980). Toxin in the culture fluid was precipitated by adjusting the pH to 4.0. Extraction and storage were as described above for type A. The toxin concentration of this stock solution was  $5.6 \times 10^6$  LD<sub>50</sub>/ml.

Type E. Strain E43 of C. botulinum was grown as previously reported (Siegel, 1981). After 5 days of incubation, the pH of the culture was adjusted to 6.0, and 313 g/l ammonium sulfate was added to precipitate the toxin. The toxin was extracted from the precipitate with 0.2 M phosphate buffer, pH 6.0. After centrifugation, the supernatant fluid was dialyzed in 0.2 M succinate, pH 5.5. The preparation was incubated with trypsin (100 µg/ml) at 35°C for 90 min. Soybean trypsin inhibitor (200 µg/ml) was then added and incubation continued at 35°C for 15 min. The preparation ( $1.0 \times 10^6$  LD<sub>50</sub>/ml) was divided into aliquots and stored at -70°C.

Type F. The Langeland strain of C. botulinum was used for the production of type F toxin (Yang and Sugiyama, 1975). The toxin was precipitated by adjusting the pH to 4.0 and extracted and stored as described above for type A. It contained  $5.6 \times 10^4$  LD<sub>50</sub>/ml.

Toxin Assay. Samples were diluted in cold gel-phosphate buffer (0.2% gelatin, 0.4% dibasic sodium phosphate, pH 6.2). A volume of 0.5 ml of an appropriate serial 2-fold dilution was injected ip into mice; 4 mice per dilution. After 4 days of observation for deaths, the LD<sub>50</sub> per ml was calculated (Reed and Muench, 1938). All doses of toxin mentioned in this study are expressed as mouse ip LD<sub>50</sub>s.

Experimental Protocol For Studies With 3,4-DAP. Immediately prior to use, an aliquot of stock BoTx was thawed and diluted in cold gel-phosphate



buffer to contain 10, 20, or 40 LD<sub>50</sub> in 0.2 ml. At zero time, a group of mice was injected ip with 0.2 ml of BoTx. At least 30 mice were injected with each dilution of toxin. Within 3 hr, the mice showed signs characteristic of botulism: ruffling of the fur and labored abdominal breathing (Center for Disease Control, 1974). At 3 hr, treatment with 3,4-DAP was begun for half of the group of mice. The drug (phosphate salt) was administered at a dose of 4 or 8 mg/kg, in a volume of 0.2 ml sterile saline, injected ip at hourly intervals. All mice were checked at 15-min intervals for survival. With type A toxin, experiments continued for 16 hr, or in longer term studies, for 32 hr. For type B, E, or F toxin, experiments were terminated only when all mice had died.

Data Analysis. The pattern of survival of untreated versus treated groups was compared by using a computer statistical program, Survival Analysis (SAS Institute, Inc., 1982). Statements concerning statistically significant differences refer to the use of the Lee-Desu statistic (SAS Institute, Inc., 1982). A P value of 0.05 or less was considered statistically significant.

Animals. Male Swiss mice, ranging in weight from 16 to 22 g, were used. Laboratory chow and water were available ad libitum.

Drug. 3,4-DAP (98% pure) was purchased from Aldrich Chemical Company, Inc., Milwaukee, WI. The drug was recrystallized as the phosphate salt. Since the molecular weight of the phosphate salt is about twice that of the drug (211 and 109, respectively), the doses of 4 and 8 mg/kg body weight used in these experiments are approximately equivalent to 2 and 4 mg/kg of 3,4-DAP.

Chemicals. Trypsin (EC 3.4.21.4, type III, twice recrystallized from bovine pancreas) and trypsin inhibitor from soybean (crude type II S) were purchased from Sigma Chemical Company, St. Louis, MO.

## RESULTS

### Type A BoTx

The effect of 3,4-DAP on the survival times of mice injected with 10, 20, or 40 LD<sub>50</sub> of type A BoTx was examined. Results from a typical experiment with 20 LD<sub>50</sub> of type A BoTx are shown in Fig. 1. By 15 hr, all of the untreated mice were dead. In contrast, all mice treated with 3,4-DAP survived. Similarly, with 40 LD<sub>50</sub> of toxin, all 15 of the untreated mice were dead by 8 hr, while 14 of the 15 mice treated with 3,4-DAP were still alive 15 hr after injection of the toxin. With 10 LD<sub>50</sub> only 4 of 15 untreated mice were alive at 15 hr; with 3,4-DAP treatment, 14 of 15 mice survived. The control group (n=15), which received 3,4-DAP but no toxin, remained alive and free from apparent adverse effects.

The results of several such experiments with type A BoTx are summarized in Table 1. At each dose of toxin employed, treatment with 3,4-DAP definitely prolonged the survival of the mice. When the patterns of survival of untreated versus treated groups of mice were compared, statistically significant differences were apparent at all doses of type A toxin tested (Table 2).

Mice were treated for a longer period (29 hr). With 20 LD<sub>50</sub> the 3,4-DAP extended survival time (Fig. 2). After 32 hr, 50% (10/20) of the mice treated with 3,4-DAP were still alive. All of the untreated mice were dead in 10.5 hr (n=20). A similar pattern was obtained with 40 LD<sub>50</sub> of type A. Hourly administration of the drug for 29 hr did not adversely affect mice that had not received BoTx.

### Type E BoTx

The effect of 3,4-DAP on mice receiving type E BoTx was also examined.

As shown in Fig. 3, 3,4-DAP treatment of mice given 20 LD<sub>50</sub> of type E toxin did not prolong survival. Statistical analysis of the survival pattern of treated (n=20) versus untreated (n=20) mice for each experiment for 20 or 40 LD<sub>50</sub> of type E toxin did not demonstrate significant differences (Table 2). In one experiment with 10 LD<sub>50</sub>, treatment with 3,4-DAP shortened survival time ( $P < 0.05$ ). Increasing the dosage of the drug to 8 mg/kg did not increase the survival time of mice receiving 10, 20, or 40 LD<sub>50</sub> of type E toxin (Table 2).

#### Type B BoTx

For 10, 20, or 40 LD<sub>50</sub> of type B BoTx, graphs of the number of survivors versus time for treated and untreated mice were similar to those obtained with type E toxin (e.g., Fig. 3). In three experiments, there was a statistically significant difference ( $P < 0.05$ ) in the pattern of survival (Table 2). In two of these, treatment with 3,4-DAP prolonged survival, but in the third, the drug shortened the time to death. However, the differences in the median survival time of treated versus untreated mice (Table 2) were, at most, one hour, and treatment did not consistently prolong survival.

#### Type F BoTx

Treatment with 3,4-DAP did not prolong the life of mice receiving 10, 20, or 40 LD<sub>50</sub> of type F BoTx (Table 2). In two experiments with 20 LD<sub>50</sub> of toxin, use of 3,4-DAP actually decreased survival time (Table 2).

### DISCUSSION

The effectiveness of 3,4-DAP in prolonging the survival of mice receiving type A BoTx has been previously described (Lewis, 1981). Sixteen hr after toxin injection, 3,4-DAP was administered ip at a dose of 1 or 4 mg/kg. This single treatment reportedly increased muscle tone and relieved paralysis for 2

to 3 hr. The drug was injected every 3 hr for 48 hr, but each subsequent treatment was less effective in restoring mobility. After 48 hr of therapy, 8 of 14 mice that had received doses of 4 mg/kg 3,4-DAP were still alive, as were 4 of 15 mice that had received 1 mg/kg. None of the 10 untreated mice and only 2 of 16 injected with a placebo (phosphate buffered saline) survived. This investigation was limited to a single experiment with a small number of mice, and the dose of type A toxin employed was not indicated.

In a subsequent study (Lewis and Wood, 1981), six cynomolgus monkeys (4.1-6.0 kg) were given type A BoTx iv at a dose of 100-350 mouse LD<sub>50</sub>/kg. After 3 to 6 hr, 3,4-DAP was administered iv (1.1-2.6 mg/kg). Within 2 to 10 min, there was dramatic clinical improvement: increased muscle tone, reduced ptosis, improved respiration, and restored mobility. However, the signs and symptoms of botulism returned within 2 hr after treatment. A second dose of 3,4-DAP (2 to 5 hr after the first) was less effective in reducing the symptoms than the initial treatment. Administration of 3,4-DAP as a continuous iv drip (0.78 - 0.95 mg/kg/hr) also failed to prevent the recurrence of paralysis.

3, 4-Diaminopyridine (4  $\mu$ M) was shown to be effective in restoring neuromuscular transmission in rat extensor digitorum longus (EDL) muscles previously paralyzed with type A BoTx (Molgo et al., 1980). Compared to results obtained with type A, the drug (at concentrations of 1, 10, and 100  $\mu$ M) was less efficacious in reversing paralysis by type B (Sellin et al., 1983b) or by type F BoTx (Kauffman et al., 1985). However, these investigations required the use of large quantities of types B (Sellin et al., 1983b) and F (Kauffman et al., 1985) toxins to produce paralysis equivalent to that obtained with type type A.

In the studies reported here, we have used comparable doses of types A, B, E, and F BoTx in mice to determine the efficacy of 3,4-DAP as a mode of chemotherapy for botulism. 3, 4-Diaminopyridine (phosphate salt, 4 mg/kg) was effective in prolonging the survival of mice receiving 10, 20, or 40 LD<sub>50</sub> of type A toxin. The drug was administered ip beginning at 3 hr after the toxin and at hourly intervals thereafter (Fig. 1 and Table 1). Comparing the pattern of survival of treated versus untreated mice yielded statistically significant differences at all doses of type A toxin tested (Table 2). When hourly treatment with 3,4-DAP was continued for an extended time period, 50% of the mice survived for 32 hr after the administration of 20 LD<sub>50</sub> of type A neurotoxin, whereas all of the untreated mice were dead by 11 hr (Fig. 2). In marked contrast, 3,4-DAP was not effective against 10, 20, or 40 LD<sub>50</sub> of types B, E, or F toxins with the same concentrations of drug and dosage schedule (Table 2). Increasing the drug dose to 8 mg/kg did not prolong survival of mice receiving 10, 20, or 40 LD<sub>50</sub> of type E toxin (Table 2). Thus, 3,4-DAP prolonged survival of mice that had received type A BoTx, but was ineffective against type B, E, or F toxin at the concentrations of drug and toxin tested.

Perhaps the differences in efficacy of 3,4-DAP against types A, B, E, and F reflect variations in the molecular mechanism of action among these neurotoxins. It has been reported that different amounts of types A, B, E, and F toxins were required to produce equivalent paralysis in isolated rat EDL muscle preparations. A dose of 565 LD<sub>50</sub> of type E (Sellin et al., 1983a) or 200-2,000 LD<sub>50</sub> of type F (Kauffman et al., 1985) was necessary to yield paralysis equivalent to that obtained with 2-3 LD<sub>50</sub> of type A toxin; 5,000 LD<sub>50</sub> of type B was required for an effect comparable to 20 LD<sub>50</sub> of type A toxin (Sellin et al., 1983b). Clinical differences in human cases of botulism

caused by type A versus type B toxin have been described (Hughes et al., 1981). In a review of the clinical records from 55 cases of botulism, they noted that individuals with type A botulism sought medical attention sooner, had a higher probability of requiring ventilatory support, and had a longer hospital stay. The combination of these previous research findings, the clinical observations, and the data reported here suggest that the antigenically distinct types of BoTx differ in pharmacological activity. Results of our investigation demonstrate that extrapolating information obtained with one serotype of BoTx to other serotypes is unwarranted, and emphasize the need for comparative pharmacological studies with these neurotoxins.

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Table 1.      Effect of 3,4 DAP on the Survival of Mice Receiving  
40, 20 or 10 LD<sub>50</sub> of Type A Botulinum Toxin

<u>LD50 A</u>	<u>Untreated</u>		<u>3,4-Diaminopyridine</u>	
40	5/70 <sup>a</sup>	7%	49/70 <sup>a</sup>	71%
20	4/70	6%	51/70	73%
10	8/30	27%	26/30	87%

<sup>a</sup> Survivors/number injected with toxin, after 15 hr.

Table 2. Effect of 3,4-Diaminopyridine Treatment on Survival of Mice Injected Intraperitoneally with Botulinum Toxin

		Survival Time (hr)				
Botulinum Type	Toxin LD50	Untreated <sup>a</sup>		3,4-Diaminopyridine <sup>a</sup>		P
		Mean $\pm$ SE <sup>b</sup>	Median	Mean $\pm$ SE	Median	
A <sup>c</sup>	40	5.7 $\pm$ 0.23	5.2	14.5 $\pm$ 0.47	>15.0	<0.001
		8.5 $\pm$ 0.53	8.6	14.7 $\pm$ 0.95	>16.0	<0.001
		6.2 $\pm$ 0.58	5.8	10.8 $\pm$ 0.98	10.5	<0.002
	20	9.1 $\pm$ 0.78	8.3	15.0 $\pm$ 0	>15.0	<0.001
		10.2 $\pm$ 0.87	9.2	15.1 $\pm$ 0.85	>16.0	<0.001
		7.7 $\pm$ 0.67	6.8	10.7 $\pm$ 0.95	12.1	<0.05
	10	12.3 $\pm$ 0.62	11.9	14.8 $\pm$ 0.22	>15.0	<0.001
		13.1 $\pm$ 0.72	13.9	14.3 $\pm$ 1.1	>16.0	<0.05
B <sup>c</sup>	40	6.1 $\pm$ 0.46	6.0	6.9 $\pm$ 0.62	6.8	NS <sup>e</sup>
		6.6 $\pm$ 0.59	6.4	5.5 $\pm$ 0.50	5.6	<0.05 DF <sup>f</sup>
	20	7.0 $\pm$ 0.56	7.2	7.6 $\pm$ 0.38	8.3	<0.05
		7.8 $\pm$ 0.74	7.4	7.5 $\pm$ 0.46	7.6	NS
	10	9.7 $\pm$ 0.51	9.7	8.4 $\pm$ 0.51	8.7	NS
		8.8 $\pm$ 0.48	9.1	10.4 $\pm$ 0.57	10.2	<0.05
E <sup>c</sup>	40	5.8 $\pm$ 0.54	5.8	4.5 $\pm$ 0.34	5.2	NS
	20	6.9 $\pm$ 0.76	6.3	5.9 $\pm$ 0.31	6.7	NS
		6.4 $\pm$ 0.28	7.2	7.0 $\pm$ 0.46	7.2	NS
	10	7.5 $\pm$ 0.51	8.1	8.2 $\pm$ 0.63	8.3	NS
		9.3 $\pm$ 0.28	9.6	7.5 $\pm$ 0.56	7.5	<0.05 DF
F <sup>c</sup>	40	4.6 $\pm$ 0.35	4.9	4.6 $\pm$ 0.18	5.2	NS
		5.7 $\pm$ 0.48	5.7	5.6 $\pm$ 0.54	5.3	NS
		5.1 $\pm$ 0.34	5.4	6.1 $\pm$ 0.61	5.6	NS
	20	7.0 $\pm$ 0.45	7.2	5.6 $\pm$ 0.40	5.8	<0.05 DF
		7.1 $\pm$ 0.36	7.4	5.7 $\pm$ 0.28	6.1	<0.01 DF
		6.7 $\pm$ 0.32	7.2	6.1 $\pm$ 0.30	6.5	NS
	10	7.7 $\pm$ 0.38	8.3	7.1 $\pm$ 0.26	7.3	NS
E <sup>d</sup>	40	4.3 $\pm$ 0.34	4.7	4.4 $\pm$ 0.34	4.8	NS
	20	5.3 $\pm$ 0.45	6.0	5.7 $\pm$ 0.30	6.0	NS
		5.5 $\pm$ 0.49	5.5	5.7 $\pm$ 0.62	5.8	NS
	10	7.8 $\pm$ 0.36	8.5	7.2 $\pm$ 0.40	8.1	NS
		6.4 $\pm$ 0.55	6.6	6.6 $\pm$ 0.46	7.4	NS

<sup>a</sup> Number of mice in each of the treated and untreated groups

<sup>b</sup> Mean survival time (Kaplan-Meier)  $\pm$  standard error of the mean

<sup>c</sup> 3,4-DAP (phosphate salt) at 4 mg/kg

<sup>d</sup> 3,4-DAP (phosphate salt) at 8 mg/kg

<sup>e</sup> Not significant (P>0.05)

<sup>f</sup> 3,4 DAP treatment shortened survival time

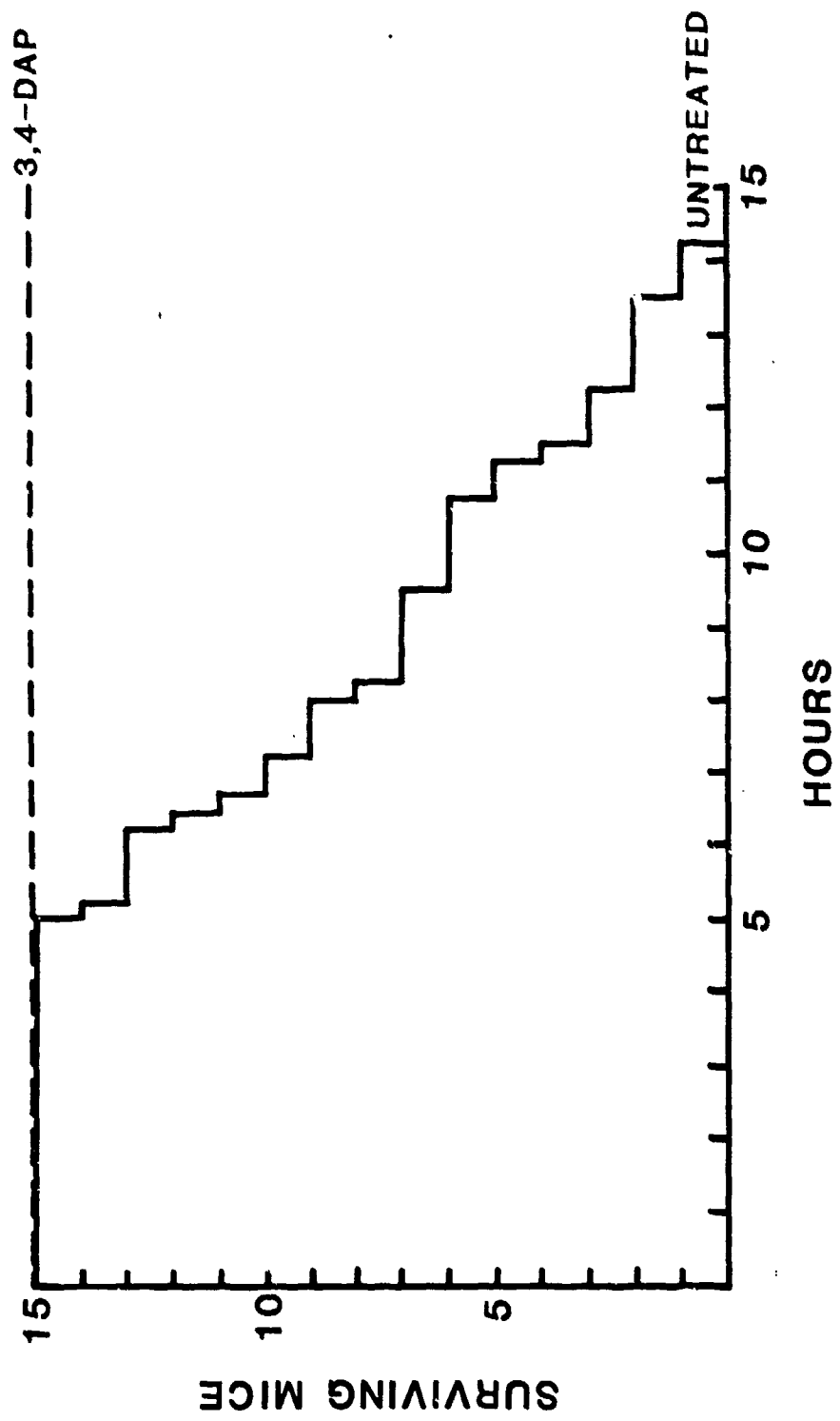
## FIGURE LEGENDS

Fig. 1. Number of surviving mice, injected with 20 LD<sub>50</sub> of type A BoTx at zero time, plotted as a function of time for 15 hr. Untreated (———) and treated (— — —) with 4 mg/kg 3,4-DAP (phosphate salt) beginning at 3 hr and at hourly intervals thereafter.

Fig. 2. Number of surviving mice, injected with 20 LD<sub>50</sub> of type A BoTx at zero time, plotted as a function of time for 32 hr. Untreated (———) and treated (— — —) with 4 mg/kg 3,4-DAP (phosphate salt) beginning at 3 hr and at hourly intervals thereafter.

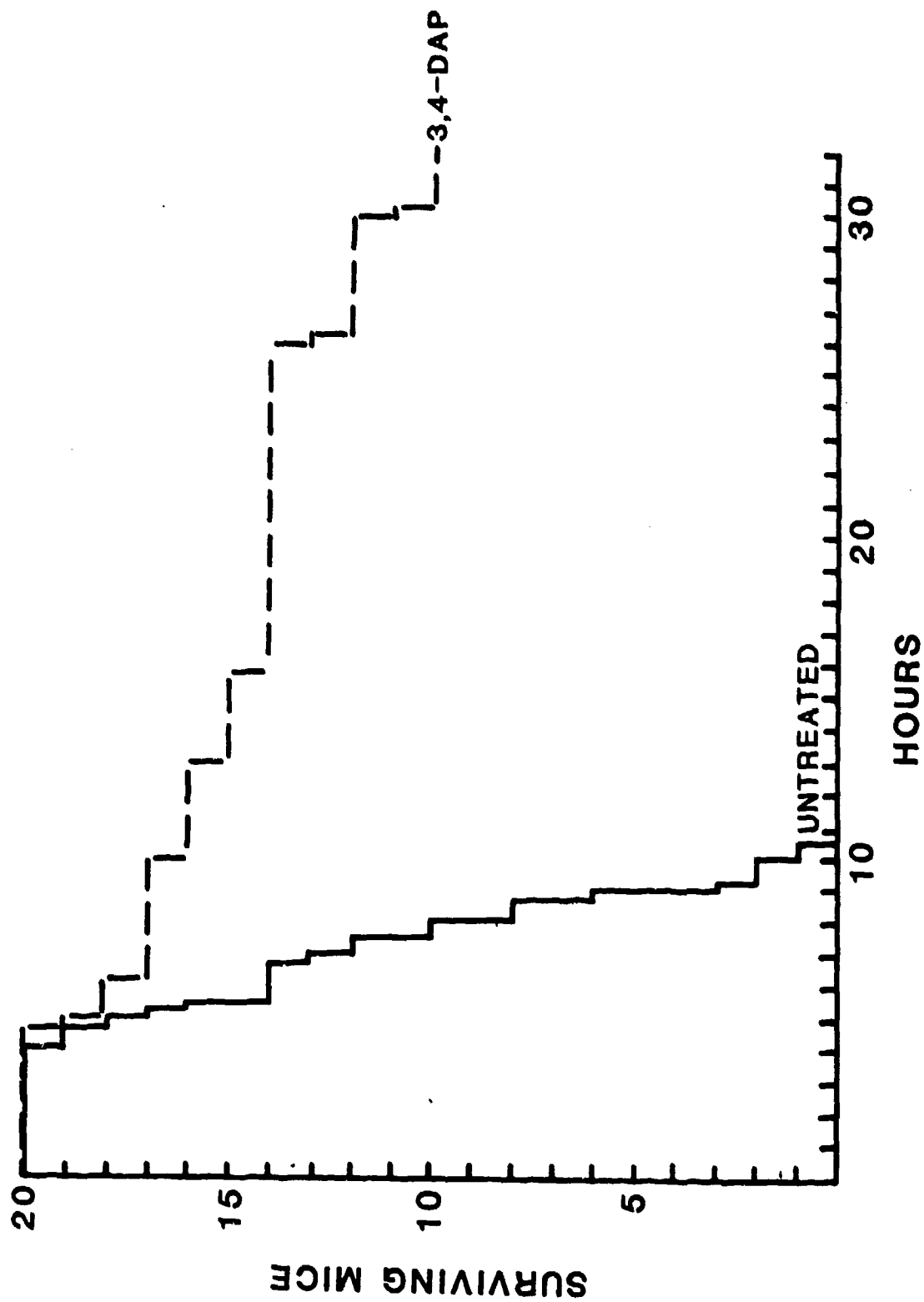
Fig. 3. Number of surviving mice, injected with 20 LD<sub>50</sub> of type E BoTx at zero time, plotted as a function of time for 10 hr. Untreated (———) and treated (— — —) with 4 mg/kg 3,4-DAP (phosphate salt) beginning at 3 hr and at hourly intervals thereafter.

TYPE A 20 LD<sub>50</sub>





TYPE A 20 LD50



TYPE E 20 LD<sub>50</sub>

