CAUSE OF IMMEDIATE DEATH BY LARGE DOSES OF BOTULINUS TOXIN.

By JACQUES J. BRONFENBRENNER, Ph.D., MONROE J. SCHLESINGER, Ph.D., and PAUL F. ORR.

(From the Department of Preventive Medicine of Harvard Medical School, Boston.)

(Received for publication, February 27, 1924.)

Ever since the discovery of the existence of soluble toxins in the culture filtrates of certain bacteria, it has been observed that these toxins differ radically from metallic or alkaloidal poisons in that they produce no immediate effect on experimental animals. The symptoms of intoxication by bacterial toxins appear only after a comparatively protracted period of latency. Within certain limits the duration of the period of latency in the action of toxins (so called period of incubation) was found to be in inverse relation to the amount of toxin administered. Thus, for instance, the symptoms of poisoning do not appear in experimental animals (mice) for several days after the injection of small amounts of tetanus toxin, but this period of latency can be shortened to 12 hours if 3,600 lethal doses of toxin are injected.[1] However, no matter how much toxin is injected in the case of tetanus or diphtheria toxin, the period of incubation cannot be reduced below 8 to 10 hours.

In the case of *botulinus* toxin, the period of incubation seems to be considerably shorter, but it may vary within very wide limits. In our own experience, among seven persons involved in a single outbreak which terminated in six deaths and one recovery, the time of the onset of symptoms varied from 4 to 54 hours after the ingestion of poisonous food.[2,3] When this toxin is introduced parenterally into experimental animals in small amounts, the first symptoms may

[1] De Waele, H., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 148.

[2] Sisco, D. L., *J. Am. Med. Assn.*, 1920, lxxiv, 516.

[3] Sisco, D. L., An epidemiological study of food poisoning, Thesis, Harvard University, 1920.

not appear before the expiration of 24 hours. When larger amounts of toxin are administered, one might expect a shortening of the incubation period comparable to that observed in the case of other bacterial toxins. However, in the course of study of toxicity of various food products experimentally infected with *Bacillus botulinus*, one of us (Orr) has observed that the injection of massive doses of such material (0.5 to 1 cc.) may cause death of animals in from 5 to 10 minutes after injection. Since this apparent absence of incubation period is inconsistent with the established conceptions concerning the mode of action of bacterial toxins, we thought it advisable to study the subject more closely.

A culture of *B. botulinus*, Type A, was grown at 37°C. for 5 days on minced meat glucose broth medium. At the end of this time, the liquid portion of the culture medium was decanted, forced through a sterile Berkefeld filter, diluted with physiological salt solution, and injected intraperitoneally into a series of mice in the amounts indicated in Table I. The animals were carefully observed and the character of the symptoms of intoxication, the time of their appearance, and the time of death noted. The experiment was repeated many times, with several batches of toxic culture filtrate. The results obtained varied somewhat with different filtrates and with different batches of mice. In the composite Table I we have entered two sets of results for each dose of toxin, the first figure indicating the shortest and the second the longest interval elapsing between the time of injection of toxin and death.[4]

As may be seen from these results, the interval of time elapsing between the intraperitoneal injection of the toxic filtrate and the death of mice is reducible from over 24 hours to about 1 hour by increasing the amount of toxin injected from a dose of 3×10^{-6} cc. to that of 3×10^{-2} cc. The animals receiving 3×10^{-2} cc. of toxin show at first no effects of injection and for about $\frac{1}{2}$ hour remain apparently normal, perhaps somewhat more quiet than control mice. Later, however, their respiration becomes progressively more labored, at first increasing in rate and then in depth. This change comes

[4] While it is customary to consider as the incubation period the time elapsing between the injection of the toxin and the appearance of the first unmistakable symptoms of intoxication, nevertheless in true *botulinus* poisoning in mice, the onset of symptoms is not very well defined and it varies considerably with individual animals. For this reason, we have recorded instead the time of death, which is more uniform.

on so gradually and is so mild at first that it is difficult to establish the exact time of its onset. In the next few minutes abdominal muscles become completely relaxed and flaccid; the apparent constriction at the diaphragm gives the animal a wasp-like appearance; it lies motionless on its abdomen and does not respond to external stimuli. The respiration rate falls very rapidly and may become as low as 5 per minute. The inspirations become extremely labored. The majority of animals present at this stage a marked exophthalmus. They soon go into coma which is not interrupted until death occurs, in about 1 hour from the time of the administration of toxin. But if the amount of the filtrate injected is much greater than 3×10^{-2} cc., the picture

TABLE I.

Period of Survival after Graded Doses of botulinus Toxin.

Amount of toxin injected, *cc.*...	10^{-0} 1	3×10^{-1} 0.3	3×10^{-2} 0.03	3×10^{-3} 0.003	3×10^{-4} 0.0003	3×10^{-5} 0.00003	3×10^{-6} 0.000003	3×10^{-7} 0.0000003
Minimum interval before death.....	5 min.	5 min.	1 hr.	1 hr.	2 hrs.	7 hrs., 45 min.	24 hrs.	Survived.
Maximum interval before death.....	7 "	1 hr.	1½ hrs.	2 hrs., 15 min.	6½ "	20 hrs.	55½ "	"

is strikingly different. The animals show definite restlessness and marked contraction of the abdominal muscles almost before the entire contents of the syringe have passed into the peritoneal cavity. Within a minute or two, they give an increased response to external stimuli, especially to sharp sound. Shortly they become prostrated, though the increased excitability persists a few minutes longer. Respiration increases in depth and decreases in frequency. The animal goes into coma interrupted by sharp convulsive seizures with clonic contraction of the extensor muscles throughout the body. Death occurs in 5 to 15 minutes after the injection and during one of these convulsive seizures. In several instances, particularly among the

larger mice receiving moderate amounts of toxin,[5] the animals exhibited most of the acute symptoms just described, but recovered temporarily, only to succumb later, that is to say within an hour or two, with symptoms of *botulinus* poisoning similar to those occurring in mice receiving smaller amounts of toxin.

Specificity of the Toxic Filtrate.

The observations just mentioned suggest the possibility that the filtrate contains two toxic substances,—one, acting slowly, apparently present in high concentration so that its presence can be demonstrated even in high dilutions of culture filtrate; the other causing acute immediate symptoms and detectable only when comparatively large amounts of filtrate are injected.

That *botulinus* toxin may have a composite nature seems quite possible, but,—by analogy with tetanus toxin, which is composed of tetanospasmin and tetanolysin,—it might be expected that if both the toxic components of *botulinus* filtrate are of the nature of true toxin, they should both be neutralized by the antitoxin. This was investigated in the following experiment.

Five test-tubes each received 0.2 cc. of the filtrate of a 5 day old culture of *B. botulinus*, Type A. This amount was selected, since the results of a preliminary experiment indicated it to be the largest amount of this culture filtrate which can be injected intraperitoneally in mice without causing acute immediate death. To each of these tubes containing toxin was introduced respectively 0.1, 0.05, 0.02, and 0.01 cc. of specific antitoxin, the fifth tube receiving no antitoxin (control). Similarly, another series of two test-tubes received each 0.5 cc. of the same culture filtrate,—an amount selected because it is certain to cause the symptoms of acute intoxication. To the contents of one of the latter tubes was added 0.5 cc. of antitoxin, that is to say at least four times as much as is necessary to neutralize the specific toxin present (Table II, Tube 2), while the other tube received no antitoxin (control). A third series of three tubes received respectively 0.3, 0.5, and 1 cc. of uninoculated sterile culture medium (minced meat glucose broth). The contents of each one of the ten tubes were brought to a uniform volume of 1 cc., by the addition of physiological salt solution, and incubated at 37°C. for 2 hours. At this time, the contents of the respective tubes were injected intraperitoneally into normal mice of 17 to 22 gm. and the results of this toxicity test were recorded in terms of time elapsing between the

[5] See second mouse receiving 0.3 cc. of toxin, Table I.

injection and death of animals. Three such experiments in all were performed. The time of death recorded in Table II represents respectively the minimum and the maximum periods of survival observed in the three experiments.

It will be seen that Guinea Pig 6, receiving a quantity of antitoxin sufficient to neutralize more than 1 cc. of specific toxin, died 5 minutes after injection of one-half this amount of the crude *botulinus* filtrate, exactly as did Guinea Pig 7 which received no antitoxin at all.

The results of this experiment indicate that the acute symptoms of intoxication observed after the injection of comparatively large

TABLE II.

Neutralizing Effect of Antitoxin on Large and Small Amounts of botulinus Toxin.

	Tube No.	Amount of toxic culture filtrate.	Amount of sterile medium.	Amount of antitoxin.	Results.
		cc.	*cc.*	*cc.*	
	1	0.2		0.1	Survived.
	2	0.2		0.05	"
A.	3	0.2		0.02	Died in 36 to 56 hrs.
	4	0.2		0.01	" " 12½ " 20 "
	5	0.2 (control).		0.00	" " 1 hr. to 1 hr., 20 min.
B.	6	0.5		0.5	Died in 5 to 8 min.
	7	0.5 (control).		0.0	" " 5 " 10 "
	8		0.3	0.0	Survived.
C.	9		0.5	0.0	"
	10		1.0	0.0	"

doses of *botulinus* culture filtrate are due to the presence in this filtrate, in addition to a specific toxin, of some other poisonous substance which is not neutralized by an excess of specific antitoxin (Table II, *B*). Such a poisonous constituent must, nevertheless, be a product of the growth of *Bacillus botulinus* on minced meat glucose broth, since the original medium is free from this poison (Table II, *C*).

Nature of Toxic By-Product of botulinus Culture Filtrate.

In order to obtain some evidence as to the nature of the poison which causes acute intoxication in mice injected with large doses of *botulinus* filtrate, we attempted to determine its resistance to heat.

3 cc. of a filtrate of 5 day old culture of *B. botulinus* were heated for 5 minutes at 80°C. in a sealed test-tube. Such an exposure is known to be amply sufficient for the destruction of true toxin. The contents were quickly cooled by immersing the test-tube in cold water and injected intraperitoneally into a series of mice in the amounts indicated in Table III, *B*. Another portion of 3 cc. of the same filtrate was placed in an open graduated test-tube and subjected to boiling over a flame for 20 minutes. At the end of this time, the evaporated water was replaced to the original volume of 3 cc. and the contents of the tube injected into a series of mice as indicated in Table III, *C*. A third 3 cc. portion of the same filtrate was sealed in a glass tube and subjected to autoclaving for 20 minutes at 15 pounds pressure, after which the tube was cooled, opened, and the contents injected into mice as indicated in Table III, *D*. As a control, a fourth portion of filtrate was injected into mice without any preliminary treatment (Table III, *A*). The results of one such experiment are presented in Table III, in terms of the time elapsing between the injection and death of the mice.

TABLE III.

Effect of Heating in Open and Closed Containers on the Toxicity of B. botulinus Culture Filtrate.

	Amounts of filtrate injected.					
	1 cc.	0.7 cc.	0.5 cc.	0.3 cc.	0.2 cc.	0.00002 cc.
A. Untreated filtrate (control).....	6 min.	8 min.	10 min.	14 min.	1 hr., 10 min.	8 hrs.
B. Filtrate heated at 80°C., 5 min.....	5 "	5 "	10 "	10 "	Survived.	Survived.
C. Filtrate boiled 20 min. in open tube.	12 "	15 "	Survived.	Survived.	"	"
D. Filtrate autoclaved (sealed)....	5 "	5 "	6 min.	8 min.	"	"

These results show that the substance which is responsible for the toxicity of minute amounts of the filtrate; which acts only after an incubation period of not less than 1 hour (Table I); and which is capable of being neutralized by *botulinus* antitoxin (Table II, *A*) possesses still another property of true bacterial toxins—it is thermolabile (Table III, *B*).

As to the second toxic constituent of *botulinus* culture filtrate, active when it is given in large amounts and responsible then for immediate acute symptoms of poisoning and the rapid death of animals, it acts without any noteworthy incubation period (Table I),

is not neutralized by an excess of *botulinus* antitoxin (Table II, *B*), and is thermostable (Table III, *B*). The fact that this poison is weakened when boiled in an open vessel (Table III, *C*), while it retains its activity in a sealed container, even after autoclaving (Table III, *D*), may be taken to indicate that it is a chemical poison capable of volatilization.

Since *Bacillus botulinus* is known to possess marked proteolytic power, it was suspected that the immediate toxicity of the filtrates might be due to accumulation in the culture of some toxic products of putrefaction. In order to investigate this possibility, we have prepared filtrates from cultures of other putrefactive bacteria grown on minced meat glucose broth and tested their toxicity on mice. We found that the filtrates of cultures of *Bacillus proteus*, *Bacillus aerogenes*, and of atoxic strains of *Bacillus botulinus* were all able to elicit in mice symptoms of acute intoxication identical with those already described. Moreover, the substances responsible for the toxicity of these filtrates were also found to be relatively thermostable and volatile.

Some time ago, while studying the nature of "toxin" produced by *Bacillus œdematis maligni*, Barger and Dale[6] concluded that the toxicity of the filtrates of this organism is due to accumulation of ammonium salts. In order to ascertain whether the toxic substances accumulating in the cultures of *Bacillus botulinus* could be ammonium salts, a small portion of the filtrate was made alkaline by the addition of an excess of NaOH solution, subjected to boiling, and the reaction of the vapor tested with litmus paper. This test showed that the filtrate contained an appreciable amount of volatile bases. When the filtrate was subjected to aeration by the method of Folin, it was found that a considerable portion of the volatile bases could be identified as ammonia. The aeration of various samples showed that 1 cc. of the filtrate may contain from 0.01 to 0.05 gm. of ammonia, depending upon the age of the culture.

In order to determine whether this amount of ammonia could alone be responsible for the acute intoxication of animals, we injected mice intraperitoneally with solutions of ammonium chloride and

[6] Barger, G., and Dale, H. H., *Brit. Med. J.*, 1915, ii, 808.

ammonium sulfate, the ammonium content of which was nearly equivalent to that present in the filtrate. The results of this experiment are shown in Table IV, in terms of period of survival after injection.

The results of this experiment indicate that the toxicity of ammonium salts is comparable to that of *Bacillus botulinus* filtrates when the doses are selected so as to contain approximately equivalent amounts of ammonia. Indeed this similarity and the similarity of symptoms elicited are so striking that one may be allowed to conclude that the acute toxicity of the large doses of culture filtrates of *Bacillus botulinus* is due directly to the ammonium salts present in them.

TABLE IV.

Ammonium chloride.			Ammonium sulfate.			*Botulinus* toxin.		
Amount of salt injected.	Amount of NH_3 calculated.	Result of injection.	Amount of salt injected.	Amount of NH_3 calculated.	Result of injection.	Amount of toxin injected.	Amount of NH_3 calculated.	Result of injection.
gm.	*gm.*		*gm.*	*gm.*		*cc.*	*gm.*	
0.1	0.032	2½ min.	0.1	0.025	4 min.	1	0.010	5 min.
0.05	0.016	4 "	0.05	0.012	5 "	0.5	0.005	7 "
0.025	0.008	6 "	0.025	0.006	6 "	0.25	0.002	65 "
0.01	0.003	50 "	0.01	0.002	Recovered.	0.1	0.001	55 "
0.005	0.001	Recovered.	0.005	0.001	"			

SUMMARY AND DISCUSSION.

Parenteral introduction of amounts of the culture filtrate of *Bacillus botulinus* greatly in excess of the minimum lethal dose has been observed to cause the practically immediate death of mice. This result is due to the presence in the filtrates of a chemical poison possessing properties distinct from those of the contained *botulinus* toxin which itself acts only after a well defined period of incubation. This chemical poison is not neutralized by *botulinus* antitoxin; it is effective only when large amounts of the culture filtrate are given; it is thermostable, not being destroyed when heated in the autoclave in a sealed tube, though when it is heated in an open container its toxicity diminishes with a coincidental volatilization of basic material. The volatile substance can be identified as ammonia.

Death resulting from the injection of comparatively large amounts of ammonium salts (0.1 gm.) is easily distinguished from that due to botulism, both through the character of the symptoms and the absence of an incubation period. However, when the amount of toxic salts injected is smaller (0.01 gm.), the symptoms of poisoning are not so characteristic and death may be delayed long enough to suggest a period of incubation similar to that observed in botulism (Table IV). This circumstance is of importance in connection with the examination of partly decomposed food products in which the presence of *botulinus* toxin is suspected. As a rule such suspected material is injected in massive doses (0.5 to 1 cc.) in mice.[7,8] It is conceivable that such spoiled foods may be contaminated with common putrefactive bacteria yielding ammonia during their growth and thus may cause death of the test animals. If in such tests mice passively protected by the preliminary injection of an excess of antitoxin be used in addition to normal animals, the chances of an error in the interpretation of the results will be materially reduced, though not ruled out. Unfortunately for such a procedure, *botulinus* antitoxin is not readily available, while furthermore, recent findings[9] indicate that it may not always be effective owing to the existence of a group of toxin-producing bacteria very similar to *Bacillus botulinus,* but not homologous immunologically with either of the known types of the latter.

The test of thermostability of the toxic constituents of suspected food may conceivably help to determine the true nature of the poison.

[7] Orr, P. F., *J. Infect. Dis.*, 1921, xxix, 287.
[8] Bengtson, I. A., *Pub. Health Rep., U. S. P. H.*, 1921, xxxvi, 1665.
[9] Bengtson, I. A., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 340.