THE NATURE OF THE TOXIN-ANTITOXIN FLOCCULATION PHENOMENON.

By J. J. BRONFENBRENNER, Ph.D., and PHILIP REICHERT, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Botulinus antitoxin is a specific remedial agent of experimentally demonstrable therapeutic value. However, in clinical cases of botulism the absence of any reliable criteria for early diagnosis precludes the timely application of the serum therapy. The clinical diagnosis of *botulinus* poisoning is made only from those central nervous symptoms which by their very presence foretell the probable fatal termination of the disease.

In an earlier series of experiments it was found that ingested *botulinus* toxin may be detected in the blood of experimental animals before the onset of unmistakable symptoms of poisoning.[1] The blood of larger animals to which toxin had been fed was injected into mice. The presence of the toxin could be established only after its concentration in the blood of the donor animals had reached a comparatively high level, so that the amount injected into a mouse would contain at least one minimal lethal dose. But the recent publications of Ramon would seem to indicate that it may be possible to detect lower concentrations of toxin by means of precipitation with a specific antitoxin (1–3).

With the problem of early diagnosis of botulism in view, we set out to study the mechanism of the Ramon test. Attempts were made to precipitate 4 day toxin by homologous antitoxin. Ramon had shown that when a series of varying amounts of diphtheria toxin and antitoxin are combined and incubated at an appropriate temperature, there appears a flocculent precipitate in a zone of dilutions which corresponds roughly with those mixtures that are neutral in guinea pig tests.

[1] Bronfenbrenner, J., and Weiss, H., unpublished results, 1922.

Having previously determined that our strain of *B. botulinus* yields its most active toxin in the filtrates of 4 day cultures (4), we prepared such filtrates, and found them to contain approximately 100,000 guinea pig M.L.D. per cc. When incubated for a week with the addition of 0.7 per cent formalin, this so called anatoxin (5), was found to be devoid of all toxicity. The rabbits immunized with the anatoxin yielded sera of high antitoxic value. When pooled rabbit serum was titrated in mice, 0.1 cc. protected the animals against approximately 6000 fatal doses of toxin. Precipitation tests were made with a constant amount

Protocol I.

Flocculation of Pooled Antitoxic Serum by Toxic Filtrates of B. botulinus.

	Amount of antigen	.02 cc. of pooled antitoxic serum			Amount of antigen	.02 cc. of pooled antitoxic serum	
		Filtrate of 3 day culture M.L.D. = .00001	Filtrate of 16 day culture M.L.D. = .001			Filtrate of 3 day culture M.L.D. = .00001	Filtrate of 16 day culture M.L.D. = .001
1	.8	—	—	15	.001	—	—
2	.6	—	—	16	.0008	—	—
3	.4	—	—	17	.0006	—	—
4	.2	—	—	18	.0004	—	—
5	.1	—	—	19	.0002	—	—
6	.08	—	—	20	.0001	—	—
7	.06	—	—	21	.00008	—	—
8	.04	—	—	22	.00006	—	—
9	.02	—	—	23	.00004	—	—
10	.01	—	—	24	.00002	—	—
11	.008	—	—	25	.00001	—	—
12	.006	—	—	26	Control of antigen	—	—
13	.004	—	—	27	" " serum	—	—
14	.002	—	—				

of the serum and a series of dilutions of antigen (4 day toxin) so that the zone of precipitation, if narrow, might not be missed. Toxins of various ages and activity were tested, and the tests were repeated a number of times. All were negative, as shown in Protocol I.

Suspecting some error in technique, we made parallel tests with diphtheria toxin and antitoxin obtained from the New York City Department of Health. The typical flocculation of these mixtures followed, as shown in Protocol II, but the parallel series with *botulinus* toxin-antitoxin remained clear. We varied the hydrogen ion con-

centration, the concentration of electrolytes, and the degree of dilution, but no specific precipitation resulted within the limits of changes not affecting the serum controls. One sample of *botulinus* antitoxin received from the Department of Health gave a very weak and irregular precipitation, but only when this serum was combined with the filtrate of a 24 day culture.

Protocol II.

*Flocculation of Diphtheria and Botulinus Toxins with Their Respective Antitoxins.**

		Diphtheria toxin		*Botulinus* toxin	
		.5 cc. of a 4 L+ toxin	.2 cc. of a 11 L+ toxin	.25 cc. of 4 day filtrate	.25 cc. of 16 day filtrate
Amount of homologous antitoxin	.3000	−	−	−	−
	.2000	−	−	−	−
	.1500	−	−	− (N.)	−
	.1000	−	−	−	−
	.0750	−	−	−	−
	.0500	−	−	−	−
	.0300	−	−	−	−
	.0200	−	−	−	−
	.0150	−	−	−	− (N.)
	.0100	<+	−	−	−
	.0075	<+	<+	−	−
	.0050	++ (N.)	+ (N.)	−	−
	.0030	++	++	−	−
	.0020	−	++	−	−
	.0015	−	+	−	−
	.0010	−	<+	−	−
	Serum control	−	−	−	−
	Antigen "	−	−	−	−

(N.) is the calculated neutral point.

* In this, as well as in other experiments, ++ = copious precipitation; +, fair amount of precipitation; <+, slight amount of precipitation; −, no precipitation.

Having previously observed (6, 7) that precipitation occurs when *botulinus* antitoxin (obtained from the New York City Department of Health) is combined with bacterial autolysates or with extracts of foods infected with *B. botulinus,* we suspected that the present failure might be attributed to the purity of our antigen, since a filtrate of a 4 day culture could be presumed to contain relatively a low concen-

tration of bacterial protein. The precipitate in Ramon's tests might result, we thought, not from actual antitoxin content of the serum, but from the presence in the serum of an antibacterial antibody. In order to test this notion, we prepared an antiserum which, in addition to antitoxin, might contain antibacterial antibody.

Protocol III.

The Influence of Antibacterial Antibody upon the Flocculation of Antitoxin by Toxins.

Amount of antigen (filtrate)	.05 cc. of antiserum to the 4 day toxic filtrate (no antibacterial antibody).		.05 cc. of antiserum to the 24 day toxic filtrate (antibacterial antibody present)	
	Toxic filtrate of 4 day culture 100,000 M.L.D./cc.	Toxic filtrate of 16 day culture 100 M.L.D./cc.	Toxic filtrate of 4 day culture 100,000 M.L.D. per cc.	Toxic filtrate of 24 day culture 100 M.L.D. per cc.
cc.				
1.000	−	−	−	−
.750	−	−	−	−
.500	−	−	−	−
.300	−	−	−	−
.200	−	−	−	−
.150	−	−	−	<+
.100	−	−	−	+
.075	−	−	−	+
.050	−	−	−	+
.030	−	−	<+	++
.020	−	−	+	+
.015	−	−	<+	<+
.010	−	−	<+	<+
Control of antigen	−	−	−	−
" " serum	−	−	−	−

Immunization with Filtrates of Old Cultures.

For this purpose, we immunized a new set of animals with the filtrates of 24 day cultures of *B. botulinus*. Although the filtrates contained only 100 guinea pig M.L.D. of toxin per cc., as determined by preliminary titration, they were assumed to contain the products of bacterial autolysis.

The filtrates were incubated as previously with 0.7 per cent formalin for a week, and then employed for subcutaneous injection in rabbits. When the pooled serum obtained from these animals was tested against the filtrate of the 24 day

culture or against the anatoxin prepared from it, the typical flocculation zone was brought out without difficulty. However, when it was tested against the filtrates of the 4 day cultures which we had used earlier (Protocol I), the second serum gave a definite flocculus, but it was less in amount and restricted to a narrower zone (Protocol III) (8).

The best results were obtained when the ingredients were measured directly, with a micro pipette, and without dilution. The racks were placed in the water bath at 55°C. for 20 minutes, in the 37° incubator for 18 hours, and then in the ice box for 24 hours. Readings were made at the end of each interval, and finally the flocculus was stained and examined for the presence of bacterial growth.

Immunization with Washed Bacteria.

Preceding experiments having shown that an antiserum containing antitoxic antibodies alone will not cause precipitation of a toxic filtrate, but that an antiserum that contains antibacterial antibody in addition to the antitoxin will cause such precipitation, it seemed worth while to determine whether an antibacterial serum wholly devoid of antitoxin would cause precipitation of a toxic filtrate.

A 24 hour culture of a rapidly growing strain of *B. botulinus* was filtered from a beef heart medium through a paper filter to remove the meat particles. The cloudy filtrate was centrifuged and the sediment of bacteria resuspended in saline, and then centrifuged again. Washing was repeated a sufficient number of times to insure the elimination of toxin by dilution. The final suspension of bacteria in saline was then shaken with glass beads with a few drops of chloroform in a shaking machine for 48 hours and filtered. The final filtrate up to 1.0 cc. of autolyzed bacteria was found non-toxic by intraperitoneal injection in mice. This material was used for the subcutaneous immunization of rabbits.

The serum thus obtained did not contain enough antitoxin in 0.1 cc. to protect a mouse against even a single M.L.D. of a Type A toxin. Yet the serum, when combined with the toxic filtrates of cultures of various ages, gave in each case definite precipitations in a comparatively wide zone. It yielded similar reactions in approximately the same zone with anatoxins made from toxins that had originally contained one-hundredth the number of M.L.D. per cc. (Protocol IV). As there was no antitoxin in the system, the precipitation could not have been influenced by antitoxic antibodies.

Immunization with Filtrates of an Atoxic Variant.

The experiments described above suggest that the precipitation of toxic filtrates of *B. botulinus* may be wholly independent of the antitoxin content of the precipitating serum. In order to determine this point, an antiserum was prepared by the immunization of animals with a homologous atoxic strain of the bacillus.

Protocol IV.

Precipitation of Antibacterial Serum Type "A" by Toxin.*

		.05 cc. of Type "A" antibacterial serum		
		Toxic filtrate of 9 day Type "A" culture (100,000 M.L.D. per cc.)	Formalinized filtrate of 16 day Type "A" culture (originally 1000 M.L.D per cc.)	Formalinized filtrate of 24 day Type "B" culture (control)
Amount of antigen (filtrate)	1.000	−	−	−
	.750	−	−	−
	.500	−	+	−
	.300	−	+	−
	.200	+	++	−
	.150	+	++	−
	.100	++	++	−
	.075	++	+	−
	.050	++	+	−
	.030	++	+	−
	.020	+	+	−
	.015	+	−	−
	.010	+	−	−
	Control of antigen	−	−	−
	" " serum	−	−	−

* This serum contains no antitoxin.

Not every individual organism in a culture of *B. botulinus* is a toxin producer. Single cell cultures produce toxins of various strengths, and occasionally a culture will yield no toxin at all (9). By means of single cell culture (Barber's method), a Type B strain was isolated which failed to produce toxin, but which by morphology, staining reaction, and by agglutination was identified with the mother and with sister strains. When the non-toxic filtrate of this strain was combined with a known antitoxin, flocculation resulted (Protocol V).

Filtrates of 9 day old cultures of this non-toxic strain were used to immunize guinea pigs. Since these filtrates were wholly non-toxic, it was not necessary to formalinize them. After an appropriate succession of subcutaneous injections, the animals were tested and found not to be resistant even to a single lethal dose of Type B toxin, and

Protocol V.

Precipitation of a Type "B" Antitoxin by the Filtrate of an Atoxic Type "B" Variant.

Amount of antigen (atoxic filtrate)	.05 cc. of Type "B" antitoxin	Amount of antigen (atoxic filtrate)	.05 cc. of Type "B" antitoxin
cc.		cc.	
.500	+	.050	+
.300	+	.030	−
.200	+	.020	−
.150	++	.015	−
.100	+	Control of antigen	−
.075	+	" " serum	−

Protocol VI.

Precipitation of Antifiltrate Serum (of Type "B" Atoxic Variant) by the Atoxic Filtrate Itself and by a Type "B" Toxin.

Amount of antigen	.05 cc. of antifiltrate serum Type "B"		Amount of antigen	.05 cc. of antifiltrate serum Type "B"	
	Toxic filtrate of Type "B" 24 day culture 100 M.L.D. per cc.	Atoxic filtrate of Type "B" variant 9 day culture		Toxic filtrate of Type "B" 24 day culture 100 M.L.D. per cc.	Atoxic filtrate of Type "B" variant 9 day culture
.500	−	−	.050	−	−
.300	<+	+	.030	−	−
.200	<+	+	.020	−	−
.150	+	++	.015	−	−
.100	<+	+	Control of antigen	−	−
.075	−	+	" " serum	−	−

the serum of the animals failed to protect mice against the homologous toxin. However, the immune serum agglutinated the non-toxic variant and also the bacteria of the toxic mother strain. It flocculated the filtrate of the atoxic variant and at approximately the same point flocculated an old toxin (Protocol VI).

Immunization with Washed Atoxic Variant.

The flocculation of Type B toxin with the serum of animals immunized by the 9 day filtrate of the atoxic variant of this strain was weak and confined to a narrow zone. If the phenomenon is, as our experiments suggested, actually an antibacterial precipitation, it should be possible to increase the precipitating power of the serum by immunizing the animals with the washed culture of the variant.

Accordingly, animals were immunized with the washed bacteria of the atoxic variant, and the serum obtained was found to precipitate the homologous Type B toxin, but more abundantly and in a wider zone (Protocol VII). This serum contained no protective antibodies.

Protocol VII.

Precipitation of Antibacterial Serum (of Type "B" Atoxic Variant) by a Type "B" Toxin.

Amount of antigen, Type "B" toxin 300 M.L.D. per cc.	.05 cc. of Antibacterial Serum "B"	Amount of antigen, Type "B" toxin 300 M.L.D. per cc.	.05 cc. of Antibacterial Serum "B"
.500	−	.050	+
.300	−	.030	+
.200	<+	.020	−
.150	+	.015	−
.100	+	Control of antigen	−
.075	+	" " serum	−

Is the Antitoxin Precipitated?

In specific precipitations, the bulk of the precipitate has repeatedly been shown to be composed mainly of the antibody-carrying globulin. If the precipitate which occurred in the Ramon test took place at the expense of the antitoxin, the latter must have been appreciably diminished in concentration in the supernatant fluid.

We tested quantitatively the antitoxic content of the supernatant fluid of the "indicating tube," at the center of the zone (1), using as antigen a formalinized filtrate, paralleling this with a tube that contained the same amount of antitoxin, but in which a heterologous flocculation was made to take place, since it was considered possible that some of the antitoxin might be carried down by adsorption on the precipitate formed. A third tube contained antitoxin diluted with broth

to the same extent. The quantities of the reagents used were those that in preliminary tests had been determined as optimum.

After the usual incubation a heavy precipitate appeared in Tubes A and C; Tube B remained clear (Protocol VIII). The flocculus was centrifuged thoroughly, and the supernatant fluids were titrated for comparison of their antitoxic value by intraperitoneal injection in mice, each of which received a single M.L.D. of toxin. The results are given in Protocol VIII.

It is evident that there is practically no difference in the protective power of the serum before (B) and after its flocculation with its own

Protocol VIII.

		Tube A	Tube B	Tube C
		3 cc. anatoxin *Botulinus* A 1 " antitoxin " "	3 cc. broth 1 " antitoxin *Botulinus* A	1.5 cc. broth 1.0 " horse serum .5 " anti-horse serum 1.0 " antitoxin *Botulinus* A
		Each tube centrifuged at high speed and supernatant fluid carefully removed, combined with toxin in doses shown, and injected intraperitoneally in mice. Effect on the mice:		
Toxin "A"	Amount of the supernatant fluids			
.00001	.01000	Survived	Survived	Survived
.00001	.00500	"	"	"
.00001	.00250	"	"	"
.00001	.00200	"	"	"
.00001	.00150	"	"	"
.00001	.00100	Died in 20 hrs.	Died in 80 hrs.	Died in 40 hrs.
.00001	.00075	" " <36 "	" " 44 "	" " <16 "
.00001	None	" " <16 "	" " <16 "	" " <16 "

antigen (A), except for such small amount of antitoxin as may be carried down with the precipitate by physical means (see Protocol VIII, Tube C). The antitoxin as such appears to take no part in the precipitation phenomenon of Ramon.

Removal of the Precipitable Substance.

It has been shown (17) that acidification of toxic filtrate is followed by precipitation of the protein without reduction of the toxin content. The effort was made to separate the toxic elements of the

filtrate from the protein by this technique in order to see what effect such treatment might have on the precipitation by homologous sera.

To the toxic filtrate of an old culture, normal hydrochloric acid was added until a precipitate appeared. This was removed rapidly by centrifugation and resuspended in normal salt solution. The hydrogen ion concentrations of both the supernatant liquid and of the resuspended precipitate were carefully adjusted to neutral. At this point both solutions were clear. The acid and alkali used diluted somewhat the original toxin, so that a similar quantity of water was

Protocol IX.

Effect of Acid Coagulation on Specific Precipitation of Toxic Filtrates.

			.05 cc. of antitoxic-antibacterial serum		
			Supernatant fluid neutralized after acid coagulation	Acid coagulum redissolved in normal saline and neutralized	Control dilution of the original toxin
Titration by precipitation	Amount of antigen	.50	−	+	+
		.30	−	+	+
		.20	−	+	+
		.15	−	−	+
		.10	−	−	+
		.075	−	−	+
		.050	−	−	−
		.030	−	−	−
		.020	−	−	−
		.015	−	−	−
		.010	−	−	−
	Control of antigen		−	−	−
	" " serum		−	−	−
Titration of toxicity in mice	Amounts given intraperitoneally	.0003	Died in <21 hrs.	Died in <21 hrs.	Died in <21 hrs.
		.0001	" " <21 "	" " <21 "	" " <21 "
		.00003	" " 42 "	" " 40 "	" " <21 "
		.00001	Survived	Survived	Survived

added to a sample of the original toxin to serve as a control. The three mixtures were then titrated in mice and by flocculation (Protocol IX).

The supernatant fluid lost only a small amount of its toxin and this toxin was adsorbed on the precipitate and was demonstrable there. However, the supernatant fluid did not precipitate the antitoxic-antibacterial serum. The acid coagulum, redissolved and readjusted to the neutral point, did produce the specific precipitate with the antitoxic-antibacterial serum.

DISCUSSION.

A method for the titration of diphtheria toxin and antitoxin *in vitro* would constitute so great an improvement over the biologic one, both because of the elimination of many uncertain factors connected with all tests on animals, and from the point of view of economy, that the publications of Ramon immediately attracted wide attention and stimulated investigation of its reliability. Reports of its usefulness because of close agreement with titrations by the Ehrlich biologic technique soon appeared in the literature (10, 2, 3), and the method was adopted as a routine procedure in many laboratories.

Our study of the phenomenon in the case of the toxin of *B. botulinus* has yielded results that show the precipitation to be entirely independent of either the toxin content of the antigen or the antitoxic content of the serum. It is found that atoxic filtrates precipitate antitoxic sera, and purely antibacterial sera are precipitated by active toxins, the width of the zone and the amount of the precipitate depending apparently upon the amount of bacterial protein present in the antigen used to produce the immune serum. The conclusion, therefore, that the precipitation in this instance is not due to union of toxin and antitoxin, but that it is a purely antibacterial precipitation is unescapable.

How then are we to account for the close agreement that so many workers have reported between the *in vitro* and *in vivo* methods of titration in diphtheria? The answer to this question is, we believe, to be found in the fact that the production of diphtheria toxin has been standardized to such an extent that almost all laboratories follow the same technique. The preparation of the medium, its reaction, the age of the cultures before filtration, and other factors are almost identical (11), and even the strain is the well known standard Park and Williams No. 8.

As the result of this uniformity of preparation, the relation between the toxin content and the concentration of bacterial protein in the culture filtrates tends to be constant. Consequently, the concentrations of antitoxin and of antibacterial antibody in the sera produced by immunization of animals with these filtrates bear sufficiently fixed relations to one another to secure comparable results by both methods

of titration. When the composition of the bacterial filtrate changes, the results of titration by precipitation fail to agree with those of the animal test.

We have not undertaken to repeat with diphtheria toxin the tests made with the *botulinus* toxin, but as our experiments progressed, we noted in the literature that discrepancies, giving indirect support to our contention, had actually occurred in the practical use of the Ramon test (12–14). Moreover, Moloney and Weld (15) investigated the neutrality of the indicating tube and reported that a deviation of 300 per cent in either direction from the neutral point is possible; and they observed incidentally that the toxin-antitoxin precipitation in diphtheria bears a relation to the concentration of agglutinins in the serum, and thus confirm our preliminary results (8).

Zingher (16) found that after he had removed a precipitate induced by the formalinization of toxins containing tricresol, the anatoxins gave absolutely no precipitate with corresponding sera, although they remained highly antigenic, a condition which he explains in the light of our findings (8).

The demonstration that the phenomenon of Ramon is a specific antibacterial precipitation explains many discrepancies which have been reported. It indicates also the inapplicability of the Ramon test to the detection of the toxin of *B. botulinus* in the blood of animals fed with the toxin, since it is the toxin itself that is absorbed from the intestinal tract, the accompanying bacterial protein having been digested by the alimentary enzymes.

SUMMARY AND CONCLUSIONS.

1. Animals immunized with the formalinized filtrates of young toxic cultures of *B. botulinus* produce an antitoxic serum poor in precipitins.

2. Animals immunized with the formalinized filtrates of old and partly autolyzed toxic cultures produce an antitoxic serum containing precipitins.

3. Animals immunized with toxin-free autolyzed bacteria produce a serum free from antitoxin but rich in specific precipitins.

4. Animals immunized with the filtrates of an atoxic variant produce a serum free from antitoxin but rich in precipitins for the homologous toxin.

5. Animals immunized with the washed bacteria of the atoxic variant produce a serum that contains no antitoxin, but is rich in precipitins for the homologous toxin.

6. Removal of the precipitins by flocculation with a non-toxic antigen does not materially reduce the antitoxic value of a serum.

7. Removal of the proteins of the antigen by acid coagulation removes the specific precipitable substance.

8. All the sera that contain precipitins produce the specific flocculus when combined with homologous toxins, anatoxins, or with the filtrates of the atoxic variant. The flocculation is restricted within the type. The amount of the precipitate and the width of the zone vary approximately with the estimated amount of bacterial protein in the antigen that is used for the immunization of animals.

We conclude, therefore, that the toxin-antitoxin flocculation is a specific bacterial precipitation phenomenon.

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