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By A. L. Barron[2] and G. B. Reed[3]

Abstract

A strain of *Clostridium botulinum* Type E in a beef heart infusion – peptone broth yields moderate amounts of toxin when grown at 30° C. but fails to produce toxin at 37°. When grown in a cellophane sac suspended in a similar medium, at 30° C., there is a 5- to 10-fold increase in the yield of toxin. Toxoid has been prepared from the high potency cellophane produced toxin by clarification with charcoal, Mandler filtration, and detoxification with 0.3% formalin at 30° C. for 20 days. Three doses of the fluid toxoid protects mice against 500 M.L.D. of homologous toxin. One dose of the same toxoid adsorbed on alum affords the same level of protection as three doses of the fluid toxoid. Mixing Type E toxoid with equal amounts of Types A and B *in vitro* augments the antigenic action of the Type E toxoid.

Introduction

A recent survey by Dolman and Chang (2) reports some 20 isolations of *Clostridium botulinum* Type E since this type was first described in 1936 by Gunnison, Cummings, and Meyer (6). In addition to those listed by Dolman and Chang (2) two other isolations were reported in 1937 (10, 11). During the past six years three outbreaks of human Type E botulism have been reported in British Columbia (1, 3, 4) and one in Alaska (12, 1). The Type E organism is probably widespread throughout the world. The organism shows some predilection for fish or other marine products (1, 7, 14).

The severe toxemia and large proportion of fatalities in outbreaks of human botulism caused by Type E suggests that the toxin produced by this type is of a high order of potency. However, all reports of laboratory cultures of Type E organisms indicate very low yields of toxin as compared with cultures of Types A, B, C, and D organisms. The highest toxin titer reported by Dolman *et al.* (3) was 4000 mouse M.L.D. per ml. of crude culture. This is in line with other reports. In contrast Stevenson *et al.* (18) regularly obtained at least 1,000,000 mouse M.L.D. per ml. of crude cultures of Type A. As suggested by Dolman and Kerr (4), man may be more susceptible to this toxin than the test animals which have been used with laboratory cultures. On the other hand the laboratory conditions may not have been conducive to high toxin production.

An attempt has been made to increase the yield of Type E toxin in order to provide material of sufficient potency to permit the preparation of an efficient toxoid.

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Contribution from the Department of Bacteriology, Queen's University, Kingston, Ontario, and the Defence Research Board Kingston Laboratory. Portion of a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

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1. Toxin Production

Strains of Type E

In a preliminary comparison of six strains of Type E the VH strain kindly sent to us by Dr. Dolman (Dolman *et al.* (3)) gave consistent yields of toxin. This strain was isolated from pickled herring responsible for an outbreak of human botulism.

Stock cultures were maintained in Robertson's meat as suggested by Stevenson *et al.* (18) for Types A and B.

Media

Several types of fluid media have been tested for growth and toxin production, Table I, at 30° and 37° C. The most satisfactory of those tested both from the point of view of growth and toxin formation was Difco heart infusion supplemented with additional peptone, 1-2%, and glucose, 0.5%. Several different peptones appeared equally effective, Table I. In these media growth is about the same at 30° and 37° C. The pepticase – corn-steep – glucose medium used in this laboratory for the production of Types A and B toxin (Stevenson *et al.* (18)) failed to support active growth of Type E.

TABLE I

GROWTH OF *C. botulinum* TYPE E, VH STRAIN, 24 HR. AT 30° AND 37° C. ON VARIOUS MEDIA

Medium	30° C.		37° C.	
	Growth, 24 hr.	pH 5 days	Growth, 24 hr.	pH, 5 days
Robertson's meat	+++	6.8	+++	6.8
Pepticase – corn-steep – glucose	Nil (48 hr.)		Nil (48 hr.)	
Heart infusion – tryptose – Difco	Nil (48 hr.)		Nil (48 hr.)	
Heart infusion – tryptose – glucose with:				
Difco peptone	+++		+++	5.7
Proteose peptone No. 2	+++		+++	5.3
" " No. 3	+++		+++	5.3
" " No. 4	+++		+++	5.3
Tryptone	+++		+++	5.3
Casitone	+++		+++	5.4
Neopeptone	+++		+++	5.4
Tryptone + 0.5% $CaCO_3$	+++	6.0	+++	6.0

Temperature and Toxin Production

Dolman *et al.* (3) reported yields of toxin by the VH strain up to 4000 lethal mouse doses per ml. of crude culture incubated at 37°. We have been unable to duplicate these yields at 37° in any media tested but good yields have been obtained in cultures incubated at 30° C. In Robertson's chopped meat medium, for example, at 30° the order of 4000 lethal mouse doses of toxin were produced whereas in similar media incubated at 37° though the growth was similar no detectable toxin was formed. This is in agreement with results reported by Gunnison *et al.* (6) and Hazen (8).

Cultivation in Cellophane

The yields of toxin by several species of bacteria have been greatly increased by cultivation in cellophane sacs (Sterne and Wentzel (17), Fredette and Ginet (5), Koch and Kaplan (9)). It has been found in this laboratory that *C. botulinum* Type A grown in pepticase – corn-steep – glucose medium regularly produces the order of a million lethal mouse doses of toxin per ml. of crude culture but when the same strain is grown in a cellophane sac suspended in a similar medium, 40 to 50 million lethal doses of toxin per ml. are produced. Moreover, since the medium used by the growing organism must diffuse through the cellophane, large molecule constituents of the medium are excluded. The crude toxin is therefore not only more concentrated but in purer form.

The usual procedure has been to form a cellophane bag by tying off one end of a length of cellophane tubing which is filled with water or saline and suspended in the medium. After sterilization the content of the bag is inoculated. Sterne and Wentzel (17) improved the procedure by intussuscepting a length of cellophane tubing which somewhat increases the surface of cellophane. In this device the authors obtained yields of Type C toxin some 20 times greater and Type D toxin 100 times greater than they obtained in ordinary culture flasks without cellophane.

Type E toxin has been developed in Sterne and Wentzel's modification of the cellophane technique. Fig. 1 indicates an arrangement to contain 500 ml. of fluid inside the cellophane and 6000 ml. outside the cellophane bag. This cellophane bag unit consisted of $2\frac{1}{4}$ in. seamless cellophane tubing well soaked, invaginated, and tied to the large rubber bung support. A similar but larger form consisted of $5\frac{1}{2}$ in. seamless cellophane tubing invaginated and attached to a stainless steel cover to a large pyrex cylinder. The vessel held 25,000 ml. of culture media and the cellophane sac 2500 ml. of fluid.

The most satisfactory yields of toxin were obtained in these cellophane culture vessels with the following medium:

Heart infusion – tryptose broth, Difco	25 gm.
Glucose	10 gm.
Tryptone	10 gm.
Calcium carbonate	5 gm.
Distilled water	1000 ml.

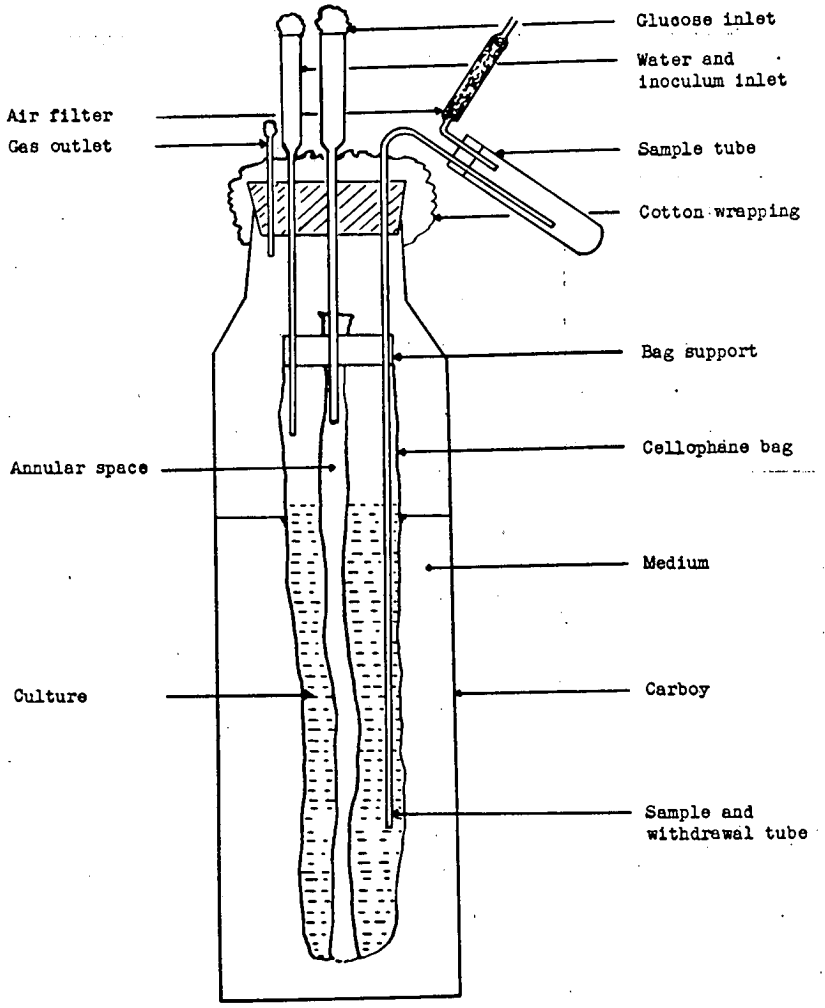

FIG. 1. 'Invaginated' cellophane bag unit.

This medium, less the amount of water to be added to the cellophane bag, was placed in the glass jars, the empty cellophane bags arranged, and the apparatus autoclaved. Sterile water was then added aseptically to the cellophane container and inoculated. In some runs the calcium carbonate was included with the water on the inside of the container but this did not significantly influence the pH of the growing culture. In the results here shown the calcium carbonate was in the medium outside the cellophane.

Cultures in this cellophane apparatus grow rapidly and reach a peak at 36 to 48 hr. In contrast to ordinary cultures, in the cellophane there is little or no autolysis of the cells for a period up to 20 days. The pH of the culture in the bag rapidly becomes acid as indicated in Table II and Fig. 2, while the outside solution, which remains sterile, shows little pH change.

TABLE II

YIELD OF TYPE E BOTULINUM TOXIN IN CELLOPHANE BAG CULTURE

Days' incubation	30° C.	25° to 29° C.	pH
	Toxin, M.L.D./ml.	Toxin, M.L.D./ml.	
½			6.3
1			5.9
3			5.5
5	6×10^3		
6	9×10^3		
7	1.5×10^4	1.5×10^3	5.5
9	1.5×10^4		
10	3×10^4	4×10^3	5.3
15		8×10^3	
18		2×10^4	5.5
20		3×10^4	5.7
24		5×10^4	5.7

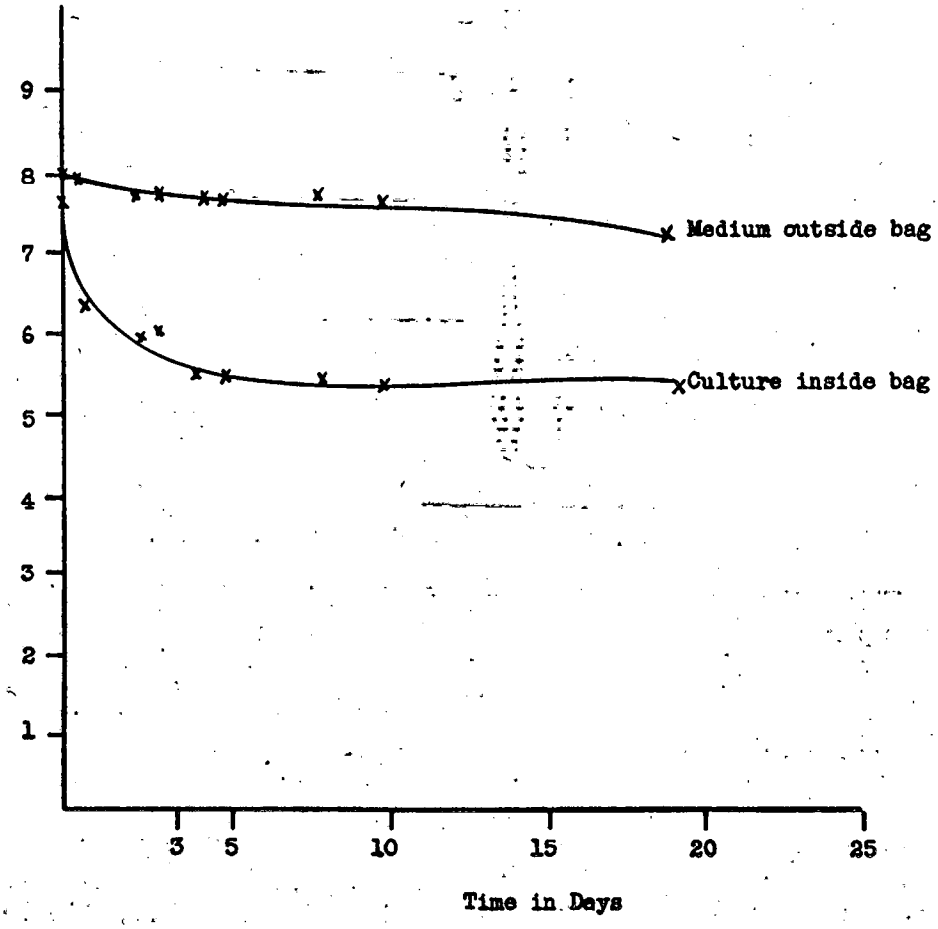

FIG. 2. Changes in pH of culture during growth in 'invaginated' cellophane bag.

Toxin Yields

Toxin assays, Table II, of the crude cellophane bag cultures show an appreciable yield of toxin after five to seven days' incubation which gradually increases to a maximum in 10 to 24 days. Titers ranging from 10,000 to 50,000 M.L.D. for the mouse per ml. were regularly obtained, with an average yield well over 20,000. This yield amounts to at least a fivefold increase in yield over that obtained by conventional culture procedures.

2. Type E Toxoid

Toxoids have been prepared for *C. botulinum* Types A and B (Rice and Reed (16), Nigg *et al.* (13)) and Sterne and Wentzel (17) have produced Type C and D toxoids. Very little has been reported on toxoids for Type E. Gunnison *et al.* (6) mention a Type E toxoid in connection with the classification of strains. Prévot and Huet (14) formalized the toxin from their strain of Type E and used it in the production of anti E serum in rabbits. Aside from these references there does not appear to have been any work done on toxoid for this type.

The preparation of toxoid from the high potency Type E toxin produced in cellophane cultures has been undertaken.

Toxoid from Crude Cultures

The crude cultures from cellophane bags with toxin content of 20,000 M.L.D. per ml. and containing large numbers of intact cells have been formalized and held at 30° C. up to six weeks. Detoxification was not complete, though greatly reduced, in this period. The material showed a very low immunizing value.

Clarification of Cellophane Cultures

Two methods of clarification have been used.

(*a*) Ten-day cultures from cellophane, rich in intact cells and containing 25,000 M.L.D. of toxin per ml. was centrifuged at low speed and the supernatant centrifuged in a Servall vacuum type instrument at 15,000 r.p.m. for 30 min. The final supernatant was clear and free from cells. There was little or no loss in toxicity. As this procedure is not practical for large lots a second method of clarification was undertaken.

(*b*) To cellophane produced toxin, activated charcoal (Darco) was added to a concentration of 2.5%, well mixed, and the mixture filtered through Whatman No. 4 filter paper on a Buchner funnel under slight suction. The filtrate was free of cells, clear, and exhibited little or no loss in toxicity when assayed immediately after clarification. The filtrate was, however, unstable. After several days storage at 4° or 20° C. there was an appreciable loss in toxicity.

Filtration

Toxins clarified by the above methods were sterilized by Mandler filtration. The clarified toxins filtered readily and in sharp contrast to Types A and B toxins there was little or no loss in toxicity. As the charcoal-clarified and

Mandler filtered toxins were unstable, formalization was started immediately after completion of clarification and filtration. It should be noted that in several instances filtrates which had apparently lost a great deal of toxicity during treatment and storage made good toxoids.

Formalization

Formalin, adjusted to pH 6.0, was added to the filtered toxins to a final concentration of 0.3%. This was added slowly with constant gentle stirring by rotation of the bottles. The toxin–formalin mixtures were incubated at 30° C. with gentle rotation of the bottles daily. Detoxification was rapid, after 20 days 1 ml. doses of undiluted toxoid failed to produce any symptoms of botulism in mice.

Alum-precipitated Toxoid

Since the work on Types A and B toxoids indicated a marked improvement in antigenicity when the fluid toxoids were adsorbed on alum (16), both fluid- and alum-precipitated Type E toxoids were prepared. The procedure of alum precipitation was similar to that used by Rice with Types A and B. A 10% sterile solution of aluminium potassium sulphate was added to the fluid toxoid to final concentrations of 0.5% or 1.0%. The precipitate was washed three times with sterile saline and the final washed precipitate suspended in a volume of sterile saline equal to the original volume of fluid toxoid.

Antigenic Response to Fluid and Alum Toxoids

The antigenic value of the toxoids was determined by giving mice a single 1 ml. dose of the toxoid or a series of doses at intervals of 10 days. Twenty-one days after the single dose or after the last dose the mice were challenged with a dose of Type E toxin. Table III summarizes results obtained with the immunization of mice with fluid toxoid. It is apparent that the fluid toxoid prepared by charcoal clarification is as good as the more laborious centrifuge clarified preparation. It is also apparent that one or two doses of the fluid toxoid produces a relatively low immune reaction in mice whereas three doses produce a good level of protection.

TABLE III

IMMUNIZATION RESPONSE OF WHITE MICE TO FLUID TYPE E BOTULINUM TOXOID CHALLENGED WITH HOMOLOGOUS TOXIN

Toxin clarified by	No. doses toxoid	Days after last dose	Challenge M.L.D.	No. mice surviving
Charcoal adsorption	1	21	10	1 of 15
	2	21	100	3 of 11
	3	21	100	7 of 7
	3	21	500	11 of 12
Centrifugation H. S.	2	21	10	7 of 8
	3	21	100	4 of 6

Table IV summarizes results obtained with alum-precipitated toxoids. It is apparent that, as in the case of the fluid toxoids, there is no significant difference between toxoids prepared by high speed centrifuging and by charcoal clarification. Animals given a single dose of alum-precipitated toxoid and challenged 10 days later showed a relatively low level of immunity whereas similarily immunized animals challenged 21 days after immunization had a very much higher level of immunity. It is also evident from the table that the toxoids precipitated with 1% alum are somewhat superior to those prepared by precipitation with 0.5% alum.

TABLE IV

IMMUNIZATION RESPONSE OF WHITE MICE TO TYPE E BOTULINUM ALUM TOXOID CHALLENGED WITH HOMOLOGOUS TOXIN

Toxin clarified by	% alum	No. doses	Days after last dose	Challenge M.L.D.	No. mice surviving
Centrifugation H. S.	0.57	1	10	10	1 of 8
		1	21	10	8 of 8
		1	21	50	3 of 8
	1.0	1	10	10	0 of 10
		1	21	500	10 of 10
		2	21	5000	1 of 10
Charcoal adsorption	0.5	1	21	50	11 of 11
		3	29	10000	1 of 11
	1.0	1	10	1000	19 of 35
		1	21	1000	9 of 10
		1	21	5000	4 of 13
		2	21	10000	1 of 12

A comparison of Tables III and IV indicates that a single dose of alum-precipitated toxoid provides as high or a higher level of immunity than provided by two or three doses of similar toxoid in the fluid state.

Polyvalent Toxoid

Rice (15) found that Type A toxoid provided a high-level specific immunity; the Type B toxoid produced only a low specific immunity. When the A and B toxoids were mixed in equal proportions and injected into mice the immune response to A toxin was similar to that in animals receiving the A toxoid alone but the immunity to B toxin was much greater in the animals receiving the mixed A and B toxoids than in animals receiving B toxoid alone.

Alum-precipitated A, B, and E toxoids were mixed in equal proportions and 1 ml. doses injected into mice. Another group of mice were given E toxoid only from the same lot as used in the A–B–E mixture. Ten and 21 days after the last dose both lots were challenged with Type E toxin. The results are shown in Table V. It is apparent from the table that the animals receiving the polyvalent A–B–E toxoid exhibited a somewhat higher specific Type E immunity than the animals receiving the E toxoid only. The

difference is most conspicuous in the animals challenged 10 days after the immunization. It seems therefore apparent that the A toxoid or the A and B toxoids somewhat improve the immunizing value of the E toxoid. This is in line with Rice's finding that Type A toxoid improves the immunizing value of B toxoid.

TABLE V

COMPARISON OF IMMUNIZATION WITH TYPE E ALUM TOXOID AND MIXTURES OF BOTULINUM TOXOIDS AGAINST TYPE E TOXIN

Toxoid	No. doses toxoid	Days after last dose	Challenge M.L.D.	No. mice surviving
Type E	1	10	1000	8 of 15
	1	21	5000	4 of 13
	2	21	10000	1 of 12
(A – B) – E mixed *in vitro*	1	10	1000	13 of 15
	1	21	5000	4 of 10
	2	21	10000	3 of 15
(A – B) – E mixed *in vivo*	1	10	1000	1 of 15
	1	21	5000	1 of 15

In Vivo Mixture of Toxoids

In a third group of mice, immunized at the same time as the two groups mentioned in the previous paragraph, the animals were given 0.66 ml. of the mixture of A and B toxoids and five hours later were given 0.33 ml. of Type E toxoid. These are the same amounts of the same lots of toxoids as given when the toxoids are mixed before injection. These animals were challenged with E toxin in the same way as those given the mixture. The results are shown in the last section of Table V. It is apparent from the table that this *in vivo* mixing of toxoids results in complete loss of the adjuvant effect of *in vitro* mixing.

Conclusion

It is apparent from the results shown in this paper that the nutritional and environmental conditions supporting growth and toxin production of *C. botulinum* Type E differs widely from Types A and B.

Growth of Type E in cellophane sacs greatly augmented toxin production, as in the case of Types A, B, C, and D. The toxic crude culture has been clarified by centrifuging or by charcoal. Clarified cultures passed a Mandler filter without loss of toxicity. The filtered cultures were readily converted to

toxoid with formalin at 30° C. The toxoid was highly antigenic but adsorption on alum improved the antigenicity. Mice immunized with the toxoid exhibited a high level of specific immunity to Type E toxin.

When Type E toxoid was mixed with Types A and B toxoids, a higher level of immunity against Type E toxin was produced in mice than when the Type E toxoid was administered alone. This adjuvant effect was apparent when the toxoids were combined *in vitro* but not when the toxoids were injected individually at five-hour intervals.

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