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# Boticinogeny and Actions of the Bacteriocin[1]

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The bacteriocin, boticin E, was produced by only a few strains of those clostridia which are nontoxigenic but otherwise identical to *Clostridium botulinum* type E. Boticin preparations from four different strains had identical spectra against indicator cultures. Experiments with bacterial lawns showed boticin to be sporostatic for all tested nonproteolytic *C. botulinum* (types B, E, and F) and nontoxigenic type E-related strains which included the producing strains as well as those different from type E in the fermentation of one to three carbohydrates. Boticin had no detectable effect on vegetative cells of boticinogenic strains but killed those of all other strains whose spores were sensitive. Cultures that were growing in an agar medium were more sensitive to the bacteriocin than those growing in broth. Vegetative cells of indicator strains adsorbed boticin, but cells of a boticin-resistant mutant did not. Boticin did not lyse suspensions of vegetative cells which had been killed previously by exposure to air but lysed actively growing protoplasts and L-forms of a strain whose normal vegetative cells are susceptible to lysis. Sporostasis resulted from inhibition of germination rather than of outgrowth. Proteolytic strains of *C. botulinum* (types A, B, and F) were resistant to boticin E.

Nontoxigenic organisms resembling *Clostridium botulinum* type E exist in environments where the food poisoning species may be present. Some of these clostridia are considered to be nontoxigenic forms of type E since the two cannot be distinguished in comparisons of morphology and biochemical activities (12) and antigens of the vegetative cells (13) and spores (16).

Among the nontoxigenic isolates are those which produce boticin E, a bacteriocin which acts on type E by clearing broth cultures and exerting sporostasis. The boticin is inactivated by trypsin but not by 1 hr of heating at 100 C (12). About 80% of the activity in a crude preparation is associated with a substance with a molecular weight less than 30,000 daltons; the remainder is associated with a particle weight greater than 40 $\times 10^6$ daltons (6).

The present communication reports on the survey for boticinogeny among *C. botulinum* type E and type E-related organisms and on some of the specific actions of the bacteriocin.

## MATERIALS AND METHODS

**Cultures.** Strain designations are *C. botulinum* type E for those producing the identifying neurotoxin; "E-like" for those not producing toxin but whose other physiological properties are indistinguishable from type E (12); and "unidentified E-like" for nontoxigenic clostridia which differ from type E in the fermentation of sucrose, sorbitol, or mannitol but which produce "pearly iridescence" (8) on liver veal-egg yolk-agar. "Type E-related strains" include all the nontoxigenic clostridia.

The cultures used are given in Table 1. The first 14 listed type E and the Langeland type F strains were isolates obtained from foods (or same lot of food) which had caused botulism in humans; these were collected from different laboratories. The boticin-resistant mutant of the Beluga type E strain (not included in Table 1) was an isolate of D. A. Kautter. The type E and E-related strains whose laboratory origin is not given were isolated in this laboratory.

**Cultural procedures.** TPGY (5% Trypticase, 0.5% peptone, 0.4% glucose, 0.1% yeast extract, 0.2% sodium thioglycolate) and LVA (liver veal-agar, Difco) were the media routinely used. Anaerobic incubation of plated cultures was in Brewer jars flushed three times with 95% $N_2$-5% $CO_2$. Incubation of the proteolytic cultures was at 35 C; the nonproteolytic cultures were held at 30 C. Optical density (OD) was measured at 600 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.).

Spore suspensions were obtained by washing 4- to 5-day-old TPGY cultures with water. The washing procedure kills most of the oxygen-sensitive vegetative cells of the nonproteolytic strains. Spore suspensions of the proteolytic strains were heated at 80 C for 15 min. Viable spore counts were estimated from tables of most-probable numbers with 5 tubes of TPGY medium used for each dilution.

Bacterial lawns were usually started with spores; in specified cases, vegetative cells were used. With spores,

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TABLE 1. *Clostridium botulinum and type E-related cultures: strains and their original sources*

| Strain | Source |
|---|---|
| Type E | |
| Alaska E43[a] | Salmon eggs, Alaska, USA |
| Beluga[a] | Beluga whale, Alaska, USA |
| 8E[a] | Sturgeon, USSR |
| 1304[a] | Lake sediment, Japan |
| Iwanai[a] | "Izushi," Japan |
| VH[a] | Herring, Vancouver, Canada |
| Kalamazoo[a] | Chub, Great Lakes, USA |
| 066B[a] | Whitefish, Great Lakes, USA |
| Minnesota[a] | Cisco, Great Lakes, USA |
| D8[a], Detroit, Can | Tuna, Pacific Ocean? |
| HS | Salmon, Labrador, Canada |
| Salmon A | Salmon, Alaska, USA |
| 3561, 3581, 3583, 3586, 3587, 3591, 3598, 4521, 4609 | Fish, sediment, or bird, Great Lakes, USA |
| E-like | |
| S5[b], 28-2[b], 6d, 3759, 6210, 15 Other strains | Fish or sediment, Great Lakes, USA |
| Unidentified E-like | |
| S9[a, b] and 15 other strains | Fish or sediment, Great Lakes, USA |
| Type A | |
| 109, 3, 73, Hall | Department of Bacteriology, Univ. of Wisconsin |
| Type B | |
| Proteolytic: 1213, 113, 13983, 32, 7949, Okra | Department of Bacteriology, Univ. of Wisconsin |
| Nonproteolytic:[c] 2B, 17B | Sediment, Pacific Ocean, USA |
| Type F | |
| Proteolytic: Langeland | Duck liver paste, Denmark |
| Nonproteolytic:[c] 83F, 202F | Sediment, Pacific Ocean, USA |

[a] Used as indicator strains in screening test.

[b] Prototype boticinogenic S5 and 28-2 (12) and non-boticinogenic S9 obtained from D. A. Kautter, Food and Drug Administration, Washington, D.C.

[c] Nonproteolytic types B (5) and F (4) isolated by M. W. Eklund, Bureau of Commercial Fisheries, Seattle, Wash.

a 3-ml volume of melted and cooled (45 C) LVA seeded with 0.1 to 0.3 ml of a spore suspension was poured as an overlay into petri plates containing a previously solidified layer of LVA. Inocula of vegetative cells were obtained by inoculating a fresh tube of TPGY from a culture incubated overnight. When the culture reached an OD of about 0.3, an inoculum of 0.3 ml was added to 3 ml of the overlay soft agar (TPGY with 0.75% agar or equal volumes of TPGY and LVA). Because of poor spore crops or death of some vegetative cells during manipulation, confluent growth was not obtained with all cultures. However, tests with known boticinogenic materials showed the "lawns" to be suitable for detecting growth inhibition by diffusible substances.

Protoplasts were obtained by adding 1,250 units of penicillin G and 100 $\mu$g of lysozyme per ml of culture which was growing exponentially in TPGY with polyethylene glycol as osmotic stabilizer (1, 2). After the 3-hr treatment, the suspension was centrifuged at 3,000 $\times$ *g* for 10 min, and the sedimented spheres were suspended in TPGY containing 1% NaCl. The Alaska E43 L-form culture (1, 2) had been transferred about 100 times in the biphasic medium (slant of TPGY agar covered with TPGY) without reversion to the bacillary form.

**Identification of cultures producing boticin E.** Type E and E-related cultures were screened by the stab-plate procedure for ability to inhibit growth of the 11 strains being tested as indicators (Table 1). LVA in plates was given a stab inoculation with up to six different cultures, covered with 3 ml of uninoculated agar, and incubated for 3 days. An agar overlay seeded with spores of an indicator strain was then poured over the covering agar layer. The plates were then incubated an additional 2 days to develop the lawns.

Cultures, whose stab growth inhibited normal development of a lawn, were grown and processed by the method that is used to obtain crude boticin E (6). The resulting heated filtrates were tested against the strains that had been inhibited in the stab-plate test. Wells of 8-mm diameter, cut with a no. 4 cork borer in a layer of LVA that had been seeded with spores of the appropriate strain, were charged with 0.5 ml of filtrate. If the lawns that developed during the subsequent 24-hr incubation had no growth around a well, the filtrate responsible for the growth inhibition was retested after it had been incubated 45 min at 37 C with a solution made of crystalline trypsin (0.005% final concentration).

A culture was considered to produce boticin E if its heated culture filtrate inhibited the growth of any strain and if trypsinization destroyed the activity of the filtrate.

**Boticin assay and preparations.** Serial twofold dilutions in 10 ml of TPGY were inoculated with 200 spores (0.1 ml) of the Alaska E43 or Minnesota strain of type E. Smaller dilution increments were used near the end points of the partially purified boticin preparations. The titer was the reciprocal of the highest dilution which prohibited growth for 60 hr. A unit was the amount of boticin E in 1 ml of the titer dilution; thus, a preparation of titer 2,560 would have 2,560 units/ml.

Crude boticin E preparations were cell-free, heated filtrates similar to the material that was used for purification of boticin (6); they usually had titers of 2,560. Alcohol precipitations (12) were used to obtain partially purified boticin from strains S5 and 28-2; these had titers of 20,000 and 40,000, respectively. Unless stated otherwise, all work on the specific actions of boticin E were done with one or both of these partially purified samples. Charging wells or spotting on nascent lawns was with 0.05-ml volumes of boticin.

## RESULTS

**Survey for boticinogenic cultures.** Like the prototype boticinogenic S5 and 28-2 strains, the E-like 3759 and 6d strains inhibited lawns of all the indicator strains in the stab-plate screening tests. Their heated culture filtrates, when used in the well-plate tests, produced inhibition zones in lawns of all indicators; the filtrates were inactive after treatment with trypsin. Thus, these cultures are producers of boticin E.

The stab growths of some of the remaining type E and E-related strains inhibited one or more indicator lawns in the stab-plate tests; one of the E-like strains was active in eight lawns. The inhibited strains did not fall into discernible patterns. Moreover, the heated culture filtrates of strains showing activity in the stab-plate tests showed no inhibition in well-plate tests. Some of these cultures, active in the stab-plate tests only, may be producers of bacteriocins other than boticin E. Others could produce boticin E but may be poor producers in a liquid medium. Although the last possibility is acknowledged, only E-like strains S5, 28-2, 3759, and 6d are considered to be boticinogenic in this work.

**Activity spectrum against spores.** The well method, using lawns developing from spores, was used to compare the spectra of cultures that were susceptible to the crude 3759, 6d, S5, and 28-2 boticin preparations. Each preparation was active against all type E and type E-related cultures except the S5 boticin-producing strain. The four filtrates were active against all the nonproteolytic type B and F strains. The radius of the inhibition zones (periphery of well to limit of growth) was usually 7 to 8 mm, with extremes of 5 and 15 mm. Trypsinization made the preparations inactive. All proteolytic *C. botulinum*, which include type A cultures, were not affected.

Results with representative strains indicated that the two partially purified boticin samples would have the same spectrum of indicator cultures as found for the crude preparations. In addition, these higher-titered samples, tested at 1,000 units/well, had an effect on S5 lawn development in which the inhibition zone showed a lighter than normal growth instead of an essentially complete absence of growth. The comparative resistance of S5 spores probably accounts for these observations: 50 boticin units/well produced a growth inhibition radius of 5.0 mm on the Minnesota strain and 3.6 mm on 28-2 but produced no noticeable zone in that of S5. The partially purified boticins, when used undiluted, had no effect on lawns of the proteolytic strains.

Spores of the four boticin-producing strains were not affected when they were in a liquid menstruum. Spore suspensions, treated with ethanol to kill any viable vegetative cells present (11), were washed with water and then diluted to contain 50 viable spores/ml of TPGY. Each strain was tested by inoculating 0.2 ml of its diluted spore suspension into 10 control tubes of TPGY and 10 tubes of TPGY which had 2,000 units of boticin/ml. In every case, growth developed in 9 to 10 tubes of both control and test sets during incubation of 48 hr.

TPGY soft agar overlays prepared with spores of the boticinogenic strains 3759, 6d, or 28-2 were poured on base layer of TPGY-1.5% agar and spotted with 100 units of boticin. The resulting lawns showed inhibition of growth where the boticin had been applied. Thus, agar or some substance introduced with it must be responsible for the differing sensitivities of the spores in liquid and agar media.

**Activity spectrum against vegetative cells.** Boticin E kills cells and/or lyses broth cultures of type E (6, 12). The turbidity of cultures of six type E strains which had been isolated in this laboratory decreased when the crude boticin preparations were added to final 200 units/ml. The similar action of the partially purified S5 boticin preparation on all the nonproteolytic type B and F cultures is illustrated for two strains (Fig. 1).

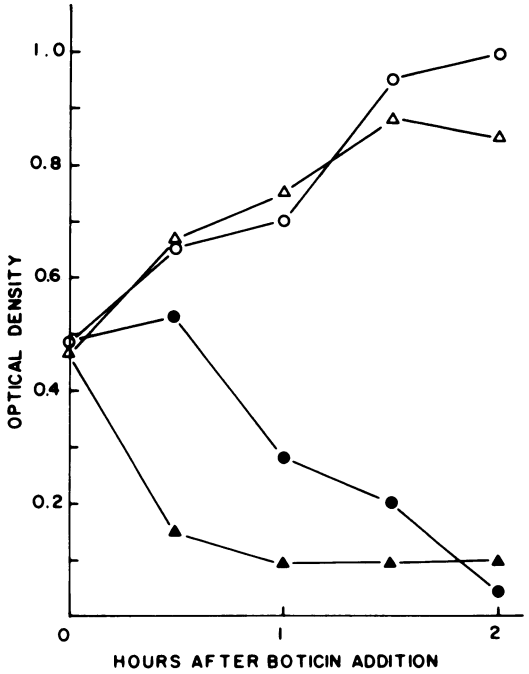


FIG. 1. *Optical density changes in cultures of nonproteolytic type B (strain 2B) and nonproteolytic type F (strain 83F) after addition of 200 units of boticin/ml of culture. Type B, with (▲) and without (△) boticin; type F, with (●) and without (○) boticin.*

Lawns that were started with vegetative cells of the six type E strains had no growth where they had been spotted with 50 units of partially purified boticins. An additional test with type E strain 066B showed it to be sensitive to 20 units of boticin, although vegetative cells of this strain were not killed when boticin was added to broth cultures (12).

Boticin had no detectable action on vegetative cells of the four boticinogenic strains in tests which involved spotting 1,000 units on LAV-TPGY overlays seeded with actively growing cultures. Cells in broth were also not affected, since an estimated 10 spores initiated growth in TPGY containing 2,000 units/ml (*see above*). Culture development could not have occurred unless the vegetative cells, originating by outgrowth from the few spores, were resistant.

The 24 nonboticinogenic, type E-related strains that were tested did not have growth where their nascent lawns, started with actively growing cultures, had been spotted with 200 units of boticin. However, young TPGY cultues (OD of about 0.3) of 9 of the 12 E-like strains and 5 of the 12 unidentified E-like strains showed normal increase in turbidity after the addition of 200 units of boticin/ml of culture. When 0.05 ml of similar young cultures was inoculated into 10 ml of TPGY having the same boticin level, half (4 of 9 and 3 of 5, respectively) of the seemingly boticin-resistant strains did not initiate new growth. All strains except 6210 could not be subcultured when the same inoculation was made into broth with 2,000 units/ml. Thus, type E-related strains growing in TPGY are sensitive to boticin but they vary considerably in sensitivity. Even strain 6210 might be killed if the ratio of boticin E to cells could be increased sufficiently.

Proteolytic *C. botulinum* strains were resistant to boticin. The resistance is probably due to their proteolytic enzyme(s). All the activity in a crude S5 boticin preparation was destroyed when it was incubated for 1 hr at 37 C with an equal volume of a cell-free filtrate that had been prepared from a 24-hr-old culture of the type A strain 109. Similarly, a highly purified, trypsin-like enzyme obtained from the Okra strain of type B inactivated boticin E (B. R. Das Gupta, Bacteriol. Proc., p. 100, 1970; *personal communication*).

Cell viability is needed for boticin-induced lysis. A portion of an actively growing culture of the Alaska E43 strain was transferred to a flask to give a depth of 2 to 3 mm, and the flask was swirled occasionally during 2 hr of holding at room temperature. Boticin (final 250 units/ml) was added to this flask and to a portion of the culture which had been handled without unnecessary exposure to air. The growing culture lysed but the air-exposed one did not (Fig. 2). That the air treatment had killed most of the cells was shown when, in spite of the total cells indicated by the OD, an average of only 75 colonies developed in LVA deep tubes that were inoculated with 0.1 ml of the suspension (not treated with boticin).

The same conclusion was drawn from experiments with lawns. Plates with an overlay seeded with spores of the Alaska E43 or Minnesota strain were incubated for 15 hr. At this time, when only occasional spores were found by microscopic examination of the growth, the plates were exposed to air for 1 hr. The density of the lawn growths was not affected when 1,000 to 2,000 units of boticin were spotted.

**Activity against protoplasts and L-forms.** A newly prepared protoplast suspension of the Alaska E43 strain (1, 2) was divided into two portions. One portion was treated with a final 200 units of boticin/ml, and the control was treated with the same amount of boticin that had been inactivated with trypsin. The latter produced gas vigorously throughout the 8-hr observation period, but the test suspension stopped producing gas in 1.5 hr, at which time microscopic examination showed only ghost forms. The same boticin preparation had no discernable effect on a protoplast suspension of the boticin-resistant mutant of the Beluga type E strain.

L-form cultures of the Alaska E43 strain were lysed by the partially purified boticin preparations. Within 2 hr of adding 50 units/ml (calculated for the broth portion of the biphasic culture medium) to an actively growing culture, cessation of gas production accompanied clearing of the culture. Spotting 50 units on the incipient L-form lawns resulted in clear areas of growth inhibition. A bacteriophage virulent for the normal vegetative cells of this culture (*unpublished data*) did not attack the L-form culture. The stable L-form culture of type A (1, 2) grew normally in the presence of 2,000 units of boticin/ml.

When boticin (25 units/ml) was substituted for penicillin in the protoplasting procedure, about half of the rods in the Alaska E43 culture were converted into spherical bodies. The spheres that developed by the entire cytoplasmic mass of the cells escaping through a break in the cell wall lysed within 5 min of becoming free.

**Sporostatic effect.** The sporostatic action of boticin E was deduced from experiments (12) in which most of the spores could have been killed. The following experiment indicated that boticin does not kill significant numbers of spores. Each of 10 control tubes of TPGY was inoculated with an estimated 10 viable spores of the Alaska E43

strain; the same inoculum was given another set of tubes containing 200 units of boticin/ml. All the control tubes showed growth after 24 hr of incubation. When the tubes with boticin showed no growth at 72 hr, trypsin (final 0.005%) was added and incubation was resumed. All tubes developed turbidity within 30 hr.

Whether the sporostasis resulted from inhibition of germination or of outgrowth was examined with the principle that spores whose growth is arrested at the outgrowth stage have the heat resistance of vegetative cells (7). A 0.2-ml amount of a Minnesota spore suspension was inoculated into 6 ml of TPGY containing 2,000 boticin units/ml and into a control containing trypsin-inactivated boticin. The control showed growth after overnight incubation. When the test showed no growth after 3 days, 1-ml amounts of the inhibited spore suspension were transferred to screw-cap tubes. The boticin was inactivated with trypsin, and the content in each tube was made to 5 ml with water. Three tubes were sealed with the caps and heated for 30 min while submerged in a 58 C water bath; the other three tubes were left unheated. The particular heat treatment was chosen because it would kill vegetative cells but not type E spores that are of low natural heat resistance (3). The average estimates of most-probable-number of viable spores in the heated and unheated tubes were not significantly different: unheated, 142,000; heated, 134,000. Thus, the spores that were incubated in a sporostatic level of boticin still had the heat resistance of ungerminated spores.

The OD of the Alaska E43 spore suspension did not change during 48 hr of incubation in TPGY containing 200 units of boticin and 30 $\mu$g of chloramphenicol/ml. The OD of the control suspension, incubated in TPGY with chloramphenicol only, decreased from 0.385 to 0.230 overnight, showing that the spores could germinate but not develop further in the presence of the antibiotic. Since spore germination is accompanied by loss of light refractility (7), the absence of OD decrease in the test suspension supports the conclusion from the heat-resistance experiment that boticin E acts at a germination level. The conclusion agrees with the microscopic observation that spores prevented from growing into vegetative cells by boticin maintain their light refractility (12).

**Specific adsorption of boticin.** Actively growing TPGY cultures with OD of about 0.9 were centrifuged, and about 90% of the supernatant fluid was decanted. The cells were promptly resuspended and added to an equal volume of a boticin solution of 4,000 units/ml. After 1 hr of incubation at 35 C, when cell lysis was not significant, the cells were removed and the supernatant fluids were assayed for boticin (Table 2). Cells, but not a cell-free filtrate, of the boticin-sensitive strains decreased the boticin activity in the solutions; cells of the boticin-resistant mutant did not.

Although not lysed by boticin E, air-killed cells also adsorbed boticin. The mixture of boticin with the suspension of air-killed cells (experiment shown as Fig. 2) was centrifuged after the 3-hr incubation. The boticin in the recovered supernatant fluid was 40 units/ml as compared to the starting 250 units/ml. A suspension of Alaska E43 cells which had been heated for 1 hr at 100 C did not adsorb boticin E.

**Trypsin rescue.** Boticin E induces a rapid, irreversible lethal response in actively growing cells (6). When the culture is susceptible to clearing (OD decrease) by boticin, contact between cells and boticin results in a similarly rapid, irreversible commitment to cellular degeneration (Fig. 3)

## DISCUSSION

The survey among type E *C. botulinum* and type E-related strains indicates that boticinogeny is limited to a few of the nontoxigenic clostridia which are characterized in this report as E-like strains. Based on the action of lawns that were

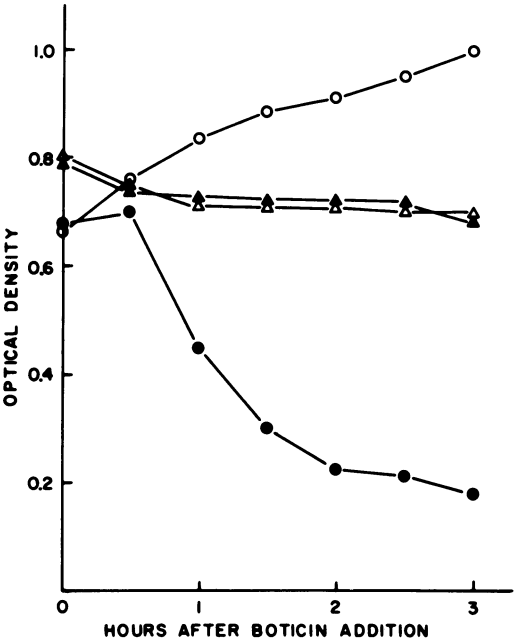


FIG. 2. *Lytic action of boticin E (250 units/ml) on growing but not air-killed cells of Alaska E43 during incubation at 30 C. Living cultures, with active (●) and trypsin-inactivated boticin (○); air-killed cells, with active (▲) and trypsin-inactivated boticin (△).*

TABLE 2. *Absorption of boticin E by cells of Clostridium botulinum type E*[a]

| Adsorbing culture | Boticin units/ml (corrected for dilutions) |
|---|---|
| None (control) | 2,000 |
| Alaska E43 | 400 |
| Beluga | 200 |
| Beluga mutant[b] | 2,000 |
| Culture filtrate[c] | 2,000 |

[a] Boticin and cells incubated for 1 hr at 35 C; cell-free filtrates assayed for boticin.

[b] Boticin resistant.

[c] Of Alaska E43 culture from which the adsorbing cells for this experiment were obtained.

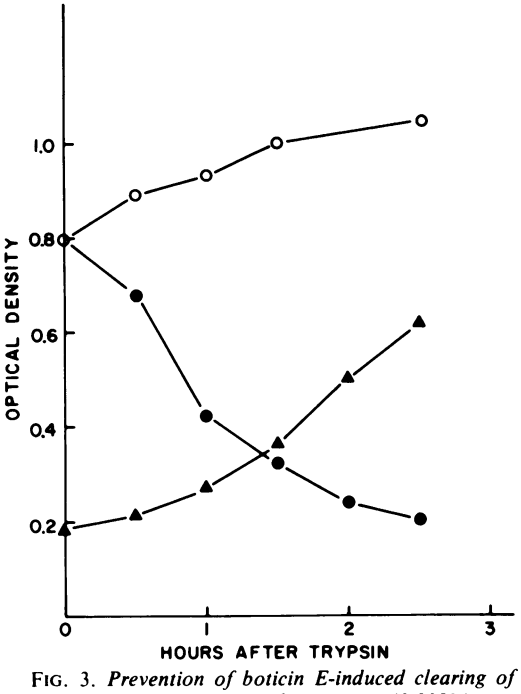


FIG. 3. *Prevention of boticin E-induced clearing of Alaska E43 broth cultures when trypsin (0.005%) was added at 30 sec (○ and ▲) but not 5 min (●) after boticin (200 units/ml). Trypsin added at time zero. Two different starting turbidities result from adding boticin to subcultures incubated for 1 and 5 hr.*

started from spores, the four boticinogenic strains produce the same boticin. The indicator cultures include all the tested nonproteolytic *C. botulinum*, irrespective of the antigenic type of neurotoxin the cultures produce, and all of the nontoxigenic, nonproteolytic type E-related clostridia, including the boticin-producing strains.

Except for the producing strains, boticin acted on vegetative cells of all cultures whose spores are sensitive to its action. The correlation is less apparent with broth cultures, since strains vary in sensitivity and boticin E is less effective when the test system lacks agar. The wide activity spectrum of boticin E resembles that found for megacin A (9).

The action on vegetative cells is a lethal effect (6) which may be followed by clearing of the cultures. As with megacin A (10), dead cells are not lysed. The action of boticin E on spores is not lethal but sporostatic. The reason for the different effects on the two cellular forms is not understood, but the observations suggest, as with certain bacteriocins (14), that boticin kills only cells that are active metabolically.

Evidence has been presented that resistance of the proteolytic *C. botulinum* strains may depend on enzyme(s) that inactivates boticin. Immunity may explain the inability to demonstrate an action of the bacteriocin on vegetative cells of the producing strains, but an enzymatic factor could also be operative; i.e., in obtaining crude boticin, heat must be used to destroy a boticin-inactivating factor. The turbidity fluctuations observed in cultures which are producing boticin E could mean that boticin can lyse the producing cells (6) from an internal location.

The action of boticin E on protoplasts and L-forms shows that somatic antigens that are common to the nonproteolytic *C. botulinum* and type E-related strains (13; F. R. K. Lynt, Jr., H. M. Solomon, D. A. Kautter, and T. Lilly, Jr., Bacteriol. Proc., p. 18, 1969) are not the sole explanation for the spectrum of indicator strains. This susceptibility of the wall-less cellular forms to boticin E is comparable to the persistence in "protoplasts" of the parent form's sensitivity to megacin A (10) and in stabilized L-form cultures of the parent's colicin sensitivity (15).

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