PHYSIOLOGY OF TOXIN PRODUCTION BY *CLOSTRIDIUM BOTULINUM* TYPES A AND B

IV. Activation of the Toxin

PETER F. BONVENTRE[1] AND LLOYD L. KEMPE

Department of Bacteriology, University of Michigan, Ann Arbor, Michigan

Received for publication June 12, 1959

Kindler *et al.* (1956) reported that a strain of *Clostridium parabotulinum* type A synthesized large quantities of toxin in a medium where cell multiplication was inhibited by high concentrations of penicillin. This interpretation of synthesis *de novo* of protein was based on the observations that the toxicity of the extracellular fluids increased in the absence of cell multiplication. It is a well established fact that growth of *Clostridium botulinum* reaches a maximum and is followed very rapidly by a period of cellular degeneration and autolysis which results in the liberation of large quantities of toxin (Boroff, 1955; Kindler *et al.*, 1955; Bonventre and Kempe, 1959*a*). At the end of the exponential growth phase the sum of the intracellular and extracellular toxin is approximately 10 per cent of that found in the culture filtrates which are allowed to incubate until autolysis is complete (Bonventre and Kempe, 1960). If synthesis *de novo* of protein is responsible for the increase in toxicity noted during the autolytic process then it must be assumed that 90 per cent of the toxin is synthesized during a period of cellular degeneration. Since this period in the life of microorganisms is usually characterized by catabolic rather than anabolic processes, the synthesis of such large amounts of protein would be a rather unique biological situation. In an attempt to investigate this phenomenon further, mechanisms other than synthesis *de novo* were considered. It has been known for some time that some of the protein bacterial toxins can be altered by proteolytic enzymes so that their activity is increased (Turner and Rodwell, 1943; Cohen *et al.*, 1942). More recently, however, Duff *et al.* (1956) showed that culture filtrates of *C. botulinum* type E could be activated by trypsin and postulated the existence of a type E "protoxin." The toxins of *C. botulinum* types A and B have also been shown to be capable of activation provided that filtrates of very young cultures are used. On the other hand, the toxicity of filtrates from older cultures which had autolyzed could not be increased by the action of proteolytic enzymes (Bonventre and Kempe, 1959*b*). Type E strains of *C. botulinum* are considered to be only slightly proteolytic (Duff *et al.*, 1956) and types A and B usually possess a full complement of proteolytic enzymes (Elberg and Meyer, 1939; Maschmann, 1939). These data suggested the possibility that the toxin of the former organisms can be activated at any time after synthesis of protoxin has occurred since they do not possess the enzymes required to do so. On the other hand, the toxins of types A and B, which produce an array of proteolytic enzymes, may be activated during the normal growth cycle of the organisms. If this were the case, then the activation phenomenon could account, at least partially, for the increased toxicity noted in nonproliferating and autolyzing cultures of *C. botulinum* types A and B. Therefore, the efforts of this investigation were directed toward obtaining experimental evidence which would support synthesis *de novo* of protein during the period of cellular degeneration and the activation of toxin precursors.

MATERIALS AND METHODS

The general experimental conditions, the media employed for the cultivation of the organisms and the toxin assay system have already been described (Bonventre and Kempe, 1960). The organisms used in the investigation are designated as *C. botulinum* type A strain JTD-IV; *C. botulinum* type B strain B-201 (both obtained from J. T. Duff, Fort Detrick, Maryland); and

[1] Present address: Department of Microbiology, College of Medicine, University of Cincinnati, Cincinnati, Ohio.

Part of this work was carried out during the tenure by one of us (P. F. B.) of the Novy Research Fellowship, Department of Bacteriology, University of Michigan.

C. parabotulinum type A strain 457-A (obtained through the courtesy of Dr. L. S. McClung, Indiana University). All stock cultures were stored in a beef infusion medium containing particles of meat and held at −20 C. To insure uniformity of response, all experiments were initiated from a frozen culture which was allowed to thaw at room temperature. The initial inoculum was made into thioglycolate medium (BBL) and then to the complete medium for at least two further transfers. This procedure was used throughout the investigation to eliminate any extraneous factors introduced from the initial inoculum and also to eliminate the adaptive mechanisms of the organisms to their previous environment. Cultures were incubated at 35 C unless otherwise stated. Periodically, microscopic examinations were made to determine the purity of the cultures and the toxin elaborated was checked by neutralization tests with specific A or B antitoxin.

Demonstration of inactive or protoxin. The presence of an inactive form of the toxin was demonstrated by treatment of the supernatant culture fluids or cell-free extracts with proteolytic enzymes. Crystalline trypsin and pepsin were obtained from the Armour Research Foundation. Samples to be treated with the enzymes were adjusted to pH 4.0 in the case of pepsin and pH 6.0 when they were to be incubated with trypsin. Equal volumes of sterile enzyme solution and sample were mixed in screw capped tubes (final enzyme concentration, 0.005 per cent) and incubated in a 37 C water bath. Samples with an equal volume of sterile distilled water were incubated as controls. A control tube and a tube of the incubation mixture were removed from the water bath at desired intervals and assayed for toxin. Any difference between the titer of the control and the enzyme treated sample was interpreted as being due to the action of the proteolytic enzyme.

Preparation of resting cells. In this investigation, the term "resting cells" is used in the same sense as used by Kindler *et al.* (1956); i.e., organisms which are unable to proliferate due to the presence of a metabolic inhibitor in a medium which is favorable for the continuation of synthetic processes. The organisms were harvested during the logarithmic growth phase, washed thoroughly in gelatin phosphate buffer (pH 6.8) or in fresh medium, and then resuspended to an arbitrarily selected turbidity in the complete medium. Metabolic inhibitors were added to samples of the suspension in concentrations high enough to inhibit cell multiplication and reincubated at 35 C. Toxin determinations were carried out after specified time intervals.

Proteolytic activity of cultures and extracts of *C. botulinum* was measured using the method described by Northrup *et al.* (1948). The method of Lowry *et al.* (1951) was used for the protein determinations.

EXPERIMENTAL RESULTS

Toxin production by resting cells in the absence of glucose. The adaptation of *C. botulinum* to growth in the absence of an exogenous supply of glucose results in a marked inhibition of toxin synthesis (Kindler *et al.*, 1956; Bonventre and Kempe, 1959*a*). Consequently, in an attempt to distinguish between synthesis *de novo* of protein and activation of a toxin precursor, a comparison was made of toxin production by nonmultiplying cultures of *C. botulinum* strain JTD-IV adapted to growth with and without an exogenous glucose supply. The glucose adapted cells were harvested after 24 hr, washed five times in gelatin phosphate buffer (pH 6.8), and resuspended in the complete medium containing no glucose. Penicillin was added to a final concentration of 200 units per ml and the resting cell suspension divided into two equal parts. Upon addition of 0.5 per cent glucose to one part, both portions of the original suspension were reincubated at 35 C. The increase in toxicity of the filtrates of both resting cell suspensions during the 48-hr incubation period with penicillin was of the same order of magnitude (figure 1). This indicated that the initial 24-hr growth period in the presence of glucose had satisfied the organism's requirements for maximal toxin synthesis The removal of the carbohydrate during the remainder of the incubation period had no effect on the ultimate toxicity. When the same organism was initially adapted to growth without glucose, however, it was found that incubation of the resting cells in the absence of glucose resulted in only a slight increase in the toxicity of the filtrates (figure 1). The fact that glucose is essential for toxin synthesis in actively growing cultures (Bonventre and Kempe, 1959*a*), and that, in the case of the glucose adapted cells, removal of the sugar after maximum growth was attained did not inhibit production of toxin,

would seem to indicate that synthesis of protein takes place during the initial 24-hr growth period, whereas maximum toxicity is not demonstrable until 48 hr later.

Effect of metabolic inhibitors on toxin production. A 16-hr culture of *C. botulinum* strain JTD-IV was harvested, washed in gelatin phosphate buffer and resuspended in freshly prepared medium. The suspension was transferred to sterile screw capped tubes containing sterile solutions of a metabolic inhibitor. The final concentrations of the antibiotics used were: 2,4-dinitrophenol (0.0005 M), chloramphenicol (200 μg per ml), ethylenediaminetetraacetate (Versene 0.001 M), and penicillin (1000 units per ml). Growth was measured turbidimetrically (figure 2) and samples were taken periodically for toxin assay of the filtrates (figure 3). These results when interpreted on the basis of what is known concerning the modes of action of the inhibitors employed would tend to support the activation of presynthetized protoxin rather than synthesis *de novo* of toxin. Considering the cases individually, the data may be interpreted as follows. (a) Control; the original cell suspension increased in optical density from 0.25 to a maximum of 0.57 before autolysis became apparent. During the 72-hr incubation period, the toxicity of the filtrates increased approximately three logs. The maximum toxin titer of 2×10^6 MLD per ml can be assumed to be that amount of toxin which a culture of strain JTD-IV is capable of producing in a favorable environment. Under no conditions were toxin titers higher than this ever attained. In addition, filtrates of this potency can not be activated further by proteolytic enzymes (Bonventre and Kempe, 1959*b*). (b) Chloramphenicol; if this antibiotic effectively inhibits protein syn-

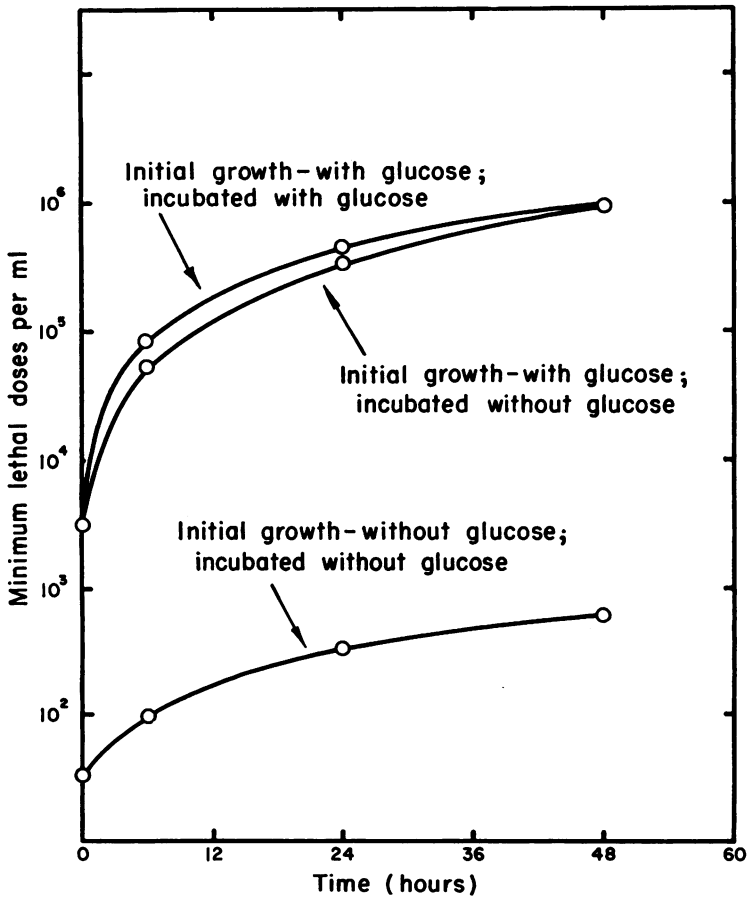

Figure 1. Toxin production by washed cells of *Clostridium botulinum* strain JTD-IV suspended in a complete medium containing high concentrations of penicillin.

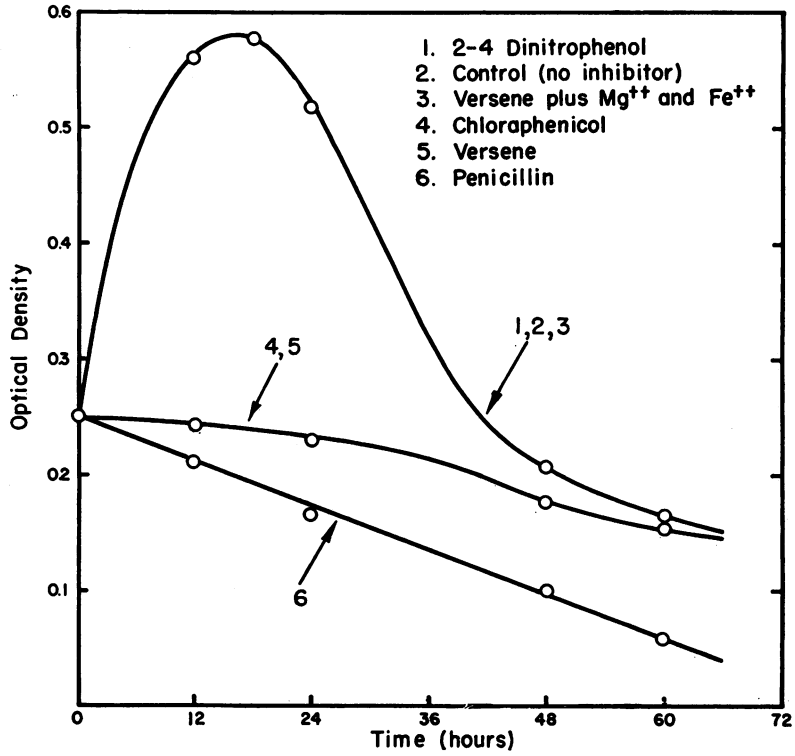

Figure 2. Effect of metabolic inhibitors on the growth of 16-hr washed cells of *Clostridium botulinum* strain JTD-IV suspended in a complete medium.

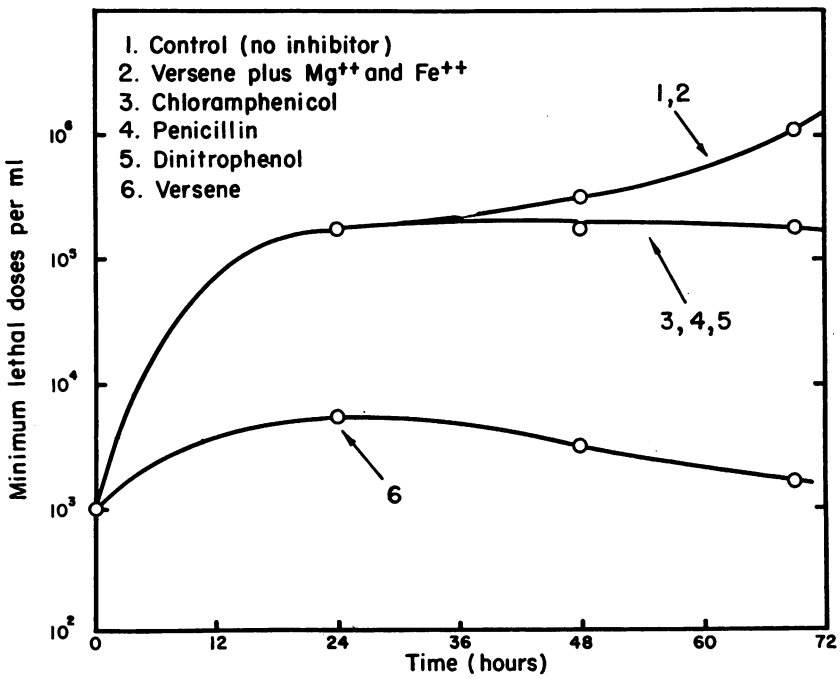

Figure 3. Effect of metabolic inhibitors on toxicity of 16-hr cells of *Clostridium botulinum* strain JTD-IV suspended in a complete medium.

thesis, then the approximate 200-fold increase in the toxicity noted in the absence of cell multiplication may not be attributable to protein synthesis *de novo*. The data suggest, however, that a less active form of the toxin is gradually altered to the most active state by the bacterial enzymes synthesized during the initial growth period in the absence of any antibiotic. (c) Penicillin; the data obtained were comparable to those obtained with chloramphenicol; growth was completely inhibited and the toxicity of the filtrates increased to 2×10^5 MLD per ml. If penicillin inhibits protein synthesis indirectly by virtue of its effect on nucleotide synthesis (Park and Strominger, 1957) then the same interpretation of the data obtained with chloramphenicol may be made. (d) Versene; this chelating agent not only inhibited cell multiplication but also suppressed appreciable increases in the toxicity of the filtrates. These effects were reversed by Mg^{++} and Fe^{++} salts. The necessity for divalent cations as cofactors in many enzymatic reactions makes it appear that Versene, unlike chloramphenicol and penicillin, inhibited the enzymes responsible for complete activation of the toxin precursor.

These experiments also suggested that the potential toxicity of a culture at the terminal stage of the exponential growth phase is much greater than the toxicity demonstrable by animal assay of the filtrates. If chloramphenicol completely blocks protein synthesis, then it must be assumed that the original suspension of log-phase cells, which contained a comparatively small quantity of biologically active toxin, possessed enough synthesized inactive toxin to account for the 200-fold increase in toxicity noted during the incubation period with the antibiotic. To test the validity of this hypothesis, the toxicity of culture extracts treated with proteolytic enzymes was compared with the toxicity of filtrates of the same culture incubated with the metabolic inhibitors.

C. botulinum JTD-IV was grown in 1 L of complete medium for 16 hr. The cells were harvested, washed five times in gelatin phosphate buffer, and resuspended in freshly prepared medium to give a heavy suspension. Samples were then subjected to the treatments described in table 1 and assayed for toxin. The toxicity of the suspension was increased more than 100-fold by sonic disintegration of the cells and subsequent incubation of the resulting extract with trypsin. This increase in toxicity was accomplished in approximately 1 hr after the preparation of the cell suspension; 30 min for sonic disruption of the cells and 30 min incubation with the enzyme. Protein synthesis during this period can be discounted since

TABLE 1

Toxicity of 16-hr washed cells of Clostridium botulinum strain JTD-IV suspended in a complete medium and subsequently subjected to sonic disintegration and treatment with trypsin

Treatment of Samples of Original Cell Suspension	Toxin Titer
	MLD/ml
Washed, intact cell suspension; cells removed and filtrate assayed at 0 time.	2×10^3
Extract obtained by sonic disruption of the cell suspension. Assay represents sum of intracellular and extracellular active toxin.	2×10^4
Cell extract incubated with trypsin (pH 6.0) at 37 C for 30 min.	$>2 \times 10^5$
Same as no. 3—incubated with trypsin for 1 hr.	$>2 \times 10^5$
Same as no. 3—incubated with trypsin for 3 hr.	5×10^4

TABLE 2

Changes in the optical density and toxicity of 16-hr washed cells of Clostridium botulinum strain JTD-IV suspended in a complete medium and incubated with metabolic inhibitors

Treatment of Samples of Cell Suspension	Time	Optical Density	Toxin Titer
	hr		*MLD/ml*
Cell suspension control (no inhibitor)	0	0.55	2×10^3
	24	0.44	5×10^5
	48	0.13	10^6
	72	0.11	2×10^6
Cell suspension plus penicillin	0	0.55	2×10^3
	24	0.27	2×10^5
	48	0.16	4×10^5
	72	0.11	4×10^5
Cell suspension plus chloramphenicol	0	0.55	2×10^3
	24	0.55	2×10^5
	48	0.13	4×10^5
	72	0.13	5×10^5
Cell suspension plus chloramphenicol and Versene	0	0.55	2×10^3
	24	0.54	5×10^3
	48	0.51	5×10^3
	72	0.50	5×10^3

the temperature during sonic treatment was maintained at 10 C. In addition, incubation of the cell-free extract in a complete medium did not increase toxicity.

Samples of the same suspension used in the above experiments were also incubated with metabolic inhibitors so that the increase in toxicity obtained in this case could be compared with the degree of activation observed by treatment of the extracts with trypsin. Table 2 indicates the growth and the increase in toxicity of the filtrates in the presence of penicillin, chloramphenicol, or chloramphenicol plus Versene. The increase in toxicity of the filtrates obtained after 48 hr incubation with chloramphenicol and penicillin was slightly greater than the increase noted after treatment of the extracts with trypsin. Or, stated in other terms, the toxicity of the filtrates after prolonged incubation with the antibiotics, was only slightly higher than the potential toxicity of the original cell suspension. The controls increased to a maximum toxicity of 2×10^6 MLD per ml. Incubation of the cells with a combination of chloramphenicol and Versene resulted in negligible increases of toxicity. In addition, a dramatic stabilization of cellular integrity was noted. This virtual abolishment of the autolytic process indicates that Versene may inhibit the activity of the autolytic enzymes as well as the enzymes required for activation of the protoxin.

To validate the hypothetical events described above as being responsible for the ultimate toxicity of culture filtrates of *C. botulinum* it was considered necessary to determine (a) if the organisms synthesized enzymes capable of hydrolyzing protein; (b) if Versene acted as an inhibitor of these enzymes; (c) if the period of cellular degeneration or autolysis was characterized by little or no protein synthesis; and (d) if chloramphenicol effectively inhibited protein synthesis.

Proteolytic activity. Twelve-hr cultures of 4 strains of *C. botulinum* types A and B were harvested, washed, and suspended in gelatin phosphate buffer containing 0.05 per cent sodium thioglycolate. One-ml samples of the suspensions were incubated with equal volumes of 1 per cent commercial casein solution at 37 C for 2 hr. The cell suspensions and 0.01 M Versene (final concentration) were also incubated with the casein solution. Crystalline trypsin was used as a means of comparison. The proteolytic activity of the organisms and the degree of inhibition by Versene are shown in table 3. All strains of *C. botulinum* demonstrated considerable proteolytic activity and Versene was found to inhibit this activity by more than 50 per cent.

TABLE 3

Proteolytic activity of Clostridium botulinum types A and B as measured by tyrosine liberation from a casein substrate

Strain	Tyrosine (μg/ml)		(Activity of Cells)/(Activity of Trypsin)	Inhibition by Versene (0.01 M)
	Cells	Cells plus Versene		
JTD-IV	62	33	0.43	0.47
B-201	62	28	0.43	0.55
62 A	71	32	0.49	0.55
213 B	65	28	0.49	0.57
Trypsin (0.01%)	145	—	—	—

TABLE 4

Changes in total protein of cultures of Clostridium botulinum strain JTD-IV grown in a defined medium

Age	Optical Density	Tyrosine per Ml of Culture
hr		*μg*
0	0	—
4	0.03	392
24	0.136	530
42	0.108	510
72	0.110	430

Total protein determination. A knowledge of the relative changes in protein content of a culture of *C. botulinum*, especially after cell multiplication ceases, was considered important so that the changes in the quantities of total protein and active toxin could be compared chronologically. Strain JTD-IV was adapted to growth in a defined medium (Mager *et al.*, 1954) containing no preformed protein. Growth and total protein were then determined during a 72-hr incubation period. Table 4 shows that after cessation of growth, the protein content of the culture decreased slightly. This evidence indicated that little or no protein synthesis occurred during the final 48 hr of incubation. Consequently, it was considered unlikely that synthesis *de novo* of protein was responsible for the tremendous rise in toxicity of culture filtrates observed during this period.

TABLE 5

Effect of chloramphenicol on total protein content of cultures of Clostridium botulinum strain JTD-IV suspended in a defined medium

Sample*	Optical Density	Total Tyrosine per Ml of Culture	Increase
		μg	%
Control, no antibiotic			
0 hr	0.152	140	—
18 hr		190	36
30 hr	0.340	184	34
50 hr	0.210	200	43
Chloramphenicol, (200 μg/ml)			
0 hr	0.144	140	—
18 hr		125	0
30 hr	0.130	140	0
50 hr	0.110	140	0

* Original cell suspension made from 12-hr culture grown in complete medium, washed, and suspended in defined medium.

Effect of chloramphenicol on total protein. To establish whether or not chloramphenicol effectively inhibited protein synthesis by *C. botulinum*, total protein determinations of cultures incubated with chloramphenicol were made. Washed cells of *C. botulinum* strain JTD-IV were suspended in the defined medium and chloramphenicol added to a concentration of 200 μg per ml. It can be seen from table 5 that growth and increases in total protein were completely inhibited.

DISCUSSION

The growth cycle of *C. botulinum* types A and B is characterized by a period of active cell multiplication followed by a rapid autolytic process. At the end of the logarithmic growth phase, the sum of the extracellular and intracellular toxin accounts for only 10 per cent of the toxicity demonstrable when autolysis is complete (Bonventre and Kempe, 1960). This situation presented two interesting possibilities; either the organism synthesized 90 per cent of its toxin during the period of active autolysis or the period of cell multiplication was characterized by the synthesis of a protoxin which was converted to active toxin during cell degeneration.

The fact that toxicity of young cultures can be enhanced by proteolytic enzymes would seem to support the latter possibility. In addition, the rise in toxicity noted during the autolytic process is accompanied by a change in the toxin molecule so that it can no longer be activated (Bonventre and Kempe, 1959*b*). This is indirect evidence that a process of activation normally occurs in cultures of *C. botulinum* types A and B. One of the reasons why the toxin is so effective when it is ingested is that the toxicity is not destroyed by the proteolytic enzymes of the gastroenteric tract. On the strength of the demonstration of activation, it is conceivable that the enzymes of the gut may hydrolyze the toxin partially, releasing toxic sites and consequently enhancing rather than minimizing lethality. Pappenheimer (1948) has pointed out that it is difficult to picture a substance with a molecular weight of 1 million being absorbed from the intestinal tract and suggested that the molecule may in fact be broken down to toxic fragments. Protein chemistry has not advanced sufficiently to explain toxicity on the molecular level. The meager evidence at hand, however, indicates that toxicity is due to simple chemical groupings, not only because of the ease with which it is inactivated by oxidizing agents and mild heat treatment, but also because of the strange behavior of the protein. Wagman and Bateman (1953) observed that when a culture of *C. botulinum* type A was precipitated at different pH levels, two separate dissociation products as toxic as the original supernatant were obtained. More surprising, they found that after precipitation, the supernatant fluid had not diminished in toxicity. Dack and Wagenaar (1956, *unpublished data*) obtained two toxic components of purified toxin by ultracentrifugation. All of these observations indicate that fragmentation of the molecule results in cumulative toxicity by virtue of more toxic sites being available for activity. Until such time as the molecule is adequately characterized, the manner in which proteolytic enzymes activate the toxin remains a matter of conjecture.

The use of metabolic inhibitors served to determine indirectly whether or not synthesis *de novo* of protein during the period of cell degeneration contributed to the ultimate toxicity of the culture filtrates. Cell suspensions, incubated in the presence of chloramphenicol, a known inhibitor of protein synthesis, increased in toxicity 200-fold. The same suspension could be increased

in toxicity to approximately the same extent by disrupting the cells and treating the resulting extract with trypsin. This showed that the potential toxicity of the original culture was far greater than initially apparent. The inhibition of toxin appearance by Versene and the reversal of this inhibition by magnesium and iron salts indicated that the chelating agent inhibited the proteolytic enzymes of the organism responsible for activation of the toxin. Further evidence showing that the strains of *C. botulinum* used in this investigation synthesized proteolytic enzymes whose activity was greatly diminished by Versene; the evidence showing that chloramphenicol effectively inhibited increases in total protein of cultures of *C. botulinum*; and finally the observation that increases in total protein were negligible during the period of time in which the greater part of the active toxin appeared, i. e., after cell multiplication ceased, all pointed to activation of a protoxin rather than synthesis *de novo* of protein as the mechanism responsible for the increased toxicity of the culture filtrates. It should be emphasized that this conclusion is based for the most part on indirect rather than on direct evidence. Until such time as a protoxin is isolated and can be shown to be capable of activation by the specific enzyme systems responsible for the phenomenon, no definite proof for activation of types A and B toxin can be claimed.

On the basis of the experimental evidence compiled in this investigation, an hypothesis describing the nature of the events contributing to the final toxicity of culture filtrates of *C. botulinum* types A and B can be schematically represented as follows:

Period of active syntheses	*Period of greatly reduced syntheses*
0 hr----------24 hr----------96 hr	
a. Cell multiplication	a. Little or no protein synthesis *de novo*
b. Synthesis of protoxin	b. Cell degeneration, autolysis of cells, and liberation of toxin
c. Accumulation of autolytic and proteolytic enzymes	c. Activation of protoxin.
d. No visible autolysis and little protoxin activation.	

SUMMARY

The potential toxicity of cultures of *Clostridium botulinum* in the exponential growth phase was found to be much greater than their apparent toxicity. The increases in toxicity noted during the period of cellular degeneration could not be attributed to protein synthesis *de novo* since this increase was noted in the presence of chloramphenicol, an effective inhibitor of protein synthesis. Furthermore, no increases in total protein could be demonstrated during autolysis. It was concluded that the toxins of *C. botulinum* types A and B are initially synthesized as large molecules with comparatively low biological activity owing to masking of active chemical groupings. These toxin "precursors" must be partially degraded, probably by the proteolytic enzymes of the organisms, before manifesting their full toxic potentialities.

REFERENCES

BONVENTRE, P. F. AND KEMPE, L. L. 1960 The physiology of toxin production by *Clostridium botulinum* types A and B. I. Growth, autolysis, and toxin production. J. Bacteriol., **79**, 18–23.

BONVENTRE, P. F. AND KEMPE, L. L. 1959*a* The physiology of toxin production by *Clostridium botulinum* types A and B. II. Effect of carbohydrate source on growth, autolysis, and toxin production. Appl. Microbiol., **7**, 372–374.

BONVENTRE, P. F. AND KEMPE, L. L. 1959*b* Toxicity enhancement of *Clostridium botulinum* types A and B culture filtrates by proteolytic enzymes. J. Bacteriol., **78**, 892–893.

BOROFF, D. A. 1955 Study of toxins of *Clostridium botulinum*. III. Relation of autolysis to toxin production. J. Bacteriol., **70**, 363–367.

COHEN, P., HALBERT, S. P., AND PERKINS, M. E. 1942 Pneumococcal hemolysin. The preparation of concentrates and their action on red cells. J. Bacteriol., **43**, 607–627.

DUFF, J. T., WRIGHT, G. G., AND YARINSKY, A. Activation of *Clostridium botulinum* type E toxin by trypsin. J. Bacteriol., **72**, 455–460.

ELBERG, S. AND MEYER, K. F. 1939 The extracellular proteolytic system of *Clostridium parabotulinum*. J. Bacteriol., **37**, 541–565.

KINDLER, S. H., MAGER, J., AND GROSSOWICZ, N. 1955 Production of toxin by resting cells of *Clostridium parabotulinum* type A. Science, **122**, 926–927.

KINDLER, S. H., MAGER, J., AND GROSSOWICZ, N. 1956 Toxin production by *Clostridium parabotulinum*. J. Gen. Microbiol., **15,** 394–403.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDELL, R. J. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193,** 265–275.

MAGER, J., KINDLER, S. H., AND GROSSOWICZ, M. 1954 Nutritional studies with *Clostridium parabotulinum* type A. J. Gen. Microbiol., **10,** 130–141.

MASCHMAN, E. 1939 Zurkenntnis der Aminopeptidase. Naturwissenschaften, **27,** 819–20.

NORTHRUP, J. H., KUNITZ, M., AND HERRIOTT, R. N. 1948 *Crystalline enzymes*, 2nd ed. Burgess Publishing Co., Minneapolis.

PAPPENHEIMER, A. M. 1948 Proteins of pathogenic bacteria. Advances in Protein Chem., **4,** 132–153.

PARK, J. T. AND STROMINGER, J. L. 1957 Mode of action of penicillin. Science, **125,** 99–101.

TURNER, A. W. AND RODWELL, A. W. 1943 The *epsilon* toxin of *Clostridium welchii* type D. I. Proteolytic conversion of *epsilon* protoxin into *epsilon* toxin by trypsin and other proteoses. Australian J. Exptl. Biol. Med. Sci., **21,** 17–25.

WAGMAN, J. AND BATEMAN, J. B. 1953 Botulinum type A toxin; properties of a toxin dissociation product. Arch. Biochem. Biophys., **45,** 375–383.