ACTIVATION OF THE TOXIN IN THE CULTURE OF *CLOSTRIDIUM BOTULINUM* TYPE A*

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(Received for publication July 12, 1963)

INTRODUCTORY

The presence of "protoxin" of *Clostridium botulinum* type E has been postulated since culture filtrates of the organisms could be activated by some proteolytic enzymes[4,5,9]. More recently, BONVENTRE and KEMPE[1] observed that toxicity of young cultures of types A and B strains could be enhanced by treatment with trypsin, whereas toxicity of filtrates from older cultures could not be increased. From the result, they postulated the presence of types A and B protoxins and additionally the occurrence of endogenous activation of the toxin in the culture[2]. If this were the case, then a certain factor which activates the protoxin to the toxin would be found in the culture.

Type E strains are considered to be non or only slightly proteolytic except a few mutants while types A and B usually possess a full complement of proteolytic enzymes[3,6,8,10]. These facts would suggest the possibility that in cultures of types A and B organisms proteolytic enzyme produced may be involved in endogenous activation of the toxin.

In this present investigation, attempts were made to prove the presence of some certain endogenous factor which would activate the protoxin to the toxin during the period of cultivation and to clarify its relationship if it does exist to the proteolytic enzyme found in the culture of *Cl. botulinum* type A.

MATERIALS AND METHODS

The strain used was *Clostridium botulinum* type A No. 38. The basal experimental conditions, the media employed for the cultivation of the organisms and the toxicity measurement have been previously described[7].

Proteolytic activity of culture filtrates was investigated by means of gelatin liquefaction as follows. Each 1 ml of serial two-fold dilutions (buffered saline pH 7.0) of the test fluid was placed in a series of test tubes, mixed by shaking with equal volume of 10 per cent gelatin solution and incubated at 37°C for 60 min. Then, all the tubes were cooled in

* This work was communicated at the 8th Symposium of the Society for Bacterial Toxins on July 21, 1961 in Koyasan.

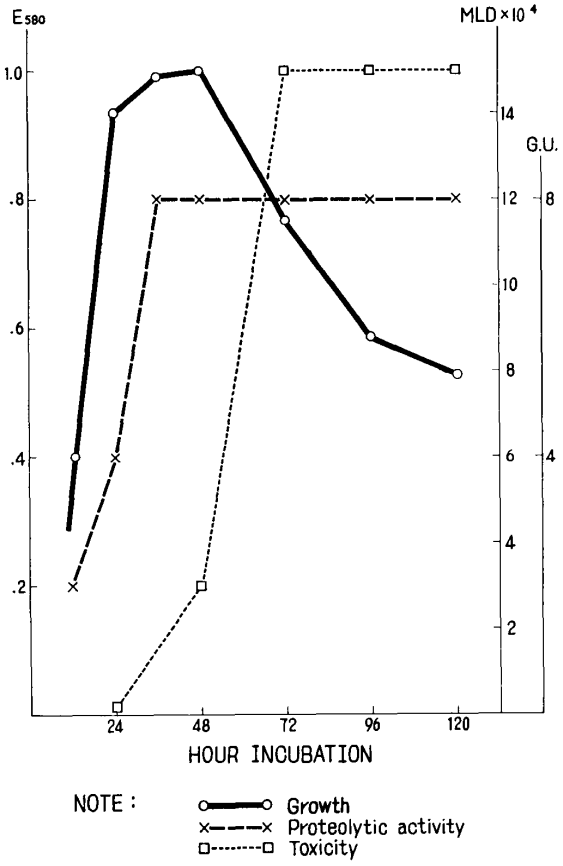
refrigerator at 2°C for 30 min. and observed for liquefaction of gelatin. Proteolytic activity of culture filtrates was expressed in terms of gelatinase unit (G.U. per ml), which was the reciprocal of the maximum dilution of test fluid that gave liquefaction of gelatin.

EXPERIMENTAL RESULTS

Growth, Toxicity and Proteolytic Activity in the Culture

Growth of the organisms, toxicity and proteolytic activity of the culture filtrates were estimated periodically. Results are shown in figure. Proteolytic activity of the culture filtrate was increased in parallel with growth of the organisms and reached maximum within 48 hours incubation. On the other hand, the toxin titre of the culture filtrate rose sharply between 48~72 hours, when autolysis began significantly, and reached maximum after 72 hours incubation. These results may be interpreted as follows. Proteolytic enzyme (s) is secreted from the cell in the anabolic phase of the organisms and the toxin is liberated rather in the catabolic phase, although both active substances are synthesized intracellularly during the exponential growth phase of the culture.

FIGURE *Growth, Toxin Production and Proteolytic Activity in the Culture of Cl. botulinum Type A*



Inhibitory Experiment for Proteolytic Activity

Preliminary experiments showed that proteolytic activity of the culture was much affected by the concentration of glucose in the growth medium. Maximum proteolytic activity was demonstrable in the culture without glucose, when it amounted to 32~64 G.U./ml. Proteolytic activity of the culture containing glucose at a concentration of 1 per cent, most favorable concentration for toxin production, was only 8~16 G.U./ml.

A 48-hr culture filtrate without glucose was used as enzyme solution. (original titre 64 G.U./ml.) Various additives were added to the enzyme solution and their effects on proteolytic activity were observed. As shown in table 1, EDTA, Cu and sera inhibited proteolytic activity at suitable concentrations whilst other additives tested were not effective.

TABLE 1. *Inhibitory Experiment for Proteolytic Activity*

ADDITIVES		PROTEOLYTIC ACTIVITY (G.U./ml)
Normal Horse Serum (× 2)		<2
Normal Rabbit Serum		<2
Trypsin Inhibitor (Soybean)	0.5%	64
*DFP	10^{-2} M	64
Cystein	"	64
KCN	"	64
$CaCl_2$	"	64
$MgSO_4$	"	64
Ba $(CH_3COO)_2$	"	64
Zn $(CH_3COO)_2$	"	64
$CuSO_4$	"	<2
**EDTA	"	<2
"	10^{-3} M	16
CH_2ICOOH	10^{-2} M	64
Control (No Additive)		64

* Diisopropyl-fluoro phosphate
** Ethylendiamine tetraacetate

TABLE 2. *Effect of Metabolic or Proteolytic Inhibitors on Appearance of the Toxin in Extracellular Culture Fluid*

INHIBITOR	TIME OF ADDITION (hr)	INITIAL TOXICITY (MLD/ml)	FINAL TOXICITY (MLD/ml)
Penicillin	20	4,000	64,000
	48	8,000	128,000
	72	128,000	128,000
	96	128,000	128,000
Chloramphenicol	20	—	64,000
	48	—	128,000
	72	—	128,000
	96	—	128,000
EDTA	20	—	8,000
	48	—	32,000
	72	—	128,000
	96	—	128,000
Cu	20	—	8,000
	48	—	16,000
	72	—	128,000
	96	—	128,000

Effect of Proteolytic or Metabolic Inhibitors on Toxin Production

If the proteolytic enzyme makes a certain contribution to toxin production of the organisms, some effect of proteolytic inhibitor on toxin production would be expected.

Cultures in various growth phases were mixed with inhibitors shown in table 2 and reincubated until the time when total incubation period came to 120 hours. (Before and after adding of inhibitor). Then, toxicity of culture filtrates was investigated. Results are shown in table 2. Penicillin and chloramphenicol, not proteolytic inhibitors but metabolic inhibitors, had no effect on subsequent appearance of the toxin in the culture environment provided that it is added after maximum growth of the organisms. This supports the suggestion by many workers that intracellular synthesis of the toxin (or protoxin) might be ceased approximately at the end of the exponential growth phase of the organisms. On the other hand, EDTA or Cu, when they were added at the time when maximum growth of the organisms was attained and less toxin could be demonstrable in the culture environment, exerted considerable inhibition upon subsequent appearance of the toxin in the culture environment. From the data, it seems that the proteolytic enzyme is related to appearance of the toxin in extracellular culture fluid. It is still uncertain, however, whether it acts on either liberation of the toxin from the cell or on activation of the toxin or on both during cultivation.

Demonstration of Protoxin

BONVENTRE and KEMPE[1,2] demonstrated the presence of an inactive form of type A toxin (protoxin) by treatment of young culture fluids or cell extracts with trypsin or pepsion. The following experiment was undertaken in order to clarify a form of the toxin in the cell.

The organisms were grown in the medium for 20 hours. The cells were harvested, washed thoroughly in buffered saline (pH 6.0) and resuspended in the same buffer (1/4 original volume). Then, a part of suspension was disrupted sonically (9 kc, 30 min.) and centrifuged (12,000 rpm, 30 min.). The resulting cell extract was assayed for toxin. If the toxin had been stored intracellularly in an active form, toxicity of the cell extract should have shown at least MLD of 10^5 order because the toxicity of the filtrate of prolonged same culture was MLD of 10^5 order. Actually, however, the titre of toxin in the cell extract was only 5,000 MLD. Then, the cell extract and cell suspension were subjected to the treatments indicated in table 3, and assayed for toxin. Toxicities of both cell suspension and extract were increased 2^5-fold by treatment with trypsin at 37°C pH 6.0 for 90 min. This would suggest the

TABLE 3. *Toxicity of 20-hr Cell Suspension and of Its Extract Before and After Treatment with Trypsin*

SAMPLE	BEFORE INCUBATION	AFTER INCUBATION			
		37°C, 1.5 hr		30°C, 20 hr	
	(MLD/ml)	Control (MLD/ml)	Trysin (MLD/ml)	Control (MLD/ml)	Trypsin (MLD/ml)
20-hr Culture Filtrate	500	<500	500	500	<500
20-hr Cell Suspension	1,000	10,000	160,000	40,000	<500
20-hr Cell Extract	5,000	10,000	160,000	20,000	<500

intracellular existence of less-active toxin or protoxin and support fully the report by BONVENTRE and KEMPE. Prolonged incubation with trypsin resulted in considerable decrease of toxicity. This would be due to further decomposition of the toxin by trypsin.

A Certain Factor Found in Culture Filtrates Which Enhances the Toxicity of the Cell Extract

As described above, toxicity of young cell extracts was increased by trypsin. On the other hand, culture filtrates, at least after 24 hours cultivation, did not increase in toxicity as a result of treatment with trypsin. These facts would suggest the presence of a certain factor in the culture which alters the protoxin to the toxin during cultivation. In addition, it would be expected that that factor is to be found in extracellular culture fluids, because the cell extract did not increase sufficiently in toxicity during incubation without trypsin. Therefore, the effect of culture filtrates on protoxin was studied.

The young cell extract described previously was used as the protoxin preparation and various culture filtrates shown in table 4 were subjected to assay for activation capacity. As shown in table 4, almost all culture filtrates tested were able more or less to enhance the toxicity of the cell extract. The facts that trypsin which hydrolyzes protein is capable of activation and that culture filtrates which activate the protoxin preparation show proteolytic activity suggest the possibility that capacity of culture filtrates to activate the protoxin might be due to the action of proteolytic enzyme produced by the organisms.

TABLE 4. *Activation by Culture Filtrates of Type A Organisms*

ADDITIVE	TOXICITY AFTER INCUBATION
96-hr Culture Filtrate (with glucose)	160,000
96-hr Culture Filtrate (without glucose)	160,000
18-hr Culture Filtrate (with glucose)	80,000
18-hr Culture Filtrate (without glucose)	160,000
Trypsin (0.1%)	160,000
Control (physiological saline)	10,000

Condition of incubation: 37°C, pH 6.0, 90 min.
Note: Culture filtrates used were detoxified previously through the heat treatment of 50°C at pH 8.0 for 15 min.

TABLE 5. *Inhibitory Experiment for Activation*

ADDITIVES	TOXICITY	PROTEOLYTIC ACTIVITY
*Control (no additive)	10,000	1
Control (no additive)	160,000	32
EDTA	40,000	1
Cu	20,000	1
Horse serum	20,000	1
DFP	160,000	32

* Culture filtrate previously heated for 10 min at 100°C was used.

Inhibitory Experiment for Activation Phenomenon

With the intention of clarifying the nature of the factor which activates the protoxin to the toxin, inhibitory experiment for activation was attempted using a system which consisted of the young cell extract and 48-hr culture filtrate without glucose. As shown in table 5, EDTA, Cu and serum, being proteolytic inhibitors, inhibited the activation of the protoxin by the culture filtrate. This would suggest the identity of both proteolytic enzyme and toxin activating factor produced by the organisms during growth.

Physical Conditions Which Affect Activation

By use of the same activation system described above, effects of temperature, pH and incubation period on activation were investigated. In consequence, it was clearly shown that optimum conditions for activation were 5.8~6.2 of pH, 37°C of temperature and 90~120 minutes of incubation period.

DISCUSSION

Culture filtrates of *Cl. botulinum* type A are not increased in toxicity by treatment with proteolytic enzyme unlike those of type E strain. The presence of an inactive or less active form of type A toxin, however, which may be called potential toxin or protoxin, was evidenced by the fact that toxicity of young cell extracts was enhanced by treatment with trypsin. Such "activation" was also observed when the culture filtrates of type A organisms were used, instead of trypsin. This suggests that type A organisms produce a certain factor which alters inactive or less-active toxin to full-active toxin.

Type A organisms are proteolytic strains and their culture filtrates, being capable of activation, possess significant proteolytic activity. Addition of EDTA or Cu to the culture filtrates resulted in complete inhibition of their proteolytic activity and simultaneously diminished their toxin activating capacity. These facts would suggest the possibility that both proteolytic enzyme and the factor which activates the toxin in the culture may be identical. This possibility was also supported by the fact that addition of proteolytic inhibitor to the culture at the terminal stage of exponential growth phase suppresses the subsequent increase of toxicity of extracellular culture fluids. Consequently, the process of toxin production by *Cl. botulinum* type A would be assumed as follows, although this is based on rather indirect evidence. That is, the toxin is synthesized intracellularly at anabolic phase of the organisms in an inactive form, then liberated from the cell at catabolic phase into environment medium and activated through the action of proteolytic enzyme which has been produced previously. Culture filtrates of type E strain are always increased in toxicity by treatment with some proteolytic enzymes. This situation may be due to the fact that the toxin remains in a form of protoxin, being inactive or less-active biologically, in the culture since the organisms do not produce a proteolytic enzyme which would alter it to active form.

Type A protoxin preparation used in this investigation showed toxicity, although it is very weak. To know whether the protoxin is perfectly inactive or not would be interesting and desirable. It should be noted, however, that if the protoxin itself does not possess any biological activity, when it is subjected to animal test it might be apparently toxic because of natural activation due to the action of proteolytic enzyme of test animals.

Proteolytic enzyme of *Cl. botulinum* type A has never been purified. Detailed investigation of such enzyme will be described in other report.

SUMMARY

The presence of "activation" reaction in process of toxin production by *Cl. botulinum* type A was evidenced experimentally. This is mainly based on the following facts: first, inactive or comparatively less-active toxin (protoxin) was demonstrable in young cell extracts. Second, a factor which alters the protoxin to the toxin could be found in extracellular culture fluides of the organisms. Additionally, the identity of the toxin activating factor and the proteolytic enzyme produced by the organisms was emphasized.

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