INFECTION AND IMMUNITY, Sept. 1973, p. 360-369

Vol. 8, No. 3
Printed in U.S.A.

Role of Zinc in the Production of *Clostridium perfringens* Alpha Toxin

HIROKO SATO AND RYOSUKE MURATA

The Second Department of Bacteriology, National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

Received for publication 24 April 1973

Clostridium perfringens was found to produce alpha toxin in a synthetic medium containing zinc; in medium containing no zinc, a little toxin was detected in the early logarithmic phase of growth and it disappeared rapidly. No intracellular accumulation of alpha toxin protein occurred whether or not zinc was present in the medium. In zinc-deficient medium, the organisms produced and released into the surrounding medium the protein specifically precipitable with alpha antitoxin in an amount comparable to that of alpha toxin produced in the zinc-containing medium. The protein combined rapidly in some unknown way with zinc to form the active and stable alpha toxin.

Clostridium perfringens alpha toxin is the main lethal toxin which has been identified as an enzyme, phospholipase C (EC 3.1.4.3) (8). *C. perfringens* requires meat digest to produce the toxin in large quantity (1, 7, 22). Factors affecting the production of the toxin were studied in a chemically defined medium (11-13). A large quantity of L-arginine (10 mg/ml) and controlled quantities of L-cystine (0.1 mg/ml or less) and of divalent (Zn^{2+}, Fe^{2+}, and Mg^{2+}) and monovalent cations (Na^{+} and K^{+}) supported production of the toxin. The toxin produced in the defined medium was indistinguishable in biological, immunological, and some physicochemical properties from that produced in a complex medium. Among these factors, zinc seemed to play the critical role. Only a small quantity of alpha toxin was produced without zinc at earlier stages of growth, and it disappeared rapidly (20). No alpha toxin was detected in zinc-deficient cultures at the end of the logarithmic phase, when the toxin activity reached its maximum in zinc-containing cultures (12). Manganese, cobalt, or nickel was partially replaceable, but the latter two gave inconsistent results (12).

The present work was undertaken to study further the role of zinc in production of alpha toxin. We have demonstrated that the antitoxin-precipitable protein of little or no toxic activity was produced without zinc and that zinc rendered the preformed protein fully active.

MATERIALS AND METHODS

Organism. *C. perfringens* strain PB6K N5 maintained in a synthetic medium composed of 19 amino acids, six vitamins, adenine, uracil, fructose, inorganic salts, and thioglycollic acid, named SM 67 (11), was used.

Culture methods. Cultures were made at 37 C usually in 4-ml portions of medium in test tubes (10 by 110 mm). For toxin production, SM 67 was modified by reducing L-leucine from 350 μg/ml to 150 μg/ml and by omitting manganese. The modified medium, named CM (complete medium), supported the growth and toxin production to almost the same extent as in SM 67. To examine the effect of zinc, zinc-less CM, named DM (deficient medium) was used with and without addition of $ZnSO_4 \cdot 7H_2O$ to 0.05 mM. To study incorporation of L-^{14}C leucine into the intra- and extracellular protein, CM or DM was supplemented with L-^{14}C leucine (named "hot CM" and "hot DM," respectively). The seed culture was an overnight culture in DM after two to three daily transfers in the medium. Centrifugation for harvesting and washing the bacterial cells was done at 2,600 × *g* for 20 min at 4 C.

Estimation of growth. The optical density (OD) at 660 nm was read with a Coleman Junior spectrophotometer with a light path of 10 mm.

Assay for alpha toxin. Alpha toxin activity was measured by the lecithovitellin method and expressed in egg unit (EU) for the direct toxicity or in Lv for the power to combine the standard antitoxin or both (13). One Lv is the smallest amount of alpha toxin producing positive egg yolk reaction in the presence of 1 IU of the Standard Gas—gangrene Antitoxin (perfringens). In practice, however, the combining power was determined with appropriate fractional units of the stand-

ard antitoxin and converted to Lv by simple arithmetic calculation. One EU corresponded to 3 mean lethal doses (LD_{50}) and the ratio of EU/Lv was approximately 10 to 20.

Alpha antitoxin and purified alpha toxin. Alpha antitoxin was prepared by peptic digestion of serum taken from a horse hyperimmunized with alpha toxin purified partially by gel filtration and concentration with ammonium sulfate (25). Purified alpha toxin was obtained by gel filtration and electrophoresis (18). It gave a single precipitin line with the alpha antitoxin by the Ouchterlony gel diffusion method (15).

Incorporation of L-^{14}C leucine into cellular protein. Cells harvested from an overnight culture in DM and suspended in hot CM or hot DM were incubated at 37 C. Cells separated from a 4-ml culture were resuspended in 2 ml of saline, to which 2 ml of 10% trichloroacetic acid was added. After heat treatment at 95 C for 15 min, the precipitate was separated and dissolved in 2 ml of 0.3 N NaOH, to which was added 4 ml of 10% trichloroacetic acid. The precipitate was redissolved in 2 ml of 0.3 N NaOH. A 0.1-ml portion was placed on a planchet, dried, and counted for radioactivity in a windowless gas-flow counter (Aloka, Tokyo).

Incorporation of L-^{14}C leucine into extracellular protein. The culture supernatant fluid containing L-^{14}C leucine was filtered through a membrane filter (HA, 0.45 μm; Millipore Corp.) and subjected to determinations of the following. (i) Radioactivity of alpha toxin: A 3.0-ml portion of the filtrate was added with 0.1 ml of purified alpha toxin (450 Lv/ml) as carrier and 0.1 ml of alpha antitoxin (550 IU/ml). The mixtures were incubated at 40 C for 1 h and then allowed to stand overnight in the cold to insure complete precipitation of alpha toxin. The precipitate was washed five times with 8 ml of chilled saline each time, dissolved in 1 ml of 0.3 N NaOH, and added with 2 ml of 10% trichloroacetic acid. The precipitate was washed with 5% trichloroacetic acid, dissolved in 80% formic acid, dried, and counted. (ii) Radioactivity of extracellular protein: A 0.5-ml portion of the filtrate was added with 0.2 ml of bovine serum albumin (10 mg/ml) and 0.7 ml of 10% trichloroacetic acid. The mixture was heated at 95 C for 15 min. The precipitate was taken and dissolved in 0.3 N NaOH. The final precipitate with 10% trichloroacetic acid was washed with 5 ml of a mixture of ethanol and ether (1:1), dissolved in 80% formic acid, dried, and counted.

Alpha toxin production by ^{14}C-labeled organisms. Cells from an overnight culture in DM were resuspended in 60 ml of hot CM or 64 ml of hot DM containing L-^{14}C leucine at 0.2 μCi/ml. The cultures were incubated at 37 C until the OD reached 0.48. The cells were centrifuged and washed once with a chilled washing medium consisting of 230 mg of Na_2HPO_4, 20 mg of $MgSO_4 \cdot 7H_2O$, and 850 mg of NaCl per 100 ml at pH 7.0. The L-^{14}C leucine-labeled cells were suspended in 60 ml of CM at an OD of 0.46. Each suspension was dispensed into tubes (4.5 ml) and incubated at 37 C. Duplicate tubes were removed at 0, 20, 40, 60, 105, and 150 min. After OD determination, each was centrifuged; the supernatant fluid was filtered through a membrane filter. Alpha toxin and radioactivities of alpha toxin and of the total protein in the filtrate were determined.

Production of alpha toxin in ^{65}Zn-containing medium. The organisms were grown in DM added with zinc at 0.025 mM and $^{65}ZnCl_2$ at 2 μCi/ml. After incubation at 37 C for 6 h, the culture was centrifuged; the supernatant fluid was filtered through a membrane filter (HA, 0.45 μm). The filtrate was concentrated with Ficoll followed by dialysis against 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.4 ("crude ^{65}Zn-toxin"). In some cases, purified alpha toxin was added to crude ^{65}Zn-toxin as carrier. Radioactivity of ^{65}Zn was determined with an NaI crystal cell scintillation counter (Aloka, Tokyo).

Gel filtration of crude ^{65}Zn-toxin. A 0.5-ml portion of crude ^{65}Zn-toxin was applied to a column (1.2 by 24.0 cm) of Sephadex G-100 in a glass tubing with the inner surface coated with hexamethyldisilazane dissolved in n-hexane. The eluant was 0.05 M Tris-hydrochloride buffer, pH 7.4. Each of the 10-drop (0.35 ml) fractions was determined for radioactivity, alpha toxin, and OD at 280 nm. The alpha toxin fractions were pooled, concentrated, and subjected again to gel filtration under the same conditions. The final preparation was named "purified ^{65}Zn-toxin."

Sucrose density gradient centrifugation of crude ^{65}Zn-toxin. A 0.2-ml portion of crude ^{65}Zn-toxin (1,140 Lv/ml and 163,000 counts per min per ml) was layered on top of 5.0 ml of 5 to 20% linear sucrose density gradient in 0.05 M Tris-hydrochloride buffer, pH 7.4. After centrifugation at 50,000 rpm for 17.5 h at 4 C in an SW65L Ti rotor with a Beckman L4 ultracentrifuge, 10-drop (0.13 ml) fractions were collected manually. The alpha toxin fraction was concentrated with Ficoll and dialyzed against 0.05 M Tris-hydrochloride buffer, pH 7.4. A 0.2-ml portion of this was subjected again to sucrose density gradient centrifugation under the same conditions.

Immunoelectrophoresis of purified ^{65}Zn-toxin. Purified ^{65}Zn-toxin was placed in the center well of a 1% agarose (Nakarai Chemicals, Kyoto) slide. Electrophoresis was performed with Veronal buffer (ionic strength 0.05, pH 8.6) at 4.0 mA/cm for 90 min at room temperature. The alpha antitoxin was poured into each trough; the slide was incubated at room temperature for 3 days.

Radioautography. Slides of double diffusion and immunoelectrophoresis were washed with saline and with deionized water and dried. A piece of X-ray film (Sakura type N; Konishiroku Photo Industry, Tokyo) was placed in contact with each slide wrapped with thin plastic film and exposed for 17 or 35 days.

Polyacrylamide gel electrophoresis. Polyacrylamide gel was prepared by the method of Williams and Reisfeld (24) or Davis (3) (pH 8.0 gel or pH 9.4 gel) in glass tubings measuring 5 by 70 mm. The running and the spacer gels were 0.95 ml and 0.19 ml, respectively. No sample gel was used. A 0.1-ml portion of purified ^{65}Zn-toxin (15 Lv/ml, 5,000 counts per min per ml) in a sucrose solution was layered on the spacer gel. Electrophoresis was carried out with a type BC apparatus (Mitsumi Scientific Industry,

Tokyo) at 4 C with Williams and Reisfeld's Veronal-tris buffer, pH 7.0, or Davis Tris-glycine buffer, pH 8.3, in the electrode vessels. With the cathode at the top, a constant current of 2.2 mA/tube for 90 min for pH 8.0 gel and 3.0 mA/tube for 70 min for pH 9.4 gel was applied. When the solvent front, indicated by bromophenol blue (BPB), reached the bottom of the gel, electrophoresis was discontinued. The gel was removed from the glass tubing by rinsing in tap water. It was cut into 2-mm segments from the top. Toxic activity was located by placing the segments on egg yolk agar plates; the radioactivity of each segment was determined with a scintillation counter.

RESULTS

Effect of zinc added to the culture in DM on the production of alpha toxin. An overnight culture in DM with an OD of 1.2 was centrifuged; the cells were resuspended in CM or DM to a concentration of an OD of 0.05. The suspension, distributed into tubes each with 4 ml, was incubated at 37 C. Growth and toxin production in duplicate tubes were examined every hour. Zinc was added to 0.05 mM to the cultures at indicated times (Fig. 1). Toxin production paralleled bacterial growth in CM, but in DM a little toxin was detected only at earlier stages of the logarithmic phase and it disappeared rapidly. The addition of zinc to the cultures in DM during the first 4 h resulted in production of alpha toxin to the levels comparable to those in CM, whereas the effect of the metal was very little if added in 5 h. The bacterial growth in CM was not different from that in DM.

Alpha toxin production by ^{14}C-labeled organisms. When the bacterial cells labeled in hot CM (Fig. 2) or hot DM (Fig. 3) were chased in CM, little or no radioactivity was detected in the alpha toxin produced in CM.

Production of alpha toxin by the organisms harvested at various stages of growth in CM or DM. Cells taken from an overnight culture in DM were suspended in 45 ml of CM or 90 ml of DM to an OD of 0.1. Each suspension was distributed into tubes in 4-ml amounts. Ten tubes of CM (group I) and 20 of DM (10 tubes were group II and the other 10 group III) were incubated at 37 C (primary cultures). Duplicate tubes were removed from each group every 30 min at OD values of 0.32, 0.46, 0.64, 0.79, and 0.90. Cells of each sample of groups I and II were resuspended in 4 ml of fresh CM; those of group III in the supernatant fluid of the parent spent cultures added with zinc to 0.05 mM. All the tubes were incubated at 37 C for 30 min (secondary cultures). The supernatant fluids of the primary and the secondary cultures were determined for toxic activities.

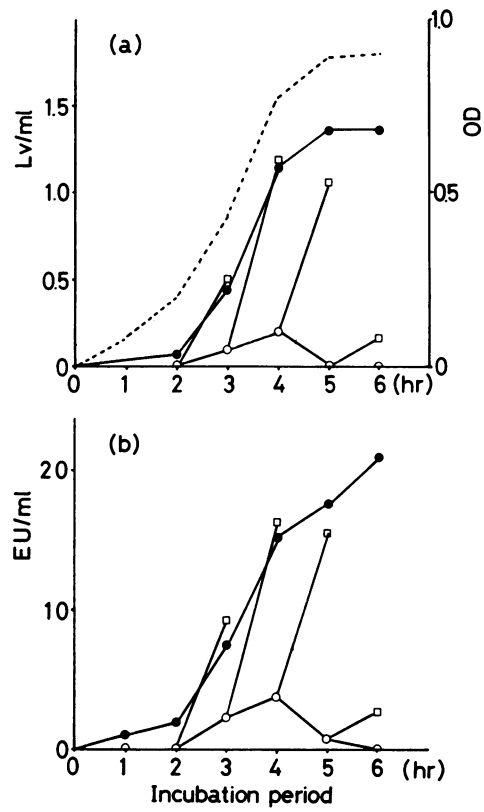

FIG. 1. *Effect of zinc added to the culture in DM on the production of alpha toxin. a, The toxin activity in Lv/ml. b, The toxin activity in EU/ml. Symbols: ●, in CM; ○, in DM; □, in DM 1 h after addition with zinc. Dotted line shows OD at 660 nm.*

Table 1 compares alpha toxin production in primary cultures (groups I and II) at harvest and that in 30 min of re-incubation (secondary cultures) of the three groups. In DM, a little alpha toxin was produced only at early stages and it disappeared rapidly. Alpha toxin production by secondary cultures of group II was lower than that by secondary cultures of group I. No explanation could be given to the fact that the later the stage of the primary cultures, the larger the difference between the two groups. The cells harvested from DM at earlier stages of growth produced more alpha toxin in the spent culture supplemented with Zn^{2+} (group III) than in fresh CM (group II). The results suggest that the primary cultures contain some extracellular substance essential for toxin production and that the more aged cultures contain smaller amounts of the substance than younger ones.

Effect of puromycin upon alpha toxin production. To the growing cultures in CM or

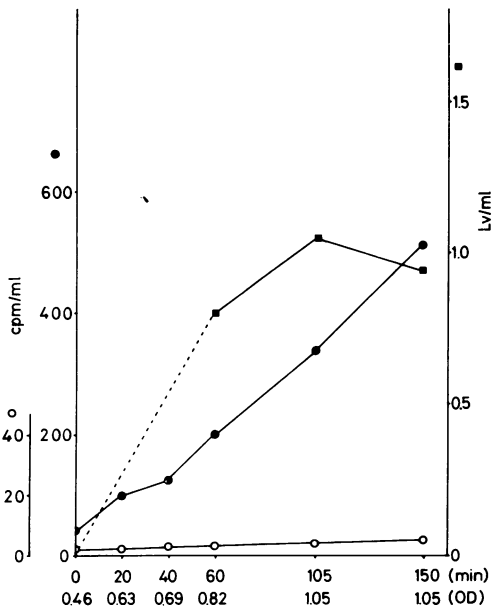

FIG. 2. *Production and radioactivity of alpha toxin in CM inoculated with the cells preincubated in hot CM. Cells grown in hot CM (OD = 0.48) were washed and resuspended in CM (OD = 0.46). Alpha toxin and the antitoxin-precipitable protein in the culture filtrate contained 0.47 Lv/ml and 61 counts per min per ml. The suspension (the cellular protein contained 31,000 counts per min per ml) was incubated. OD values during growth are also indicated. Symbols: ■, toxin (below 0.4 Lv/ml at 20 and 40 min); ○, radioactivity of toxin; ●, radioactivity of total protein in the culture filtrate.*

DM, puromycin was added to 0.4 mg/4 ml at intervals. Incubation was continued for an additional 30 min, and the toxic activities were determined. To some tubes of DM, zinc was added within 1 min after the addition of puromycin. Puromycin inhibited immediately and completely the alpha toxin production in CM. In DM, however, the addition of zinc increased alpha toxin significantly to a level comparable to that in CM detected at the time of addition of puromycin (Fig. 4). The results show that the inactive toxin protein is synthesized without zinc and that no further protein synthesis is required for the increased toxic activity if zinc is added.

Incorporation of L-^{14}C leucine into toxin protein produced in CM or DM. The organisms were grown in hot CM or hot DM containing L-^{14}C leucine at 0.25 μCi/ml, and radioactivities of the extracellular alpha antitoxin-precipitable protein and of intra- and extracellular protein were determined. As shown in Fig. 5, radioactivity of the antitoxin-precipitable protein in the two media increased at similar rates for the first 3 h, although the toxic activity was very low in DM.

Demonstration of inactive toxin protein in culture supernatant fluid. Zinc was added to the supernatant fluid of cultures in DM incubated for different periods of time. The toxic activities were determined before and after the addition of zinc. If added with zinc at earlier stages, the toxic activity increased to a level comparable to that produced in CM; while that at later stages did not increase the toxic activity (Fig. 6). All the processes of these experiments were conducted under refrigeration; other experiments conducted at room temperature gave the same results.

Incorporation of zinc into the toxin molecule. Crude ^{65}Zn-toxin containing 1,140 Lv/ml and 163,000 counts per min per ml was filtered through a Sephadex G-100 column; the alpha toxin fraction was subjected again to gel filtration under the same conditions. Most ^{65}Zn was found in the alpha toxin fraction; the peaks of radioactivity and the toxic activity coincided (Fig. 7).

The two activities sedimented to the same position in sucrose density gradient centrifugation (Fig. 8). The peak fraction, fraction no. 23, containing 32 Lv/ml and 1,783 counts per min per ml, formed a single precipitin line with alpha antitoxin in agar gel double diffusion. The precipitin line proved to be alpha toxin-

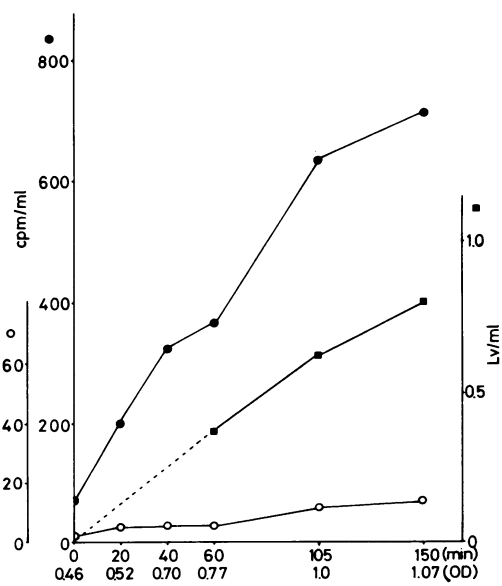

FIG. 3. *Production and radioactivity of alpha toxin in CM inoculated with the cells preincubated in hot DM. The protein fraction of the cell suspension in CM (OD = 0.46) contained 30,000 counts per min per ml. Symbols: same as those in Fig. 2.*

TABLE 1. *Toxin production by the cells harvested at various stages of growth in CM or DM*

Stage of growth in primary culture (OD)	Toxin (Lv/ml)				
	Group I[a]		Group II[a]		Group III[b] (DM + Zn^{2+})
	Primary culture (CM)	Secondary culture (CM)	Primary culture (DM)	Secondary culture (CM)	
0.32	0.24	0.34	<0.05	0.28	0.67
0.46	0.67	0.56	<0.05	0.42	1.44
0.64	1.71	0.67	0.20	0.34	1.44
0.79	2.07	0.50	0.15	0.20	0.43
0.90	1.71	0.47	<0.05	0.17	<0.05

[a] Groups I and II: cells from cultures in CM(I) or DM(II) (primary cultures) were transferred to fresh CM (secondary culture) and assayed for toxin after a 30-min incubation.

[b] Group III: cells from culture in DM were resuspended and incubated for 30 min in the separated supernatant fluid added with Zn^{2+} at 0.05 mM.

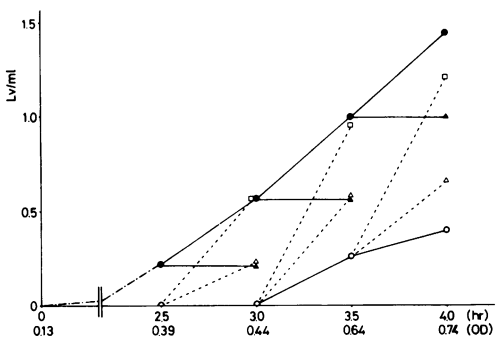

FIG. 4. *Effect of puromycin on the production of alpha toxin. Cultures in CM were added with puromycin (0.1 mg/ml) (▲). Cultures in DM were added with Zn^{2+} (□) or puromycin and Zn^{2+} (△). Symbols: ●, toxin in untreated CM; ○, toxin in untreated DM; ▲, □, △, toxin 30 min after addition of puromycin or Zn^{2+} or both. OD values during growth in untreated media are also indicated. ---, estimated from other experiments.*

antitoxin precipitate in egg yolk agar. Radioautograph of the slide showed a single black line at the location identical to that of the precipitin line (Fig. 9a and b). In immunoelectrophoresis of purified ^{65}Zn-toxin containing 835 Lv/ml and 60,000 counts per min per ml, a major and a minor arc appeared; the major one was alpha toxin-antitoxin precipitate and the minor one a contaminant (Fig. 10a). The radioautograph showed that ^{65}Zn was associated only with the major arc (Fig. 10b and c).

Role of zinc in manifestation of alpha toxin activity. Purified ^{65}Zn-toxin containing 25 Lv/ml and 838 counts per min per ml was dialyzed against 0.05 M Tris-hydrochloride buffer, pH 7.4, with or without addition of ethylenediaminetetraacetic acid (EDTA) at 3 mM. The activities of ^{65}Zn and alpha toxin determined at intervals during dialysis against the EDTA-containing buffer were expressed in percentage to the corresponding values of the specimen dialyzed against the buffer containing no EDTA (Fig. 11). The two activities decreased at the same rates by dialysis against EDTA-containing buffer, whereas they did not change appreciably by dialysis against the plain buffer for 48 h.

Dialysis of purified alpha toxin against EDTA-containing buffer also caused loss of the activity. Purified alpha toxin and EDTA-dialyzed alpha toxin (EDTA-toxin) were applied to columns of Sephadex G-100 equilibrated respectively with 0.05 M Tris-hydrochloride buffer, pH 7.4, and the same buffer containing 3 mM EDTA and eluted with the respective buffers. Eluted fractions of EDTA-toxin were assayed for the activity before and after addition of zinc. The EDTA-toxin and the native toxin were eluted in the same elution volumes (Fig. 12).

Acrylamide gel electrophoresis of purified ^{65}Zn-toxin. In pH 8.0 gel, the activities of alpha toxin and of ^{65}Zn were found at the same position (Fig. 13); whereas in pH 9.4 gel, they dissociated. ^{65}Zn migrated together with BPB, but the toxic activity was detected in the 22-mm segment (no. 11) from the top (Fig. 14). In gel filtration, however, of purified ^{65}Zn-toxin on Sephadex G-100 at pH 9.4, no dissociation occurred between the alpha toxin and ^{65}Zn. Nor decreased activity resulted from dialysis of alpha toxin against 0.05 M Tris-hydrochloride buffer, pH 9.4.

DISCUSSION

Production of *C. perfringens* alpha toxin is affected markedly with various inorganic cations in the medium; the concentrations of Fe^{2+} and Zn^{2+} must be controlled strictly. Pappen-

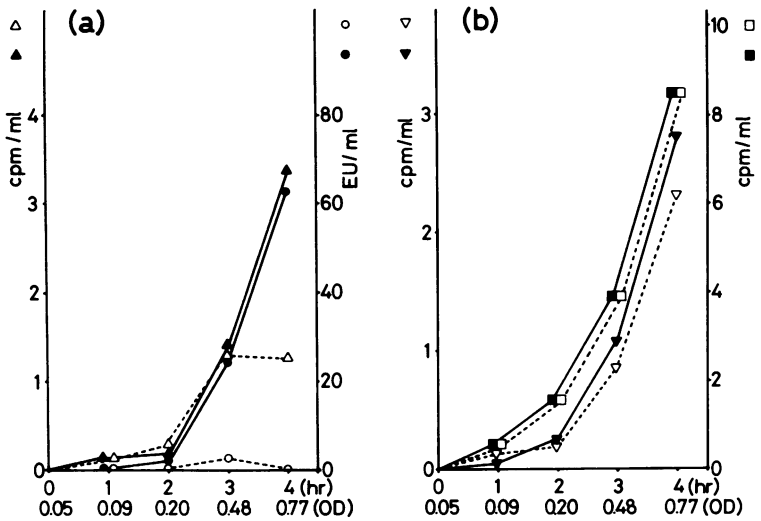

FIG. 5. *Incorporation of* L-^{14}C *leucine into antitoxin-precipitable protein in hot CM and hot DM. Filled and open symbols represent CM and DM, respectively. a,* ●, ○, *Toxin (EU/ml);* ▲, △, *radioactivity of antitoxin-precipitable protein of the culture filtrate* ($\times 10^{-2}$ *counts per min per ml). b,* ▼, ▽, *Radioactivity of the total protein of the culture filtrate* ($\times 10^{-3}$ *counts per min per ml);* ■, □, *radioactivity of cellular protein* ($\times 10^{-4}$ *counts per min per ml).*

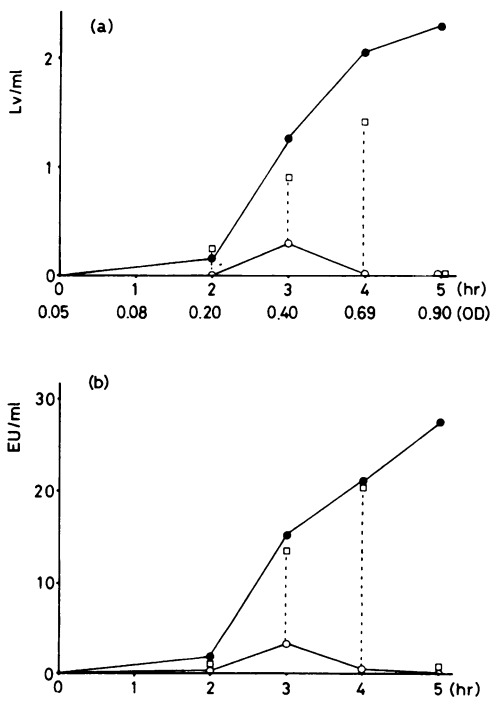

FIG. 6. *Increase in toxin activity by addition of* Zn^{2+} *to the supernatant of DM. The supernatant fluid was added with* Zn^{2+} *to 0.05 mM. Toxin activity is expressed in Lv/ml (a) and EU/ml (b). Symbols:* ●, *toxin in CM;* ○, *toxin in DM;* □, *toxin in the supernatant fluid of DM was assayed immediately after addition of* Zn^{2+}.

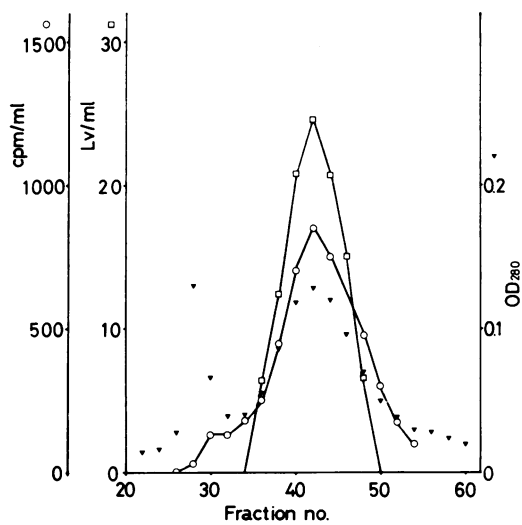

FIG. 7. *Gel filtration of* ^{65}Zn*-toxin. Crude* ^{65}Zn*-toxin was partially purified by Sephadex G-100 gel filtration. A 0.2-ml portion of the toxin (850 Lv/ml, 55,000 counts per min per ml) was applied again to the column. Each of 10-drop fractions was added with 0.5 ml of 0.05 M Tris-hydrochloride buffer for assay. Symbols:* ○, *radioactivity of* ^{65}Zn *(counts per min per ml);* □, *toxin (Lv/ml);* ▼, *protein* (OD_{280}).

heimer and Shaskan (16) suggested that Fe^{2+} affects the metabolic process of this organism. No explanation for the roles of Zn^{2+} or other metals has been given.

In agreement with the results reported previ-

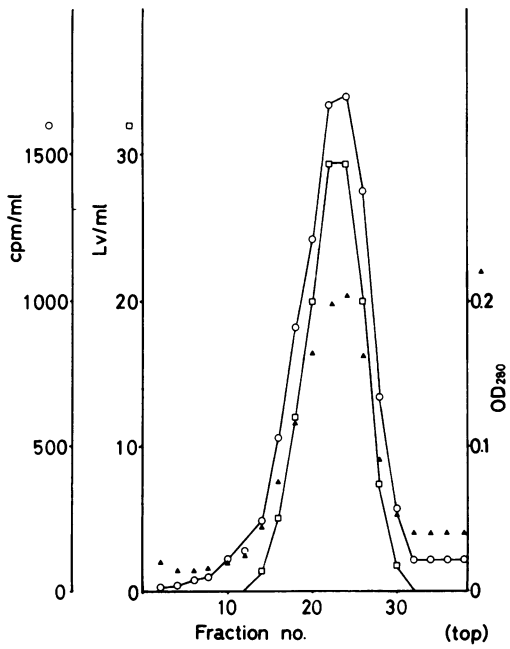

FIG. 8. *Sucrose density gradient centrifugation of ^{65}Zn-toxin. Crude ^{65}Zn-toxin was partially purified by sucrose density gradient centrifugation. A 0.2-ml portion of the toxin (800 Lv/ml, 60,000 counts per min per ml) was centrifuged again. Ten-drop fractions were collected; each fraction was added with 0.5 ml of 0.05 M Tris-hydrochloride buffer for assay. Symbols: O, radioactivity of ^{65}Zn (counts per min per ml); □, toxin (Lv/ml); ▲, protein (OD_{280}).*

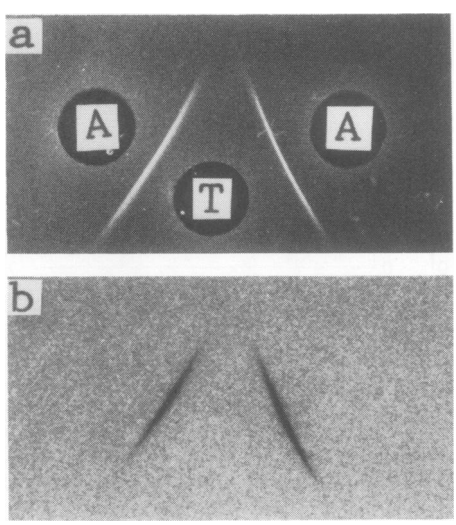

FIG. 9. *Agar gel diffusion tests and radioautography with fraction no. 23 of Fig. 8. a, Precipitation. Abbreviations: T, toxin; A, antitoxin (100 IU/ml). b, Radioautograph of Fig. 9a on X-ray film by a 17-day exposure.*

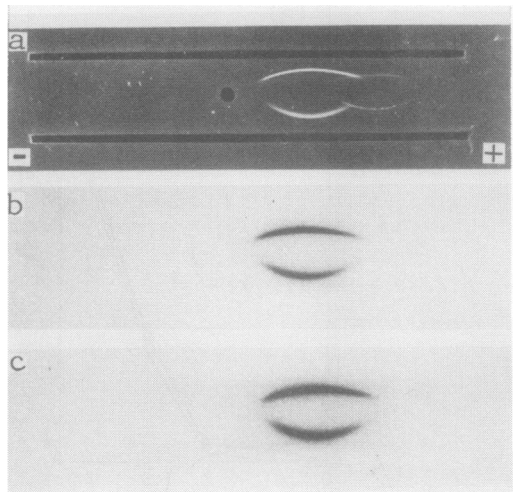

FIG. 10. *Immunoelectrophoresis and radioautography of purified ^{65}Zn-toxin. a, Immunoelectrophoresis. Antitoxin added to the troughs was 580 IU/ml. b, Radioautograph of Fig. 10a exposed for 17 days. c, The same as above exposed for 35 days.*

ously (20), in a zinc-deficient medium, only a little alpha toxin was produced at earlier stages of growth and it disappeared rapidly. It was no longer detectable at 6 to 7 h, when the toxic activity reached the maximum in the zinc-containing medium. Addition of zinc to young cultures in the zinc-deficient medium caused immediate appearance of alpha toxin activity to a level comparable to that attained in the complete medium. Zinc deficiency did not interfere with the growth of the organism at earlier stages (20). The organisms retained the toxigenicity after serial transfers in the zinc-deficient medium (13). From these facts, it is not likely that zinc, affecting greatly the metabolic process of the organism, enhances the alpha toxin production.

The rapidity with which the activity of alpha toxin appeared by the addition of zinc may indicate the presence of inactive toxin protein in the culture. However, there was no indication of intracellular accumulation of the inactive toxin protein at any stage of growth in DM or in CM. Alpha toxin protein seemed to be synthesized de novo in CM, as reported by Shemanova et al. (19). The role of zinc is not related to releasing the toxin presynthesized in the cells.

The present results indicate synthesis of an inactive alpha toxin protein even in the zinc-deficient medium. Radioactivity precipitable with alpha antitoxin in DM and that in CM were almost the same for the first 3 h, when the addition of zinc to the supernatant fluid of

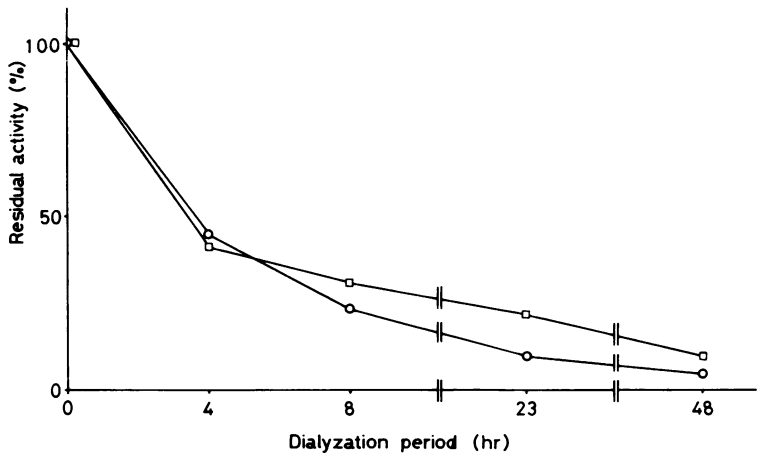

FIG. 11. *Effect of dialysis against EDTA-Tris-hydrochloride buffer upon toxin activity and radioactivity of purified ^{65}Zn-toxin. ^{65}Zn-toxin was dialyzed at 4 C against 0.05 M Tris-hydrochloride buffer (pH 7.4) with or without addition of EDTA at 3 mM. Toxin (□) and radioactivity (○) of the sample dialyzed against EDTA buffer were shown in percentage to those of the sample dialyzed against plain buffer.*

culture in DM caused immediate appearance of the toxic activity. Thus, the extracellular inactive protein was converted immediately into the active toxin upon addition of zinc. These facts show that the organisms produce the inactive toxin protein without zinc. In DM, however, the antitoxin-precipitable radioactivity did not increase in quantity in longer than 4 h, although radioactivity of the labeled total protein continued to increase at the same rate as in CM. It is not known whether synthesis of the toxin protein completed within 4 h or its synthesis and decomposition take place simultaneously, thus causing no increased toxin protein. The rapid disappearance of the toxic activity may indicate the lability of the toxin protein produced in the absence of zinc. The fact that sometimes little or no increased toxic activity resulted from the addition of zinc to the culture supernatant fluid may also be explained by the lability of the protein. Possible destructive mechanism appearing at later stages may also have contributed to the short life of the toxin protein produced in DM. *C. perfringens* theta toxin, which is also a rather unstable toxin, was destroyed sometimes very quickly in the later logarithmic phase (13). Soda, Sato, and Murata (20) demonstrated an enhanced destruction of theta toxin with Ca^{2+}. It is not known, however, whether the same is true with the protein of alpha toxin produced in DM.

Attempts to isolate the inactive toxin protein produced in DM were not successful because of its extreme lability. Zinc may also have a role in stabilizing the labile molecule. Ispolatovskaya (4–6) observed that zinc, when used in purification, increased the yield of the toxin. From loss of the activity of the purified toxin with chelating agents, regeneration of the activity by the addition of zinc, and the presence of two extinction bands at 3345 and 3202A, she suggested that alpha toxin was a zinc metalloenzyme. In this respect, Moskowitz (9, 10) stated that alpha toxin lost the activity by treatment with EDTA, and the activity was restored with Zn^{2+}, Co^{2+}, and Mn^{2+}, but not with Ca^{2+} or Mg^{2+}.

Although no direct evidence of a metalloenzyme was obtained due to the unsatisfactory purification, the present results strongly indicate the incorporation of zinc into the alpha toxin molecule. Particularly, immunoelectrophoresis, having ruled out the possibility that zinc was merely a contaminant, proved the firm binding of zinc to the alpha toxin molecule. The same result was obtained with another buffer, citrate phosphate buffer, pH 6.5. Atomic absorption spectrophotography applied to a preparation of purified toxin (specific activity 1.3 μg/Lv) confirmed the binding between zinc and the toxin molecule. The toxin lost the toxic activity and the metal by dialysis against EDTA-buffer; the toxicity was restored by addition with zinc. A similar enzyme produced by *Bacillus cereus* was inactivated with such chelating agents as EDTA and *o*-phenanthroline and reactivated with Zn^{2+} (or Mn^{2+}) but not with Ca^{2+} (14). Alkaline phosphatase of *Escherichia coli* proved to be a zinc metalloenzyme; removal of zinc with EDTA resulted in complete loss of the activity without accompanying dissociation of the molecule into subunits (17, 21). Evidences have been presented with zinc enzymes

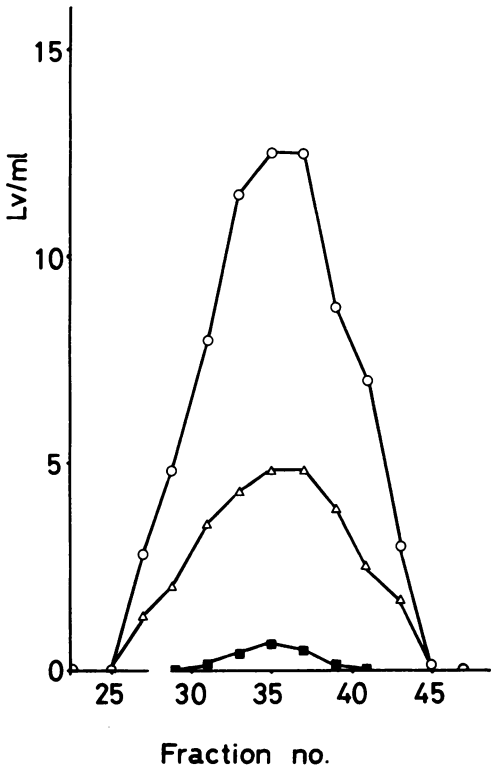

FIG. 12. *Sephadex G-100 gel filtration of purified alpha toxin after dialysis against Tris- or EDTA-Tris-hydrochloride buffer. Column: 1.2 by 24.0 cm; samples: a 0.3-ml portion of each of purified toxin (500 Lv/ml) and EDTA-toxin (96 Lv/ml). Ten-drop fractions were collected; each fraction was added with 0.3 ml Tris-hydrochloride buffer and assayed. Symbols:* O, *purified toxin;* ■, *EDTA-toxin;* Δ, *EDTA-toxin after addition of zinc.*

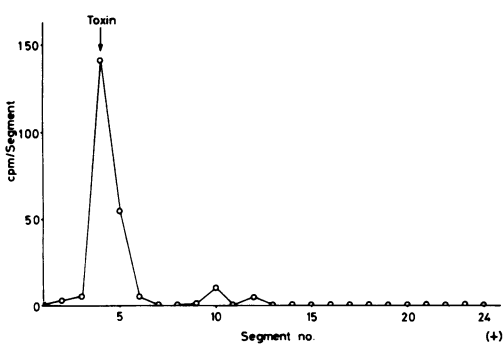

FIG. 13. *Polyacrylamide gel electrophoresis of purified* ^{65}*Zn-toxin in pH 8.0 gel.*

that the metal is important not only for manifestation of the activity but also for maintenance of the integrity of the molecule (2, 17, 21, 23). The removal of zinc from alpha toxin did not appreciably decrease the molecular size. The result of polyacrylamide gel electrophoresis

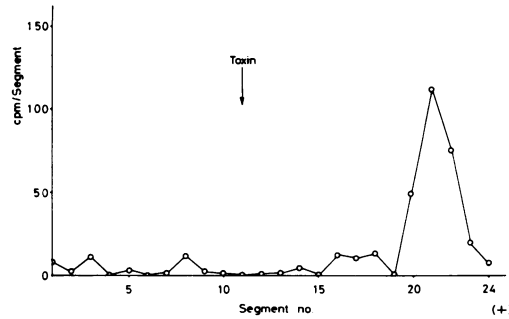

FIG. 14. *Polyacrylamide gel electrophoresis of purified* ^{65}*Zn-toxin in pH 9.4 gel.*

at pH 9.4 suggests that the toxin may have a little enzymatic activity without zinc. However, further confirmation is needed on this point.

The foregoing results show clearly that *C. perfringens* produces the inactive protein molecule of alpha toxin irrespective of the presence or absence of zinc in the medium and releases it immediately into the medium. The protein combines rapidly with the metal in the medium to form the active and stable structure of the toxin.

It has not been clarified whether the structure of the inactive toxin protein produced in DM was similar to that of alpha toxin deprived of zinc with a chelating agent. The low activity of the toxin detected at early stages of growth in DM may have been due to the toxin protein devoid of zinc. However, further confirmation is needed to rule out the possibility of contamination with a trace amount of the metal.

ACKNOWLEDGMENT

The authors wish to thank Genji Sakaguchi, College of Agriculture, University of Osąka Prefecture, for his help in preparing the manuscript.

LITERATURE CITED

1. Adams, M. H., and E. D. Hendee. 1945. Methods for the production of the alpha and theta toxins of *Clostridium welchii*. J. Immunol. **51**:249-256.
2. Coleman, P. L., I. Iweibo, and H. Weiner. 1972. Role of zinc in horse liver alcohol dehydrogenase. Influence on structure and conformational changes. Biochemistry **11**:1010-1018.
3. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. **121**:404-427.
4. Ispolatovskaya, M. V. 1970. Role of metals in the activity of lecithinase of *Cl. perfringens*. Biokhimiya **35**:434-439.
5. Ispolatovskaya, M. V. 1971. Purification and some properties of lecithinase from *Clostridium perfringens*. Voprosy Meditsinskoi Khimii **17**:137-141.
6. Ispolatovskaya, M. V. 1971. Type A *Clostridium perfringens* toxin, p. 109-158. *In* S. Kadis, T. C. Montie and S. J. Ajl (ed.), Microbial toxins, vol. II A. Academic Press Inc., New York.
7. Logan, M. A., A. A. Tytell, I. Danielson, and A. Griner.

1945. Production of *Clostridium perfringens* alpha toxin. J. Immunol. **51**:317-328.
8. Macfarlane, M. G., and B. C. J. G. Knight. 1941. The biochemistry of bacterial toxins. 1. The lecithinase activity of *Clostridium welchii* toxins. Biochem. J. **35**:884-902.
9. Moskowitz, M. 1960. Protection against *Clostridium perfringens* toxin by metal-binding compounds, p. 205-208. *In* M. J. Seven and L. A. Johnson (ed.), Metal-binding in medicine, J. B. Lippincott Co., Philadelphia.
10. Moskowitz, M., M. W. Deverell, and R. McKinney. 1956. Protection against *Clostridium perfringens* type A toxin by a metal chelating compound. Science **123**:1077-1078.
11. Murata, R., S. Soda, A. Yamamoto, and A. Ito. 1968. Further investigations on the influence of inorganic cations on growth and toxin production by *Clostridium perfringens* PB6K. Jap. J. Med. Sci. Biol. **21**:55-70.
12. Murata, R., S. Soda, A. Yamamoto, H. Sato, and A. Ito. 1969. The effect of zinc on the production of various toxins of *Clostridium perfringens*. Jap. J. Med. Sci. Biol. **22**:133-148.
13. Murata, R., A. Yamamoto, S. Soda, and A. Ito. 1965. Nutritional requirements of *Clostridium perfringens* PB6K for alpha toxin production. Jap. J. Med. Sci. Biol. **18**:189-202.
14. Ottolenghi, A. C. 1965. Phospholipase C from *Bacillus cereus*. A zinc-requiring metalloenzyme. Biochim. Biophys. Acta **106**:510-518.
15. Ouchterlony, Ö. 1953. Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. Acta Pathol. Microbiol. Scand. **32**:231-240.
16. Pappenheimer, A. M., Jr., and E. Shaskan. 1944. Effect of iron on carbohydrate metabolism of *Clostridium welchii*. J. Biochem. **155**:265-275.
17. Reynolds, J. A., and M. J. Schlesinger. 1969. Alterations in the structure and function of *Escherichia coli* alkaline phosphatase due to Zn^{2+} binding. Biochemistry **8**:588-593.
18. Sato, H., S. Kameyama, and R. Murata. 1972. Immunogenicity of highly purified α-toxoid of *Clostridium perfringens*. Jap. J. Med. Sci. Biol. **25**:53-56.
19. Shemanova, G. F., K. L. Shakhanina, E. V. Vlasova, O. K. Borisova, and R. D. Bakriova. 1968. The study of the formation of lecithinase of *Clostridium perfringens* type A. Biokhimiya **33**:561-569.
20. Soda, S., H. Sato, and R. Murata. 1969. The effect of calcium on the production of various toxins of *Clostridium perfringens*. Jap. J. Med. Sci. Biol. **22**:175-179.
21. Ulmer, D. D., and B. L. Vallee. 1971. Structure and function of metalloenzymes, p. 187-218. *In* R. F. Gould (ed.), Bioinorganic chemistry. American Chemical Society, Washington, D.C.
22. van Heyningen, W. E. 1948. The biochemistry of the gas gangrene toxins. III. Development of a medium suitable for the large scale production of the toxins of *Clostridium welchii* type A. Biochem. J. **42**:127-130.
23. Vallee, B. L., and Wacker, W. E. C. 1970. The proteins. Composition, structure, and function. Metalloproteins, vol. V. Academic Press Inc., New York.
24. Williams, D. E., and R. A. Reisfeld. 1964. Disc electrophoresis in polyacrylamide gels: extension to new conditions of pH and buffer. Ann. N. Y. Acad. Sci. **121**:373-381.
25. Yamamoto, A., A. Ito, R. Murata, N. Uematsu, K. Nagai, and K. Minomo. 1970. Hyperimmunization of horses with alpha toxoid of *Clostridium perfringens*. Jap. J. Med. Sci. Biol. **23**:111-115.