Time of Enterotoxin Formation and Release During Sporulation of *Clostridium perfringens* Type A

CHARLES L. DUNCAN

Food Research Institute and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Clostridium perfringens enterotoxin was detected intracellularly about 3 hr after the inoculation of vegetative cells into sporulation medium. The subsequent increase in intracellular enterotoxin concentration roughly paralleled but followed by 2.5 to 5 hr the increase in number of heat-resistant spores. The increase in biologically active toxin coincided with the increase in enterotoxin antigen. Enterotoxin was released from the sporangium with its lysis, concomitantly with the mature spore release.

A number of lethal proteins are produced by the various types of *Clostridium perfringens*. These accumulate as exotoxins in the culture filtrate during vegetative growth of these organisms. *C. perfringens* type A enterotoxin, which is active in human food poisoning, is unique in that it is produced only during sporulation and not during vegetative growth of this organism (4, 5). By using mutants with an altered ability to sporulate, we recently demonstrated that a direct relationship exists between enterotoxin synthesis and spore formation and that the enterotoxin is a sporulation-specific gene product (5). Previous results also indicated that biologically active intracellular enterotoxin could be detected as early as 3 hr after inoculation of vegetative cells into sporulation medium, whereas extracellular enterotoxin was present in 24-hr but not 5-hr culture filtrates (4). The present study was undertaken to determine the time of enterotoxin formation and release during sporulation. The results permit comparison of the time of enterotoxin production with that of other known sporulation-specific biochemical events and establish the release mechanism for perfringens enterotoxin.

MATERIALS AND METHODS

Cultural procedures. *C. perfringens* type A, strain NCTC 8798 was inoculated from cooked meat medium (Difco) into 135 ml of fluid thioglycolate medium (Baltimore Biological Laboratories) and incubated at 37 C for 12 hr. The entire culture was then inoculated into 13.5 liters of DS (Duncan Strong) sporulation medium (3) at 37 C and an initial

pH of 7.2. The headspace of the container was flushed with nitrogen, and anaerobic conditions were maintained by providing a water seal for gas evolution. A separate siphon outlet was used for sample collection. A magnetic stirrer was used to provide constant agitation of the culture.

Sampling. A sample volume of 1 liter was collected hourly through the first 18 hr of incubation and then at 20, 24, 36, and 38 hr. Duplicate cultures were employed so that samples could be collected for 1 through 12 hr from one culture and for 11 through 48 hr from the other culture. This procedure provided for overlapping sampling at 11 and 12 hr. Concentrated culture filtrates and extracts of cells harvested from each sample were prepared as previously described (4). Unless indicated otherwise, the data presented are the averages of results from two different complete experiments.

Protein determination. Protein concentrations of cell extracts were determined by the method of Lowry et al. (10).

Enterotoxin detection. Qualitative detection of enterotoxin antigen in crude preparations was accomplished by immunodiffusion with two-dimensional double diffusion in agar gel as described previously (13). Specific antiserum prepared against purified enterotoxin was used for immunodiffusion.

Quantitation of biologically active enterotoxin in erythemal units was based on its ability to produce erythema when injected intradermally into depilated guinea pigs as described previously (13). Samples were injected intradermally in a volume of 0.05 ml. Areas of erythema with diameters between 0.5 and 1.2 cm were measured after 18 to 24 hr. Diameters within these limits were directly proportional to the amount of enterotoxin in the sample injected. A unit of erythemal activity was defined previously as that amount of enterotoxin producing an area of erythema 0.8 cm in diameter under the conditions of

the experiment (13). The activity of an unknown sample was always compared to a standard injected into the same guinea pigs. All samples were injected in duplicate on the same guinea pig, and results from a minimum of two different pigs were averaged. The erythemal activity of samples containing enterotoxin was neutralized by immune serum prepared against purified enterotoxin but not by normal serum. Neutralized samples were always used as controls in each guinea pig. The usefulness of this skin test as an accurate, sensitive assay for biologically active enterotoxin has been documented previously (8, 13) and is confirmed by the data presented in Fig. 2 of the present communication. The standard for the assay was a crude toxin preparation obtained from extracts of 6-hr-old sporulating cells. Erythemal units were converted to micrograms of enterotoxin as determined from a standard curve relating erythemal units to micrograms of highly purified enterotoxin protein that had been obtained from 8-hr-old sporulating cells.

Determination of enterotoxin antigen was by electroimmunodiffusion. This technique has been used for accurate and sensitive determination of a variety of proteins including immunoglobulins and albumin (9), and type A botulinum toxin (11). The reliability of this technique for this particular antigen was previously determined (2).

Electroimmunodiffusion was performed in 0.6% agarose (Fisher Scientific Co.) slabs containing specific antienterotoxin serum at a final dilution of 1:100. The agarose was prepared with pH 8.5, 0.025 ionic strength barbital buffer. The buffer in the electrophoresis reservoir was similar except the ionic strength was increased to 0.1. Sample wells at the cathode end of the slide were filled with 10 μliters of appropriately diluted cell extract preparations immediately prior to electrophoresis. A current of 1 ma/cm of agarose slab was used with an electrophoresis time of 6 hr. The concentration of enterotoxin protein in the extracts of different age cells was determined by relating the distance the tip of the cone of antigen-antienterotoxin precipitate migrated, as measured from the anodal edge of the sample well, to that of a standard purified enterotoxin preparation at a known concentration.

Population monitoring. Cell multiplication as reflected by turbidity increase was measured in Klett units using a no. 66 filter. Enumeration of heat-resistant heat-shocked spores or total viable cells was by described techniques (1). The percent refractile spores was determined by phase-contrast microscopy.

RESULTS AND DISCUSSION

Growth and sporulation. Immediately following inoculation, the sporulation medium culture contained approximately 10^5 viable vegetative cells/ml. Following a short lag period (Fig. 1) the cell population increased for 1.0 to 1.5 hr, as indicated by a turbidity increase and as observed by phase-contrast microscopy. The turbidity reached a maximum of only 30

Klett units at 3 hr. Between 3 and 5 hr the turbidity of the culture decreased sharply. The decrease in culture turbidity occurred fairly consistently, and may have resulted from intracellular protein turnover associated with the synthesis of spore proteins. Such a decrease in culture density has been reported in sporulating cultures of certain other anaerobes (6) but is rarely seen in cultures of sporulating aerobes. The subsequent, less dramatic decreases in turbidity shown in Fig. 1 did not occur consistently.

Heat-resistant spores were first detected at 3 hr. The heat-resistant spore population increased sharply through the fifth hour followed by a gradual increase to the maximum spore level at about 10 hr. The spore level remained fairly constant through 48 hr of incubation.

The peculiar increase in turbidity of the culture at 12 hr was due to the formation of new vegetative cells which did not appear to sporulate. The percentage of unlysed cells containing refractile spores decreased from approximately 30% at 10 hr to 3% at 20 hr. The explanation for the vegetative cell increase is not known.

Formation of biologically active enterotoxin. Biologically active intracellular enterotoxin (1.76 μg/mg of cell protein) was first detected at 2 hr (Fig. 1). However, the presence of activity at 2 hr was variable in different experiments. At 3 hr the concentration of intracellular enterotoxin began to increase rapidly and reached a maximum at 11 hr. At 11 hr, the enterotoxin protein accounted for approximately 11% (112 μg of toxin/mg of protein) of the total cell extract protein. Since the percentage of cells containing refractile spores was about 30% at 11 hr, a considerable portion of the protein was vegetative cell protein. The contribution of enterotoxin protein to total protein in the sporulating cell apparently is considerably greater than 11%. The exact percentage is undertain due to lack of synchrony of the sporulating fraction of the cell population and variation in the sporulation frequency in different culture preparations. The increase in enterotoxin concentration roughly paralleled but followed by 2.5 to 5 hr the increase in heat-resistant spores. Following the production of a maximum level of enterotoxin at 11 hr, the intracellular enterotoxin concentration declined to a level of only 2.33 μg/mg of cell protein at 48 hr. Small increases of enterotoxin activity occurred at 13 and 17 hr. These were reproducible and not artifacts. The increases in activity may have resulted from lack of synchrony of the sporulating population or represent discrete populations of
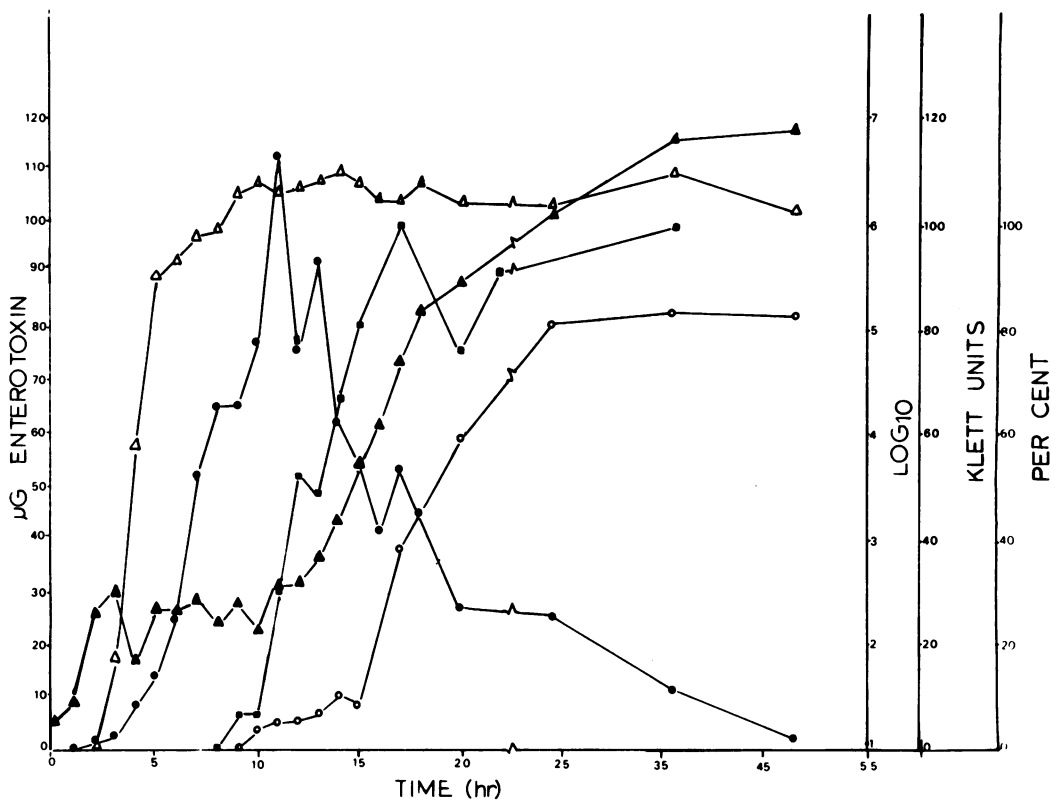
FIG. 1. *Time course of intracellular enterotoxin formation and release during sporulation of Clostridium perfringens type A. Symbols: ●, micrograms of biologically active enterotoxin/milligram of cell extract protein; ○, micrograms of biologically active enterotoxin/milliliter of culture filtrate; △, log_{10} heat-resistant spores/milliliter; ▲, Klett units; ■, percent of refractile spores that are free from sporangia. The erythemal activity assay was used for measuring biologically active toxin.*

cells in which the sporulation sequence began at a later time. The increased activity could not be related to any significant increase in the level of heat-resistant spores. However, this would not preclude an increase in toxin due to asynchronous formation of immature heat-sensitive spores. Mutants blocked at stages III, IV, and V of sporulation were previously shown to produce toxin without the formation of mature heat-resistant spores (5).

Extracellular enterotoxin was first detected in the culture filtrate at 10 hr and coincided with the first release (at 9 to 10 hr) of free mature spores from the sporangia. The concentration of extracellular enterotoxin increased and paralleled the release of mature spores during the next 14 hr. It leveled off at about 24 hr at a maximum concentration of 82 to 84 μg/ml of culture filtrate. Thus, enterotoxin is released from the sporangium with its lysis, concomitantly with the mature spore release.

At about 12 hr, a second increase in the number of vegetative cells, as indicated by an increase in turbidity and as observed by phase-contrast microscopy, occurred. The duration of this increase was 6 hr, followed by a gradual increase in turbidity to a final level of 117 Klett units at 48 hr. This second turbidity increase did not result in a subsequent appreciable increase in spore concentration. However, the increase in vegetative cell protein in effect resulted in a diluting of the enterotoxin protein of unlysed sporulated cells.

If asynchronous sporulation or discrete populations of cells result in the increases in intracellular enterotoxin at 13 and 17 hr, as described above, one might normally expect an increase in specific activity of the intracellular toxin above that present at 11 hr (Fig. 1). This increase apparently does not occur due to the diluting effect of increasing levels of vegetative cell protein in the crude cell extracts and the release of enterotoxin with sporangial lysis.

Formation of enterotoxin antigen. The appearance and increase in concentration of enterotoxin antigen coincided with that of biolog-

ically active toxin (Fig. 2). The enterotoxin could always be detected at 3 hr by using either biological activity or electroimmunodiffusion assays. By comparison, the antigen, as indicated by a precipitin line of identity with known enterotoxin, could be detected at 4 hr but not 3 hr when less sensitive immunodiffusion techniques employing diffusion from wells on agar-coated slides were used (Fig. 3). Neither the enterotoxin antigen nor biologically active toxin was present in cells at 1 hr or in cells obtained from fluid thioglycolate growth medium immediately before inoculation into sporulation medium. The increase in intracellular enterotoxin with time apparently is due to the formation of new enterotoxin antigen.

Most studies of biochemical events occurring during sporulation have been concerned with those events that precede the appearance of mature heat-resistant spores. Intracellular accumulation of perfringens enterotoxin differs from most of the biochemical events that have been studied in *Bacillus* species such as the
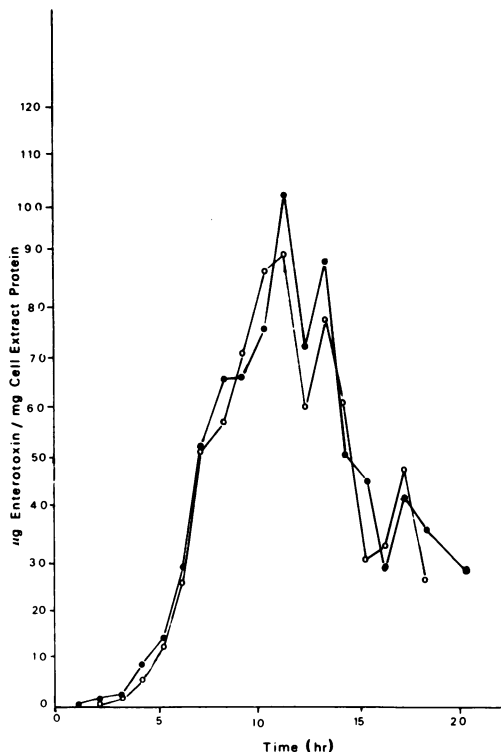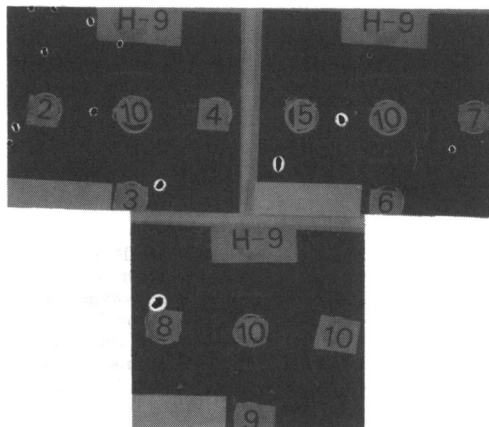

FIG. 3. *Immunodiffusion of crude extracts of different age sporulating cells of C. perfringens type A. The numbers in outside wells refer to age (hr) of the sporulating cells from which extracts were prepared. The center well contained specific antienterotoxin. The well labeled H-9 contained control enterotoxin.*

formation of proteolytic enzymes, antibiotics, dipicolinic acid, and sulfolactic acid (7, 12, 14) in that pronounced toxin accumulation follows rather than precedes the appearance of heat-resistant spores. Enterotoxin accumulation may be only a matter of overproduction of a protein that is required for sporulation or for some event concerned with sporangial lysis. Alternatively, the toxin may be a waste product of the sporulation process. If the toxin is required for sporulation, it would be expected that an increase in the intracellular level of toxin would precede, not follow, the increase in heat-resistant spores. Previous studies in our laboratory showed that Sp^- mutants blocked at stages III, IV, or V of sporulation produced enterotoxin whereas those blocked at stage 0 did not (5). This would indicate that synthesis of the enterotoxin does begin by at least stage III of sporulation and continues to accumulate in the sporangium until lysis of the sporangium releases both the free spore and enterotoxin. The previous failure to detect gene products serologically homologous to enterotoxin in Sp_0^- Ent^- mutants indicated that transcription of relevant sporulation genes is essential for synthesis of enterotoxin protein (5). Further studies are underway to clarify the relationship of this human food poisoning toxin to morphogenesis of sporulating *C. perfringens* cells.

FIG. 2. *Comparison of the time course of intracellular enterotoxin antigen formation as measured by electroimmunodiffusion and biologically active enterotoxin as measured by its erythemal activity. Symbols: O, enterotoxin concentration measured by electroimmunodiffusion; ●, concentration of biologically active enterotoxin.*

ACKNOWLEDGMENTS

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