The methods currently used for the enumeration of *Clostridium perfringens* in food are often inadequate because of the rapid loss of viability of this organism when the sample is frozen or refrigerated. A method for estimating the presence of *C. perfringens* in food which utilizes the hemolytic and lecithinase activities of alpha toxin was developed. The hemolytic activity was measured in hemolysin indicator plates. Lecithinase activity of the extract was determined by the lecithovitellin test. Of 34 strains of *C. perfringens* associated with foodborne disease outbreaks, 32 produced sufficient alpha toxin in roast beef with gravy and in chicken broth to permit a reliable estimate of growth in these foods. Alpha toxin was extracted from food with 0.4 M saline buffered (at pH 8.0) with 0.05 M N-2-hydroxyethyipipperazine-N'-2-ethanesulfonic acid and concentrated by dialysis against 30% polyethylene glycol. A detectable quantity of alpha toxin was produced by approximately 10⁴ *C. perfringens* cells per g of substrate, and the amount increased in proportion to the cell population. Results obtained with food samples responsible for gastroenteritis in humans indicate that a correlation can be made between the amount of alpha toxin present and previous growth of *C. perfringens* in food regardless of whether the organisms are viable when the examination is performed.

*Clostridium perfringens* has become increasingly recognized in the United States as a causative agent of food poisoning (10). The illness occurs upon ingestion of a large number of viable *C. perfringens* (3, 8), but the factors responsible for illness have not been elucidated. Because this organism is often present in small numbers in certain foods (14), quantitative cultural methods are used to establish that *C. perfringens* is responsible for a foodborne disease outbreak. A number of such methods have been proposed for this purpose (1, 4, 9; S. A. Shahidi and A. R. Ferguson, Bacteriol. Proc., p. 1, 1969). A rapid loss in the viability of *C. perfringens* occurs, however, when food samples are frozen or held under prolonged refrigeration (5). Since only 1% of the vegetative cells survive frozen storage for 48 hr (2), a considerable reduction in the viable count may be expected with these samples. In a preliminary report, we suggested the possibility of utilizing the alpha toxin of *C. perfringens* as an index of growth of this organism in food (S. M. Harmon, Bacteriol. Proc., p. 81, 1969). Although many foodborne disease strains have been reported to produce a small quantity of alpha toxin (6, 7, 8, 15), our results indicated that most strains associated with foodborne disease outbreaks in the United States produced an appreciable quantity of alpha toxin in roast beef and chicken broth. Growth of large numbers of *C. perfringens* in these foods could be estimated by extraction and determination of the amount of alpha toxin present. The results obtained with the proposed method in the examination of foods associated with foodborne disease outbreaks are presented.

**MATERIALS AND METHODS**

* Cultures. All *C. perfringens* strains used in this investigation were isolated from food or feces associated with foodborne disease outbreaks.

The source of these cultures and their antigenic relationship to Hobbs heat-resistant strains are as follows. Strains NCTC 8797 (Hobbs 1), NCTC 8238 (Hobbs 2), NCTC 8798 (Hobbs 9), and NCTC 10,240 (Hobbs 13) were obtained from the National Collection of Type Cultures, London, England; strains T-65 (Hobbs 10) and 79393 were obtained from D. H. Strong, University of Wisconsin, Madison, Wis.; strains NCDC 1861, NCDC 2080, NCDC 2258 (Hobbs 10), NCDC 2078, NCDC 3032 (Hobbs 12), NCDC 3131, and NCDC 3708 were obtained from V. R. Dowell, Center for Disease Control, Atlanta, Ga.; strains IU-686, IU-2825 (Hobbs 7), IU-3344, and IU-1505 were obtained from L. S. McClung, Indiana University, Bloomington, Ind.; strains B-1
(Hobbs 9), B-11, A-29, A-38 (Hobbs 9), 6867 (Hobbs 6), S-34, S-40, S-80 (Hobbs 4), and S-88 (Hobbs 10) were obtained from H. E. Hall, Food and Drug Administration, Cincinnati Laboratories, Cincinnati, Ohio; and strains FD-1, FD-2, FD-20, FD-21, FD-22, FD-23 (Hobbs 1), FD-25 (Hobbs 10), and FD-26 were isolated in our laboratory from food associated with food poisoning outbreaks. The cultural and antigenic characteristics of many of these strains were previously reported (6, 7, 8, 15). The cultures of C. novyi type A, C. septicum, C. bifermentans, C. hemolyticum, and Bacillus cereus were from the stock culture collection of the Food and Drug Administration.

**Viable counts.** A 25-g portion of food was blended with 225 ml of 0.1% peptone water, diluted serially in the same diluent, and plated in laboratory prepared sulfite-polymyxin-sulfadiazine (SPS) agar (1). After solidification, this agar was overlaid with an additional 5 ml of SPS agar and incubated for 24 hr in a Case Anaero-jar (Case Laboratories Inc., Chicago, Ill.) under an atmosphere of nitrogen. Black colonies were counted, and 10 were picked to motility-nitrate medium for confirmation as C. perfringens.

**Alpha toxin production in foods.** To determine the quantities of alpha toxin produced by the 34 food poisoning strains, 100 g of canned roast beef with gravy or canned chicken broth was placed in a glass jar, heated for 15 min to expel the oxygen, and inoculated with 1 ml of a 16-hr culture of the test strain grown in Noyes veal broth (1). The food was incubated for 4 hr at 35 C and tested for alpha toxin production.

**Alpha toxin extraction.** Alpha toxin was extracted by homogenizing a 25-g portion of the food with 100 ml of 0.4 M NaCl buffered (at pH 8.0) with 0.05 M N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid (HEPES) for 2 min at high speed in a Waring Blender or a Sorval Omni-Mixer. The homogenate was centrifuged for 20 min at 28,000 x g at 4 C, and the supernatant was decanted through a fine-wire screen to remove the fat. The sediment was discarded. The supernatant was then sterilized by filtration through a Seitz filter, and the sterile extract was concentrated to between 5 and 10 ml by dialysis against 30% polyethylene glycol (PEG; molecular weight 20,000) at 4 C. The dialysis tubing was rinsed in tap water to remove the PEG, and the concentrated extract was collected. If the supernatant contained excessive fat as indicated by turbidity, it was extracted with ethyl ether to facilitate filtration. To do this, it was mixed with 30 ml of ethyl ether and centrifuged at 18,000 x g for 15 min at 4 C, and the aqueous phase was removed by siphoning into a vacuum flask. The clarified extract was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) with a prefILTER pad, concentrated to a minimal volume by dialysis against PEG at 4 C, and dialyzed against 0.85% saline for 2 hr to rehydrate the alpha toxin. The extract was collected and the dialysis tubing was rinsed out with additional saline to bring the final volume to between 5 and 10 ml. Liquid samples such as chicken broth and beef juice were centrifuged and sterilized by filtration, but the extraction and concentration steps were omitted.

**Quantitation of alpha toxin.** The hemolytic activity of alpha toxin was measured by means of the hemolysis indicator (HI) plate described by Noyes and Earling (11), a method similar to that proposed by Sheldon, Moskwitz, and Deverell (13). The medium for the HI plates consisted of sterile, purified 1.5% agar (Difco) in 0.85% NaCl to which washed, human red blood cells were added while the medium was at 50 C to give a 5% final concentration. The plates containing 7 ml of medium were dried overnight at room temperature and stored at 4 C. Just prior to use, test wells were cut in the agar by applying vacuum to a thin-walled stainless-steel tube (3-mm diameter, no. 9 surgical tubing) plunged into the agar. The wells were spaced 3 cm apart and 2 cm from the edge of the plate with a template.

Peripheral wells of duplicate HI plates were filled with the undiluted extract and eight twofold dilutions of extract with a fine-tipped Pasteur pipette. To determine whether the hemolysis caused by the extract was due to alpha toxin, a portion of the 1:2 dilution of the extract was mixed with C. perfringens alpha antitoxin (Wellcome Laboratories, London, England) and with C. perfringens type A diagnostic antiserum containing alpha antitoxin (Burroughs Wellcome & Co., Tuckahoe, N.Y.) and placed in the two center wells. Alpha antitoxin was added to give a final concentration of 1:50 (4.5 to 7.1 units per ml.)

The plates were incubated for 24 hr at 35 C and examined for hemolytic zones surrounding the wells. A 1-mm zone of hemolysis was considered significant and was used as the criterion of hemolytic activity throughout the study. The activity of alpha toxin was recorded as the highest dilution which produced a 1-mm zone of hemolysis in HI plates. Figure 1 shows a typical titration of an extract containing alpha toxin. The antitoxin preparations were tested for their specificity with culture filtrates of C. perfringens and five other organisms. Neither preparation inhibited hemolysins or lecithinases produced by C. novyi type A, C. septicum, C. hemolyticum, or B. cereus. Although the hemolytic activity of C. bifermentans filtrates was inhibited by alpha antitoxin in the HI plate, their lecithinase activity was readily distinguished from that of alpha toxin by a negative lecithovitellin (LV) test.

The lecithinase activity of alpha toxin was determined by the LV test (12). The LV solution was prepared by mixing an egg yolk in 250 ml of physiological saline (pH 7.0), centrifuging at 15,000 x g for 15 min, and sterilizing the supernatant by Seitz filtration. The LV solution could be stored for 7 to 10 days at 4 C. The test was performed by adding 0.5 ml of the LV solution to an equal volume of serial twofold dilutions of the extract made in physiological saline (pH 7.0). The tubes were incubated overnight at 37 C and examined for turbidity or flocculation, which indicated lecithinase activity. Suppression of the reaction by alpha antitoxin or by C. perfringens type A diagnostic antiserum was considered evidence of the presence of alpha toxin in the extract. Since the lici-
thinase of *C. bifermentans* was not active above pH 5.0, the LV test was negative with filtrates of this organism.

**Correlation of viable count with alpha toxin.** The relationship between viable count and the amount of alpha toxin produced was determined with six representative strains of *C. perfringens* in chicken broth. Two hundred grams of heated chicken broth was inoculated with approximately $5 \times 10^5$ washed cells per milliliter of the test strain and incubated at 35 C. After 2 hr of incubation, samples were removed at 30-min intervals, and the viable count and the alpha toxin production were determined.

**Effect of frozen storage or cell viability and alpha toxin.** The effect of frozen storage on the viable count and alpha toxin was determined with six strains of *C. perfringens* in roast beef and chicken broth. Each food was inoculated with approximately $10^6$ cells per g and incubated for 4 hr at 35 C. The viable count and alpha toxin were determined after incubation and after 17 days of storage at -20 C.

**Examination of samples from food poisoning outbreaks.** Samples of food associated with foodborne disease outbreaks were examined for viable *C. perfringens* and alpha toxin by the procedures described previously. The strains of *C. perfringens* isolated from these samples were serotyped by the slide agglutination technique with Hobbs antisera and antisera prepared against other *C. perfringens* strains obtained from the Center for Disease Control, Atlanta, Ga.

**RESULTS**

**Alpha toxin production in foods.** The amount of alpha toxin produced in canned roast beef with gravy and in canned chicken broth inoculated with *C. perfringens* depended upon the alpha toxin-producing ability of the strain tested (Table 1). A majority of the 34 strains produced a substantial quantity of alpha toxin, and, on the basis of the amount produced, the strains tested were divided into high-, moderate-, and low-level-producing groups.

**Extraction and quantitation of alpha toxin.** The adequacy of recovery of added alpha toxin varied slightly depending on the type of food. When a culture filtrate containing a known titer of alpha toxin was added to roast beef or chicken, it was found that concentration of the extract to a final volume of between 5 and 10 ml was sufficient to compensate for any losses due to the extraction procedure. The most suitable conditions for alpha toxin extraction were determined by varying the concentrations of NaCl and pH of the extracting diluent. NaCl concentrations from 0.1 to 1.0 M and pH values from 4.0 to 9.0 were tested. The highest recovery was obtained with 0.4 M NaCl buffered (at pH 8.0) with 0.05 M HEPES.

Results obtained with food extracts indicate that the HI plate is a sensitive and reliable procedure for measuring *C. perfringens* alpha toxin. The LV test was less sensitive than the HI plate, having an end point of activity at one or two dilutions lower. Some extracts from canned chicken broth containing alpha toxin failed to give a positive LV test. However, these extracts exhibited hemolytic activity in the HI plate and were neutralized by *C. perfringens* alpha antitoxin, indicating the presence of alpha toxin.

**Correlation of viable count with alpha toxin.** The relationship between the viable count and the amount of alpha toxin produced in food was determined with six *C. perfringens* strains in chicken broth. Three strains producing heat-sensitive spores (FD-1, NCDC 1861, and S-34,
isolated in the United States) and three strains producing heat-resistant spores [NCTC 8797, NCTC 8238, and NCTC 8798, isolated in England by Hobbs et al. (8)] were employed.

The correlation between growth of *C. perfringens* and the amount of alpha toxin detected is presented in Table 2. These data represent the average of the viable counts obtained with the six strains and the corresponding titers of alpha toxin measured by the HI plate and LV test. Although some variation in the alpha toxin-producing ability was noted with individual strains, a close relationship was observed between the viable count and the amount of alpha toxin produced. Detectable alpha toxin was produced by approximately $10^6$ cells per ml, and the amount increased in proportion to the cell population.

**TABLE 2. Correlation between the viable count of *Clostridium perfringens* and the amount of alpha toxin detected in filtrates of chicken broth**

| Viable count per gram<sup>a</sup> | Alpha toxin |          |         |
|----------------------------------|-------------|----------|---------|
|                                  | Hemolysin   | Lecithovitellin | test    |
|                                  | indicator plate |        |         |
| 1.2                              | Undiluted   | No reaction |         |
| 2.5                              | 1:2<sup>b</sup> | Undiluted |         |
| 6.5                              | 1:4         | 1:2<sup>c</sup> |         |
| 9.5                              | 1:8         | 1:4      |         |
| 25                               | 1:16        | 1:8      |         |
| 55                               | 1:32        | 1:16     |         |
| 80                               | 1:64        | 1:32     |         |
| 150                              | 1:128       | 1:128    |         |
| 210                              | 1:256       | 1:256    |         |

<sup>a</sup> Average of viable counts obtained with six strains. Values to be multiplied by 10<sup>4</sup>.  
<sup>b</sup> Dilution of filtrate producing a 1-mm zone of hemolysis.  
<sup>c</sup> Highest positive dilution.

**Effect of frozen storage on cell viability and alpha toxin.** The effect of frozen storage on the viability of *C. perfringens* and the stability of alpha toxin is presented in Table 3. These data show at least a 1,000-fold decrease in the viable count of the organism after frozen storage for 17 days at $-20\,^\circ$C, whereas the alpha toxin titer remained unchanged. Similar data were obtained with strains S-34, NCDC 1861, and NCTC 8238. The titer of alpha toxin remained unchanged in some food samples stored for 3 months at $-20\,^\circ$C.

**Estimation of previous growth in food samples.** Several samples of food from foodborne disease outbreaks were examined for alpha toxin and for viable *C. perfringens* (Table 4). An estimation of previous growth of *C. perfringens* in these foods was made from the amount of alpha toxin detected by the HI plate. The data presented in Table 2 were used for this purpose. Estimates obtained in this manner were compared with the viable counts obtained by other laboratories soon after the outbreak occurred. This comparison indicated to us that the estimation of previous growth obtained by quantitation of the alpha toxin present in the food agreed in most cases with the viable count obtained before the samples were frozen. The deleterious effect of freezing on the viability of *C. perfringens* in these samples is shown by comparing the count obtained prior to freezing with that made in our laboratory.

A detectable amount of alpha toxin was demonstrated in all samples and could be correlated with previous growth of *C. perfringens* in a majority of samples. The discrepancy between original plate counts obtained in other laboratories and the estimation of previous growth by the HI plate with two of the samples (roast beef, beef hash) suggests that a reduction occurred in the viability of *C. perfringens* prior to the original plate count.

**TABLE 3. Effect of frozen storage on the viability of *Clostridium perfringens* and the stability of alpha toxin**

| Substrate  | Strain | Viable count per gram | Titer of alpha toxin |
|------------|--------|-----------------------|----------------------|
|            |        | Before freezing<sup>a</sup> | After frozen storage<sup>b</sup> | Before freezing | After frozen storage<sup>d</sup> |
| Roast beef | FD-1   | 160 20 | 128<sup>d</sup> | 128          |
|           | NCTC 8797 | 147 3 | 128 | 128          |
|           | NCTC 8798 | 162 1 | 64  | 64           |
| Chicken broth | FD-1 | 180 10 | 256  | 256  |
|             | NCTC 8797 | 140 6 | 128  | 128  |
|             | NCTC 8798 | 163 1 | 64  | 128          |

<sup>a</sup> Values to be multiplied by 10<sup>4</sup>.  
<sup>b</sup> Values to be multiplied by 10<sup>4</sup>.  
<sup>c</sup> Stored for 17 days at $-20\,^\circ$C.  
<sup>d</sup> Reciprocal titer of extract producing a 1-mm zone of hemolysis in hemolysin indicator plates.
### Table 4. Comparison of viable count with an estimation of the previous growth based on the quantity of alpha toxin detected in food samples from foodborne disease outbreaks

| Food                  | Viable count per gram | Previous growth per gram | Serotype of isolates |
|-----------------------|-----------------------|--------------------------|----------------------|
|                       | Original count<sup>a</sup> | After frozen storage<sup>b</sup> |                       |
| Roast beef (au jus)   | $3.0 \times 10^7$     | $2.7 \times 10^6$        | PS89, Hobbs 13       |
| Beef, prime ribs      | $5.5 \times 10^4$     | $1.2 \times 10^6$        | PS89                 |
| Roast beef            | $1.4 \times 10^3$     | $5.0 \times 10^4$        | Hobbs 1, PS 41       |
| Roast beef            | $6.3 \times 10^7$     | $1.0 \times 10^3$        | Hobbs 10             |
| Barbecue beef hash    | $9.0 \times 10^4$     | $1.5 \times 10^3$        | Hobbs 10             |
| Beef hash             |                       | 10                       | Nontypable           |

<sup>a</sup> Viable count determined in other laboratories prior to shipment to FDA.
<sup>b</sup> Viable count determined by FDA laboratory after frozen storage.
<sup>c</sup> Estimation of previous growth per gram of food based on the quantity of alpha toxin detected.
<sup>d</sup> Agglutinated by Hobbs antisera or *Clostridium perfringens* antisera obtained from the Center for Disease Control.
<sup>e</sup> No count available.

## DISCUSSION

The experimental data presented in Table 3 show that alpha toxin titers are not affected by freezing, whereas the viable counts are markedly decreased. Moreover, the results obtained with a limited number of foods associated with *C. perfringens* foodborne disease outbreaks indicate that the alpha toxin produced by this organism can be recovered from the food after frozen storage. The quantity of alpha toxin detected may be utilized to estimate the extent of previous growth of the organism by using the relationship between viable count and the amount of alpha toxin (Table 2).

Although the foodborne disease strains of *C. perfringens* which were tested varied somewhat in their ability to produce alpha toxin (Table 1), most of the strains produced a substantial quantity in roast beef and chicken broth. The antigenic relationship of the strains to Hobbs heat-resistant strains appeared to have little bearing on the ability to produce alpha toxin in these substrates. Our data indicate that alpha toxin production is variable but that the range of alpha toxin produced by the strains corresponding to Hobbs serotypes was approximately the same as that of the others. The results obtained with incriminated foods (Table 4) which contained different serotypes support this conclusion.

Even though our results indicate that a relationship exists between the extent of growth of the organism and the amount of alpha toxin produced in food, several factors must be considered in interpreting the results obtained by the method described. Some strains which produce small quantities of alpha toxin may not be detected by this procedure. The amount of alpha toxin produced in any food may be influenced by the nature of the substrate as well as the time and temperature at which it is being held. In addition, the recovery of alpha toxin is dependent on the extraction method used, and some extracts may interfere with the lecithinase activity of the alpha toxin. In spite of these limitations, a relatively reliable estimate of the previous growth based on alpha toxin may be obtained with foods associated with *C. perfringens* outbreaks. Our results show that detectable alpha toxin was produced in food when the growth of *C. perfringens* exceeded $10^6$ organisms per g and the amount increased in proportion to the cell population. Therefore, the presence of detectable alpha toxin in food may be useful as an indicator of improper handling and storage.

The method described provides an additional means for determining the extent of growth of *C. perfringens* in food samples which have been subjected to freezing or prolonged refrigeration prior to laboratory analysis and for which low plate count values may be expected.

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