The enterotoxigenicity of *Bacillus cereus* was at one time attributed to lecinthinase C production by the bacterium (3, 6, 16), although some workers (13, 15; A. Ottolenghi, S. Gollub, L. Lisbinsky, and A. Ulin, Fed. Proc. 22:1002, 1963) suggested that *B. cereus* might produce a toxin that was not identical to lecinthinase. A highly purified enterotoxin was recently obtained from *B. cereus* (7). This toxin does not possess lecinthinase or hemolytic activity; it is lethal for mice, rabbits, rats, and cats (10, 11); and it displays enterotropic properties when injected into cats (8, 11).

Various methods have been developed for the quantitative determination of bacterial enterotoxins; these methods differ both in sensitivity and in principle. One of the most sensitive methods of antigen determination is the passive hemagglutination-inhibition reaction (2, 4). In recent years a new and more sensitive technique, aggregate-hemagglutination, has been proposed for detection of antigen content. It is based on the use of erythrocytes sensitized by aggregated proteins of immune serum (17–19). Antibodies are introduced into three-dimensional protein complexes, so that the active sites of the antibodies are spaced some distance from the surface of the erythrocytes. Thus, the active sites become readily accessible to antigenic determinants.

The reaction of aggregate-hemagglutination was used previously for detecting antigens of different origin (1, 20). The simplicity and sensitivity of the aggregate-hemagglutination technique make it convenient for detecting of small quantities of bacterial antigens for diagnostic purposes. Data on the enterotoxin of *B. cereus*, detected by the aggregate-hemagglutination technique, are given in this communication.

**MATERIALS AND METHODS**

**Cultures and toxin production.** Toxin-producing *B. cereus* 96 was grown in a medium containing fermented casein hydrolysate and 100 to 150 mg of amine nitrogen per ml (9). The growth medium contained (grams per liter): glucose, 5.0; NaHCO₃, 5.0; CaCl₂·2H₂O, 0.0147; MgSO₄·7H₂O, 0.0197; K₂HPO₄, 0.174; KH₂PO₄, 0.136; MnSO₄·H₂O, 0.00172; adenine sulfate, 0.002; uracil, 0.007; thiamine hydrochloride, 0.0001; glycine, 0.0003; tryptophan, 0.00024; and cystine, 0.00024. The pH was adjusted to 6.8 to 7.0. The standard conditions for incubation were as follows: 37 C for 16 to 24 h on a shaker at 110 rpm. Other *Bacillus* strains used in this study were grown under analogous conditions. Strains of *B. mesentericus* (*B. cereus*), *B. cereus* var. *mycoides*, *B. anthracis*, *B. thuringiensis*, and *B. subtilis* were obtained from the Tarasevitch State Control Institute, Moscow. *B. megaterium* strains 207, 216, and 337 were kindly supplied by E. N. Levina. All the above microorganisms were grown for 24 h at 37 C, as was described previously (9). The producer of enterotoxin A, *Staphylococcus aureus* 100, was obtained from the collection of A. C. Baird-Parker. *S. aureus* 243, the producer of enterotoxin B, was kindly supplied by E. P. Casman. *S. aureus* strains 100 and 243 were grown in Casman medium (5) for 24 h at 37 C. *Escherichia coli* was kindly supplied by V. G. Petrovskaya; it was grown in peptone broth for 24 h at 37 C. The culture filtrates of *Shigella dysenteriae* and *Clostridium perfringens* were obtained from V. P. Solodovnikov; those of *Vibrio cholerae* were from G. D. Kobrinsky. The enterotoxin of *B. cereus*, produced and purified by the method of Ezepchuk and Fluer (7), was isolated by ammonium sulfate treatment followed by fractionation in Bio Gel P-150 in 0.015 M NaCl (pH 7.0) (7, 8, 11).

Foodstuffs were inoculated with 10⁶ cells of *B. cereus* 96 per g and then were kept at 4, 24, or 28 C for 16 h. Solid, infected foodstuffs were ground in a microgrinder and suspended in saline (1 g of food stuff per 1 ml of saline). After filtration and centrifugation of the suspension to remove undissolved fragments, filtrates were analyzed for enterotoxin.
The protein concentration of the purified enterotoxin was determined by the method of Lowry et al. (14). The lethal activity was determined by injections of the toxin preparation or the culture filtrates into the tail veins of white random-bred mice (weight, 15 to 20 g); responses of the mice were estimated after 30 min. The minimum dose of enterotoxin causing a reaction in response to intravenous injection in cats (weight under 4 kg) was estimated after 1 h. Various bifunctional agents may be used for aggregation of immune serum proteins, in particular the stabilized tetrazotate of 4,4'-diaminodiphenylamine and glutaraldehyde. Glutaraldehyde was used at all stages of present work by the method of Gorina and Olovnikov (12).

**Antiserum.** To obtain antiserum to a purified preparation of *B. cereus* enterotoxin, three subcutaneous injections of toxin in the footpads and in four sites on each side (5 mg per rabbit, mixed with Freund incomplete adjuvant) were made at 1-month intervals. Two weeks after the third injection, antiserum with an antibody content of about 1.5 mg/ml, as determined by a slight modification of the neutralization test (21), was obtained for use in the experiment. The antiserum had a 1:64 titer by the Ouchterlony technique, where serum double dilutions were prepared against enterotoxin (protein concentration in enterotoxin preparation, as determined by the method of Lowry [14] was 1.5 mg/ml).

**Aggregate-hemagglutination technique.** Stabilization and activation of erythrocytes were done by the method of Gorina and Olovnikov (12). Two volumes of 0.9% saline were added to 1 volume of packed erythrocytes, and then a 25% aqueous solution of glutaraldehyde (Merck) was added to obtain a final concentration of 0.25%. After incubation for 3 h at 37°C, the erythrocytes were washed four times in saline and then resuspended in saline. Glutaraldehyde was again added to a final concentration of 0.25%, and the erythrocytes were refrigerated as an 8% suspension until use. For aggregation, 0.08 ml of 2.5% glutaraldehyde was added to 1 ml of antiserum, and after 1 h of incubation at 37°C the aggregated antiserum was used for sensitization. A 1-ml volume of the 8% suspension was removed from the glutaraldehyde-saline suspension, washed with saline, and suspended in aggregated immune serum (concentration of 8%). After 90 min of incubation at 56°C and then during the 30-min period at room temperature, sensitized erythrocytes were washed three times with saline, and a 1 to 2% suspension of red blood cells in saline, preserved with merthiolate (1:10,000), was prepared (12). Agglutinations were performed by the Takatsy microtitration procedure, with 0.2% normal rabbit serum as diluent. The results were estimated after incubation for 4 h at 37°C. Erythrocytes, sensitized by aggregated proteins of normal rabbit serum, were used as controls.

**RESULTS AND DISCUSSION**

In this work we attempted to detect microquantities of *B. cereus* enterotoxin in cultures and foodstuffs by the aggregate-hemagglutination technique. The sensitivity of this method exceeded that of the Ouchterlony procedure by about 1,000 times. It is suggested, therefore, that it is a convenient means for diagnostic determination of bacterial enterotoxin.

The antiserum used was not monospecific and gave three lines of precipitation in gels against the enterotoxin preparation used for immunization. One line corresponded to a thermodabile antigen, and two other lines corresponded to thermostable antigens. The thermostable antigens seemed to contain polysaccharide; only traces of antibodies to these antigens were present in the antiserum. Before heating, the enterotoxin preparation (protein concentration of 1.5 mg/ml; see Table 1) with the thermodabile compound gave a titer of 1:262,000 by aggregate-hemagglutination and, after heat-

**Table 1. Use of the aggregate-hemagglutination technique for detection of enterotoxin of *B. cereus***

| Antigen                            | Agglutination titers |
|------------------------------------|----------------------|
| Enterotoxin of *B. cereus*, 1.5 mg/ml | 1:262,000            |
| Enterotoxin of *B. cereus*, heated 30 min 100 C | 1:2          |
| Culture filtrate of *B. cereus*    | 1:32,768             |
| Culture filtrate, heated 30 min 100 C | 1:8               |
| Culture medium (control)           | 1:0                  |
| Culture filtrate of *B. anthracis* | 1:8                  |
| Culture filtrate of *B. anthracis*, heated 30 min, 100 C | 1:4          |
| Culture filtrate of *B. megaterium* 207 | 1:16             |
| Culture filtrate of *B. megaterium* 207, heated 30 min, 100 C | 1:4          |
| Culture filtrate of *B. megaterium* 216 | 1:2               |
| Culture filtrate of *B. megaterium* 337 | 1:4               |
| Culture filtrate of *B. mesentericus* 1227 | 1:2          |
| Culture filtrate of *B. subtilis* | 1:0                  |
| Culture filtrate of *B. cereus var. mycoides* | 1:0          |
| Culture filtrate of *B. thuringiensis* var. *galleriae* 13-1 | 1:0         |
|                                | 25-2                 |
|                                | 42-2                 |
| Culture filtrate of *S. dysenteriae* | 1:4             |
| Culture filtrate of *E. coli*      | 1:2                  |
| Culture filtrate of *C. perfringens* | 1:2             |
| Culture filtrate of *V. cholerae*  | 1:2                  |

**Staphylococcus** enterotoxins purified in Bio Gel P-60: **Type-A** | 1:2 |
| **Type-B** | 1:2 |
ing at 100 °C for 30 min, it gave a titer of 1:4. The antibody titer against thermolabile antigen by the Ouchterlony procedure was 1:64, and antibody titers against two thermostable antigens were 1:4. Thus, at dilutions of the antiserum 1:8 or higher, one can obtain a single precipitation line corresponding to the thermolabile antigen.

Closely related aerobic microorganisms of the Bacillus type, such as B. anthracis, B. megaterium, etc., are known to have common thermostable antigens polysaccharide in nature. The antiserum that we used in aggregate-hemagglutination gave cross-reactions with two closely related microorganisms, B. anthracis and B. megaterium, yielding titers of 1:8 to 1:16. Other experiments (data not shown) proved by gel precipitation that both thermostable components of B. cereus enterotoxin preparations are immunologically identical to the thermostable antigens of closely related Bacillus species. The aggregate-hemagglutination technique for detecting B. cereus toxin was sensitive down to 0.004 µg/ml.

The erythrocyte diagnosticum used is a specific one and does not give in the aggregate-hemagglutination of cross-reactions with enterotoxins produced by other microorganisms such as S. aureus, C. perfringens, E. coli, Shigella dysenteriae, Vibrio cholerae (Table 1). To exclude the possibility of cross-reactions with the thermostable antigens of B. cereus, foodstuffs were heated in control experiments for 30 min at 100 °C. Purified toxin and culture filtrate of toxigenic B. cereus yielded high titers (Table 1). The titers of heated and unheated preparations were uniformly low for a number of nontoxigenic Bacillus species and culture filtrates of various other bacteria. It follows that, even if non-monospecific serum is used and the protein toxin is inactivated by heating, the specificity of B. cereus enterotoxin may be proved by this procedure.

When the purified enterotoxin of B. cereus was injected intravenously into mice, the minimum lethal dose was 300 µg per mouse (Table 2). The enterotoxenic effect in cats caused by B. cereus enterotoxin was observed after intravenous injection of 70 to 80 µg/kg (live weight). For evidence of neutralizing activity of the antiserum, the enterotoxin of B. cereus was mixed with antiserum, incubated for 1 h at 24 °C, and injected into mice. The mixture, consisting of 0.25 ml of toxin (about 3 minimum lethal doses) and 0.25 ml of homologous antiserum (antibody protein content, about 1.5 mg/ml), was not lethal for mice. It was possible to detect by aggregate-hemagglutination a quantity of toxin approximately 75,000 times lower than that detected by the biotests (Table 2).

### Table 2. Comparison of the sensitivity of the aggregate-hemagglutination technique, of immunodiffusion in gel, and of a biological test for detection of B. cereus enterotoxin

| Method of enterotoxin detection | Quantity of detected enterotoxin |
|---------------------------------|---------------------------------|
| Aggregate-hemagglutination       | 0.004 µg/ml                    |
| Immunodiffusion in gel           | 2-3 µg/ml                      |
| Test for determining of the minimum lethal dose for mice | 15 mg/kg (live weight) |
| Test for determining of the enterotoxin dose causing a vomiting reaction in cat | 75 µg/kg (live weight) |

### Table 3. Use of the aggregate-hemagglutination technique for detection of bacterial enterotoxin in some foodstuffs after incubation with B. cereus for 16 h

| Foodstuffs | Temp of incubation (°C) | Agglutination titers |
|------------|--------------------------|----------------------|
| Boiled meat^a |                         | 28 1:32,768          |
| Boiled meat^a |                         | 24 1:4,096           |
| Boiled meat^a |                         | 4 1:2                |
| Boiled meat heated for 30 min, 100 °C | | 28 1:4 |
| Meat broth   |                         | 28 1:16,384          |
| Meat broth   |                         | 24 1:512             |
| Meat broth heated for 30 min, 100 °C | | 24 1:4 |
| Meat broth heated for 30 min, 100 °C | | 28 1:4 |
| Raw meat     |                         | 24 1:128             |
| Raw meat     |                         | 4 1:8                |
| Sausage^a    |                         | 24 1:128             |
| Sausage^a    |                         | 4 1:8                |
| Milk         |                         | 24 1:32              |
| Tea          |                         | 24 1:2               |

Control for specificity with uninfected foodstuffs:

| Foodstuffs | Temp of incubation (°C) | Agglutination titers |
|------------|--------------------------|----------------------|
| Boiled meat |                         | 24 1:2               |
| Meat broth  |                         | 24 1:2               |
| Raw meat    |                         | 4 1:4                |
| Sausage     |                         | 24 1:8               |
| Milk        |                         | 24 1:8               |
| Tea         |                         | 24 0                 |

^a Cattle meat and boiled sausage were used.
The results showed the possibility of using this method for detecting enterotoxin in different foodstuffs infected by *B. cereus*. Beef, boiled sausages, and other foodstuffs were incubated at 4, 24, or 28 C for this purpose. Uninoculated foodstuffs incubated at the same temperature as inoculated foodstuffs served as controls.

The enterotoxin of *B. cereus* was detected by the aggregate-hemagglutination test in boiled meat (titer, 1:4,096), meat broth (1:512), and boiled sausage and raw meat (1:128) when these foodstuffs were kept for 16 h at 24 C (Table 3). Increase in incubation temperature of foodstuffs inoculated with *B. cereus* up to 28 C resulted in increases of the agglutination titer by 8 to 30 times. Only small quantities of enterotoxin (titer at 1:8) were found in the same foodstuffs kept at 4 C. Such reactions were considered to be nonspecific.

Controls were as follows. Erythrocytes coated with aggregated proteins of immune serum were agglutinated with extracts of inoculated foodstuffs or with cultural media at titers of 1:2 to 1:16 (Table 3). Erythrocytes coated with aggregated proteins of serum were not agglutinated by the same materials. Controls for nonspecific hemagglutination caused by noninfected products and by cultural filtrates of closely related strains, as well as controls with erythrocytes coated with aggregated proteins of nonimmune serum, were 1:2 to 1:16.

The aggregate-hemagglutination technique gives reproducible results and does not require much time for the reaction. Sensitized erythrocytes persist for a month without loss of activity, and thus this reaction is convenient for practical detection of *B. cereus* enterotoxin in foodstuffs and culture media.

LITERATURE CITED

1. Abelev, G. I., V. S. Tzvetkov, T. I. Birulina, A. M. Olovnikov, D. A. Elgort, A. I. Gusev, A. K. Jazova, S. D. Perova, I. V. Rubtsov, S. V. Shaborina, B. A. Kantarovich, V. M. Tur, A. I. Chazanov, and D. M. Levine. 1971. Use of highly sensitive methods of determining alpha-fetoprotein for the diagnosis of hepato cellular cancer and teratoblastoma. Bull. Exp. Biol. Med. 4:75. (In Russian)

2. Avrameas, A., B. Taudou, and S. Chuliun. 1969. Glutaraldehyde, cyanuric chloride and tetralsotized o-dianisidine as coupling reagents in the passive hemagglutination test. Immunochemistry 6:87-76.

3. Bonventre, P. F., and N. J. Eckert. 1963. The biologic activities of *Bacillus anthracis* and *Bacillus cereus*. Am. J. Pathol. 43:201-212.

4. Boyd, W. C. 1966. Fundamentals of immunology. John Wiley & Son, Inc., New York.

5. Casman, E. P. 1968. Serologic studies on the staphylococcal enterotoxin. Public Health Rep. 73:599.

6. Chu, H. P. 1949. The lecithinase of *Bacillus cereus* and its comparison with *Clostridium welchi* a-toxin. J. Gen. Microbiol. 3:255-273.

7. Ezepchuk, Yu. V., and F. S. Fluer. 1971. Isolation and certain properties of *Bacillus cereus* toxin. J. Microbiol. Epidemiol. Immunobiol. 7:124-131. (In Russian)

8. Ezepchuk, Yu. V., and P. S. Fluer. 1973. The enterotoxin effect. Med. Med. 5:20-25.

9. Fluer, F. S., and Yu. V. Ezepchuk. 1970. Production and accumulation of lecithinase by *Bacillus cereus*. Microbiol. Biochemistry 59:465-470. (In Russian)

10. Fluer, F. S., and Yu. V. Ezepchuk. 1972. A study of enterotrophic properties of *Bacillus cereus* toxin. Bull. Exp. Biol. Med. 1:77-80. (In Russian)

11. Fluer, F. S., and V. V. Ezepchuk. 1973. Some chemical and phisico-chemical characteristics of exo-enterotoxin of *Bacillus cereus*. Bioclass 38:136-142. (In Russian)

12. Gorina, L. G., and A. M. Olovnikov. 1975. Use of the glutaraldehyde in aggregate-hemagglutination technique for detection of antigens. Delo N 4:241-242. (In Russian)

13. Johnson, C. E., and P. F. Bonventre. 1967. Lethal toxin of *Bacillus cereus*. I. Relationships and nature of toxin, hemolysin, and phospholipase. J. Bacteriol. 94:306-316.

14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

15. Molnar, D. M. 1962. Separation of the toxin of *Bacillus cereus* into two components and nonidentity of the toxin with phospholipase. J. Bacteriol. 84:147-153.

16. Nygren, M. 1962. Phospholipase C producing bacteria and food poisoning: an experimental study on *Clostridium perfringens* and *Bacillus cereus*. Acta Pathol. Microbiol. Scand. Suppl. 160:1-88.

17. Olovnikov, A. M. 1966. Detection of antigens content by agglutination of the erythrocytes, coated with antiseraum proteins, polycondensed by dianinodiphenilamine tetraasotate. Dokl. Akad. Nauk USSR 169:1180-1183. (In Russian)

18. Olovnikov, A. M. 1967. Sensitization of erythrocytes by polycondensed proteins of immune serum and their use for determining antigen content. Immunochemistry 4:77-90.

19. Olovnikov, A. M. 1968. Detection of antigen content. Method of the agglutination PFFS-erythrocytes and method of the agglutination of particles of polycondensed antibody-immunoadsorbent, p. 68-78. In L. A. Zilber (ed.). Immunochemical analysis. Medizine, Moscow. (In Russian).

20. Olovnikov, A. M., and V. S. Tzvetkov. 1969. Detection of the embusional α-ketoprotein by the method of aggregate-hemagglutination in the sera of patients with certain forms of human cancer. Bull. Exp. Biol. Med. 94:102-104. (In Russian).

21. Zollinger M. 1969. Nachweis von Ferritin mit der un-gekehrten passiven Haemagglutinationsmethode. Pathol. Microbiol. 33:321-335.