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Ileal Loop Fluid Accumulation and Production of Diarrhea in Rabbits by Cell-free Products of *Clostridium perfringens*[1]

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The ability of cell extracts and culture filtrates of various strains of *C. perfringens* to produce ileal loop fluid accumulation and overt diarrhea in rabbits was tested. Good correlation was obtained in the ability of whole cells and a toxic factor (present in cell extracts and concentrated culture filtrates) to produce both fluid accumulation in ileal loops and diarrhea when injected into the normal ileum of the rabbit. The toxic factor was present in cell-free preparations when cells were grown in a sporulation medium, but not when they were grown in an asporogenic medium. The factor was shown to be heat labile, nondialyzable, and was inactivated by Pronase but not by trypsin, lipase, or amylase. Loss of activity occurred at *p*H 1.0, 3.0, 5.0, and 12.0.

The principal symptom of human food poisoning caused by *Clostridium perfringens* is a mild diarrhea which usually occurs 6 to 24 hr after ingestion of a food containing large numbers of cells. Although various mechanisms for production of the symptoms of food poisoning caused by the presence of this organism in food have been postulated, the mode of action still remains unknown.

Cravitz and Gillmore (cited in Dische and Elek, 4) attributed the disease to a heat-stable enterotoxin after finding that nausea, cramps, and vomiting occurred in six people fed with 75- to 100-ml doses of heated *C. perfringens* culture filtrates. The onset of the symptoms occurred as early as 45 to 80 min after the challenge, and one person had diarrhea. However, Dische and Elek concluded that the symptoms, as observed by Cravitz and Gillmore, did not constitute typical *C. perfringens* food poisoning. Dische and Elek (4) found that, whereas cell-free culture filtrates and cultures which had been heated to 100 C did not produce food poisoning symptoms when fed to human volunteers, live cultures or suspensions of one of three cultures did produce the symptoms. They concluded that the illness is caused by a mild transient infection. Dack et al. (3) failed to induce the illness in human volunteers fed culture filtrates or whole broth cultures.

Nygren (10) suggested that phosphorylcholine, a product resulting from the hydrolysis of lecithin in the presence of the α-toxin (lecithinase C) of *C. perfringens*, was the agent responsible for perfringens poisoning. However his results could not be confirmed in our laboratory (11).

Hauschild et al. (8) reported the experimental production of diarrhea in lambs after oral or intraduodenal challenge with *C. perfringens* type A. Later, they also reported (9) that fluid accumulation occurred in ligated intestinal loops of lambs when *C. perfringens* suspended in fresh medium was injected. In both cases, neither culture supernatant fluid nor medium alone caused diarrhea or produced fluid accumulation in the ligated intestinal loops. Results indicated that α-toxin was not the factor responsible for fluid accumulation in the loops.

We reported (7) results of our investigations on the ligated loop of the rabbit intestine as a possible experimental model for the study of *C. perfringens* food poisoning. A total of 14 of 29 type A strains isolated from food poisoning outbreaks consistently produced exudation of fluid in the ileal segments when the challenge was made with cultures grown for 4 hr at 37 C in skim milk. In contrast, 15 of the 18 strains derived from other sources failed to elicit a response when the challenge was prepared by this same procedure. The

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fluid accumulation was shown not to be due to α-toxin. Subsequently, we were able to experimentally produce overt diarrhea in rabbits by injection of *C. perfringens* into the normal ileum, but not by oral challenge (6). The ability to induce diarrhea was dependent on the strain, the method of preparation of cells for challenge, and the number of cells in the challenge. Good correlation was obtained between the ability of the strains to produce fluid accumulation in the ileal loops and overt diarrhea in the rabbit.

The current report describes the results of our studies on ileal loop fluid accumulation and the production of overt diarrhea through intraluminal injection of rabbits with cell-free preparations of various strains of *C. perfringens*. To our knowledge, this is the first report in the literature on the repeated production of a diarrhea response in an experimental animal by such cell-free preparations.

MATERIALS AND METHODS

Cultures. A total of 26 *C. perfringens* type A strains were used. All but two of these (strains 215b and ATCC 3624) were isolates either from feces of food-poisoning cases or from incriminated foods. By use of appropriate procedures, it has been shown that a total of 14 of these strains were capable of producing both fluid accumulation in rabbit ileal loops (7) and overt diarrhea in rabbits (6); an additional 2 strains produced fluid accumulation in ileal loops, but not overt diarrhea, while the remaining 10 strains did not produce a response under either of these conditions.

Stock cultures were maintained frozen in Cooked Meat Medium (Difco). Cultures were activated for use by transferring into Fluid Thioglycollate Medium (BBL) with subsequent incubation at 37 C for 16 to 18 hr.

Surgical procedure. New Zealand white rabbits of both sexes were 6 to 9 weeks old and weighed about 1.2 to 2.0 kg at the time of testing. The operative procedure for the preparation of ligated ileal loops and for testing for the induction of diarrhea by injection of test preparations into the normal (not ligated) ileum was previously described (6). Unless indicated otherwise, 2-ml test samples were injected into ileal loops; animals were sacrificed after 18 to 20 hr, and the loop fluid volume (ml)/length (cm) ratios (1) were determined. In testing for overt diarrhea production, 10-ml test samples were injected into the lumen of the ileum about 90 to 100 cm anterior to the meso-appendix.

Growth of cells and preparation of cell extracts and concentrated culture supernatant fluids. For preparation of cell extracts, 10 ml of an active Fluid Thioglycollate Medium culture was inoculated into 350 ml of DS-sporulation medium (5) with subsequent incubation for 3 hr at 37 C. No special precautions were taken to provide for anaerobic incubation other than the use of sodium thioglycolate in the various media. In one series of experiments, Fluid Thioglycollate Medium was used throughout rather than employing DS-sporulation medium for growth of the cells.

Cells were harvested from the 3,860 ml of culture medium by centrifugation at 4 C. The cells were resuspended in 20 ml of cold physiological saline, and extracts were prepared by using a Branson Sonifier. The extraction chamber was cooled in an ethyl alcohol-dry ice bath. The degree of cell disruption was monitored by phase-contrast microscopy. Vegetative cell disruption was achieved after 15 to 20 min; spores, when present, were not ruptured in this time interval. The extract was centrifuged at 4 C and 27,000 $\times$ *g* for 10 min and then sterilized by filtration through a Seitz filter with positive pressure. The extract was routinely stored at 4 C.

Concentrated culture supernatant fluids were prepared routinely from cells grown in DS-sporulation medium for predetermined time intervals. In one series of experiments, Fluid Thioglycollate Medium was used instead of DS-sporulation medium. The inoculation procedure was the same as that used for obtaining cells when preparing cell extracts, except that 100- and 1,000-ml volumes were routinely used. Cultures first were centrifuged to remove the cells; the supernatant fluids then were sterilized by filtration through a Seitz filter with positive pressure. Filtrates were concentrated by dialysis against Carbowax 20,000 (polyethylene glycol, Union Carbide Corp.) at 4 C. Unless indicated otherwise, the filtrates were reconstituted with saline to a concentration 33 times that of the original supernatant fluid. The concentrated filtrates were also stored at 4 C.

Lyophilized cell extracts (5-hr-old cultures) or culture filtrates (48-hr-old cultures) used in some experiments were prepared from the concentrated materials described above. Distilled water was used to reconstitute the lyophilized preparations of supernatant fluid instead of saline. The lyophilized preparations were stored desiccated at -12 C.

Effect of heat on the ileal loop fluid-producing factor. Unless otherwise indicated, lyophilized cell extract at a concentration of 5 mg/ml of saline was used; culture filtrate was the 33-fold concentrated preparation described above. The extract or filtrate contained in screw-capped test tubes was heated at selected temperatures in a constant-temperature water bath for various time intervals. On removal from the bath, the tubes were cooled in ice water.

Effect of pH on the ileal loop fluid-producing factor. Lyophilized culture filtrate of strain NCTC 8798 was used at a concentration of 31.25 mg/ml in distilled water. The solution was initially made double strength, and the *p*H was adjusted by using concentrated HCl and 4 N NaOH. Distilled water was added to give the final concentration indicated. The *p*H values of 1.0, 3.0, 5.0, 6.0, 9.0, 10.0, 11.0, and 12.0 were used. The appropriately adjusted filtrate was then stored at 4 C for 24 hr prior to challenge in ileal loops.

Effect of enzymes on the ileal loop fluid-producing factor. The following enzymes were tested for their effect on the factor present in both cell extract and culture fluid of strain NCTC 8798: bacterial α-amylase (Calbiochem), bacterial lipase (Mann Research Laboratories), steapsin (Nutritional Biochemicals

Corp.), trypsin (Fisher Scientific Co.), and Pronase (Calbiochem). Pronase was used in a final concentration of 0.05 mg/ml. All other enzymes were used in a final concentration of 2.5 mg/ml. Pronase and trypsin were tested at *p*H 7.4 with 0.05 M trihydroxymethylaminomethane buffer. α-Amylase was tested at *p*H 7.0 with 0.05 M phosphate buffer. The bacterial lipase and steapsin were tested at *p*H 6.0 with 0.05 M citrate-phosphate buffer. All buffers contained 0.001% thimerosal.

To test for the effect of the enzymes on the activity of the ileal loop fluid-producing factor, three preparations were used for challenge in each rabbit. The test preparation consisted of either the filtrate or cell extract mixed with the specific enzyme and two control preparations consisting of the filtrate or cell extract alone in the respective buffer and the enzyme alone in the buffer. Lyophilized cell extract and culture filtrate were reconstituted in the different buffers to a final concentration of 5 mg/ml and 31.25 mg/ml, respectively. All preparations were incubated for 24 hr at 37 C prior to testing for ileal loop activity.

RESULTS

Initial studies were made by using *C. perfringens* strain NCTC 8798. Table 1 shows that cell extracts prepared from cultures grown for 3, 5, 8, 11, or 24 hr contained a heat-labile toxic factor that caused fluid accumulation in ileal loops. Heating for 10 min at 60 C always inactivated the factor, whereas heating for 5 min at 55 C never prevented fluid accumulation. The ileal loop fluid volume/length ratios obtained are comparable to those previously reported for loops challenged with viable cells (7). Since a 10-min, 60 C heat treatment always inactivated the factor, preparations treated in this manner were used as controls in subsequent ileal loop tests.

Previously, we had shown that cells of certain strains of *C. perfringens* which were grown in Fluid Thioglycollate Medium would not produce diarrhea when injected directly into the ileum but would do so when grown in DS-sporulation medium (6). It had been shown also that Fluid Thioglycollate Medium cultures would not produce fluid accumulation in ileal loops (7). To determine whether cell extracts prepared from cells grown in Fluid Thioglycollate Medium would produce fluid accumulation in ileal loops, the same inoculation sequence as used previously with DS-sporulation medium was used for growing cells in this medium. Cell extracts were prepared from 3-, 5-, 8-, 11-, or 24-hr cultures and tested unheated for activity in ileal loops. None of the extracts produced fluid accumulation in the ileal loops, a result which correlates with the inability of the whole cells to produce fluid accumulation in ileal loops or diarrhea when grown in the same medium.

A test to determine the effect of storage at different temperatures on the stability of the toxic factor present in cell extracts of strain NCTC 8798 indicated that activity was still present at the end of 5 days, whether the extract was stored at 37 C, room temperature, 4 C, or −21 C. Therefore, when stored within this range, no special precaution seemed to be necessary in preventing temperature inactivation of the factor.

The time course of fluid accumulation in ileal loops injected with cell extract of strain NCTC 8798 is shown in Fig. 1. Four loops prepared in a single rabbit were injected with either 1 ml of unheated, undiluted extract, or unheated extract diluted 1:2, 1:4, or 1:6 with saline. In the same animal, a fifth loop injected with extract heated at 60 C for 10 min served as a control. Rabbits injected with these preparations were sacrificed after 3, 6, 12, and 18 hr, and the loop fluid volume/length ratio was determined.

Net fluid movement into the loop had occurred as early as 3 hr after injection of unheated extract which had been diluted 1:4 or less. In the loops that were injected with extract heated at 60 C for 10 min, about 0.75 of the volume of extract injected had been absorbed by 3 hr, and at 6 hr all the extract had been absorbed.

TABLE 1. *Rabbit ileal loop response to the injection of cell extracts of Clostridium perfringens NCTC 8798*

Age of culture grown at 37 C	Treatment of cell extract[a]				
	Unheated	Heated 10 min at 60 C	Heated 5 min at 60 C	Heated 10 min at 55 C	Heated 5 min at 55 C
hr					
3	9/10 (1.5)	0/4	1/4 (1.0)	0/4	1/1 (1.7)
5	15/15 (1.4)	0/6	1/6 (1.3)	1/6 (0.9)	3/3 (1.7)
8	5/5 (1.3)	0/3	0/3	0/3	
11	3/3 (1.0)	0/3	0/3	0/3	
24	6/6 (1.3)	0/3		3/3 (1.4)	3/3 (1.3)

[a] Number active/number tested. The numbers in parentheses are the average ileal loop fluid volume/length ratios. A standard volume of 1 ml was injected into each loop.

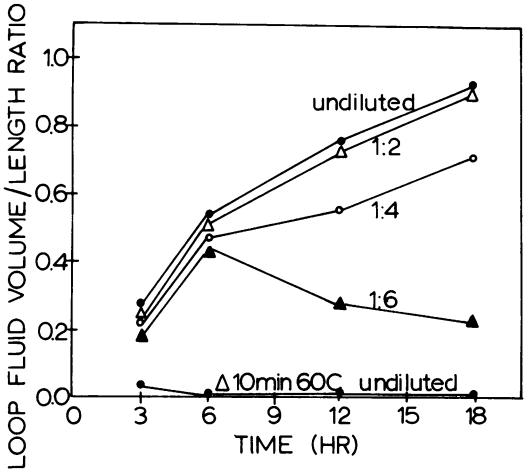

FIG. 1. *Time-course of fluid accumulation in ileal loops injected with varying dilutions of cell extract of strain NCTC 8798. Each point represents an average of values obtained from three to five different rabbits.*

An approximately linear increase in the volume/length ratio occurred with the undiluted and the 1:2 diluted extract. However, a decrease in the response to extract diluted 1:6 occurred after 12 and 18 hr, indicating some possible reabsorption of the accumulated fluid.

A replot of these data is shown in Fig. 2, in which the loop fluid volume/length ratios are plotted as a function of the log of the dilution of extract. The dose-response curves show the concentration dependence of the loop response. Although linear curves were obtained at 3 and 6 hr, the concentration dependence was not very pronounced. However, at 12 and 18 hr, the curves were very much concentration-dependent, but deviated from linearity. This deviation was apparently due in part to reabsorption of accumulated fluid at the higher dilution.

Experiments were next conducted to correlate the ability of cell extracts of *C. perfringens* to produce fluid accumulation (when injected into ileal loops) and diarrhea (when injected into the normal ileum) with the ability of whole cells to produce similar responses.

Cell extracts of 26 different strains were prepared as described in the previous section. Extract which had been heated at 60 C for 10 min was used as a negative control in both animals tested by the ileal loop technique and in those tested for diarrhea by injection of 10 ml of extract into the normal ileum. The results are presented in Table 2.

Cell extracts which produced fluid accumulation in the ileal loop were obtained from 12 of 14 of the strains which also produced both overt diarrhea and fluid accumulation in ileal loops when viable cells were injected. All the strains that were inactive when viable cells were tested also produced inactive cell extracts. When certain loop active strains were tested repeatedly, the ability to obtain an active cell extract was sometimes inconsistent. The loop active cell extracts from 7 of 10 of the strains tested produced overt diarrhea when 10 ml was injected into the normal ileum. Cell extracts heated 10 min at 60 C did not induce diarrhea. Diarrhea occurred in the rabbits injected with extract as early as 10 hr after injection and always within 18 hr. Diarrhea usually stopped within 24 to 28 hr from the time of challenge. Of the two strains tested, cell extracts which failed to produce fluid in the ileal loop also failed to produce diarrhea. Both of the strains (NCTC 8449 and NCTC 8359) which did not produce diarrhea but which did produce fluid accumulation in the ileal loop when whole cells were injected, yielded an extract which was active in the ileal loop. However, two of four different extracts prepared from each of these strains were inactive. Also, the active preparation of the one strain tested (NCTC 8449) failed to produce diarrhea.

Preliminary studies indicated that a culture filtrate of strain NCTC 8798 grown 48 hr in DS-sporulation medium at 37 C and concentrated 33-fold would produce fluid accumulation in the ileal loop. The activity of the filtrate was also inactivated by heating 10 min at 60 C. Con-

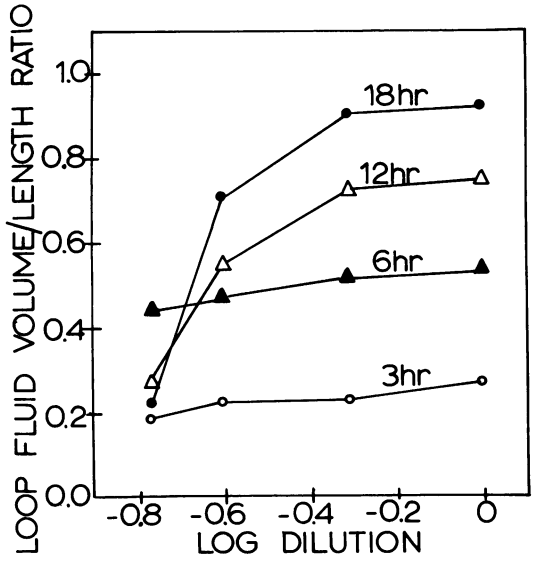

FIG. 2. *Ileal loop fluid accumulation at various times as a function of NCTC 8798 cell extract concentration. The data are replotted from Fig. 1.*

TABLE 2. *Ability of cell extracts of Clostridium perfringens to produce ileal loop fluid accumulation and overt diarrhea in rabbits*

Strain	Viable cells		Cell extract	
	Loop response[a]	Diarrhea production[a]	Loop response[b]	Diarrhea production[c]
NCTC 8798	+	+	1.4	8/9
NCTC 10239	+	+	1.9	2/2
NCTC 10240	+	+	1.3, 0.7	2/2
NCTC 9851	+	+	1.7	1/2
NCTC 8449	+	−	−, −, 0.8, 1.2	0/2
NCTC 8235	+	+	−, −	0/2
NCTC 8359	+	−	−, −, 0.13, 0.2	
NCTC 8238	+	+	−, −, 1.0, 1.6	2/2
NCTC 8239	+	+	1.5, 1.3	1/2
NCTC 8679	+	+	−, 1.25	
T-65	+	+	−, −, 0.4	
77455	+	+	−, 1.9	0/2
E13	+	+	−, 0.6, 0.5	2/2
68900	+	+	1.2, 1.1	0/2
FD5	+	+	−, −	0/2
80535	+	+	−, 1.6	
FD2	−	−	−	
FD7	−	−	−	
NCTC 8247	−	−	−, −	
NCTC 8678	−	−	−	
FD1	−	−	−	
79394	−	−	−	
6867	−	−	−	
F42	−	−	−	
215b	−	−	−	
ATCC 3624	−	−	−	

[a] The data in these columns are from a previous paper (6) and are used here for comparison.

[b] Fluid volume/length ratio. Ileal loop response was active when ratio is given. Negative symbol (−) indicates no response with the extract. Each result was obtained from different preparations of cell extract and represents the average response obtained in at least two different rabbits.

[c] Number of rabbits with diarrhea/number challenged. When both an active and an inactive ideal loop response was obtained with the cell extracts, only extract producing a positive response was used to test for diarrhea production.

centrated culture filtrates of 16 strains of *C. perfringens* grown in sporulation medium for various time periods at 37 C were prepared. All but two of the strains (NCTC 8247 and FD 5) had yielded cell extracts which induced ileal loop fluid accumulation. The ability of the concentrated filtrates to induce fluid accumulation in the ileal loop was determined. Some of the active preparations were further tested for their ability to produce diarrhea after injection of 10 ml of the preparations into the normal ileum of different rabbits (Table 3).

The age of the culture appeared to be a factor in the production of an active filtrate, since the 5-hr culture filtrates of strain NCTC 8798 were inactive in the ileal loop, yet the 24-, 36-, 48-, and 63-hr cultures were active. Therefore, the other strains were tested for filtrate activity only after at least 24 hr of growth.

Loop active concentrated culture filtrates were obtained from 11 of 14 of the strains which had loop active cell extracts. Filtrates from 4 of 6 of the strains tested, which produced an active ileal loop response, also produced diarrhea when 10

TABLE 3. *Ability of concentrated culture filtrates of Clostridium perfringens to produce ileal loop fluid accumulation and overt diarrhea in rabbits*

Strain[a]	Culture age	Loop fluid vol/length ratio[b]	No. of rabbits having diarrhea/no. challenged with filtrate
	hr		
NCTC 8798	5	−	
	24	1.2	
	36	1.0	
	48	1.8, 1.8	4/4
	63	1.5	
NCTC 10240	48	1.2	0/2
NCTC 8239	48	1.4	2/2
NCTC 8679	48	1.3	
NCTC 10239	48	1.2	2/2
T-65	48	1.2	0/2
E13	24	1.3	2/2
	48	1.3	2/2
77455	24	−	
	48	−	
	72	1.39	
NCTC 9851	24	−	
	48	−, −	
	72	−	
80535	48	0.8	
NCTC 8359	24	0.7	
NCTC 8238	24	0.6	
NCTC 8449	48	−	
68900	24	−	
	48	−	
	72	−	
FD5	48	−	
NCTC 8247	48	−	

[a] Cell extracts of all strains except NCTC 8247 and FD5 produced an active loop response (*see* Table 2).

[b] Ileal loop response was active when ratio is given. Negative symbol (−) indicates no response with the filtrate. Each ratio is the average of the responses obtained in two or more rabbits.

ml was injected into the normal ileum. After heating 10 min at 60 C, 48-hr culture filtrates of NCTC 8798 (the only strain tested) failed to induce overt diarrhea in four different animals. Strains NCTC 8247 and FD5, which had inactive cell extracts, also had inactive culture filtrates. Concentrated sterile medium had no activity either in the ileal loop or in the normal ileum.

Concentrated culture filtrates were prepared from strains NCTC 8798 and NCTC 8239 which had been grown for 48 hr at 37 C in Fluid Thioglycollate Medium instead of sporulation medium. These preparations were consistently inactive when tested in the ileal loop.

The effect of heat on the activity of the ileal loop fluid-producing factor in cell extract and concentrated culture filtrate of strain NCTC 8798 was further investigated. The samples were heated at 55 C as described. Results are presented in Table 4.

The heat lability of the toxic factor in both cell extract and culture filtrate is evident. Little inactivation of the factor occurred within 5 to 10 min of heating; however, 15- and 20-min heating times resulted in decreasing average loop fluid volume/length ratios. No activity was obtained after heating the crude preparations for 25 min.

The effect of *p*H on the activity of lyophilized culture filtrate of strain NCTC 8798 is shown in Table 5.

The activity of the factor was not changed appreciably when the filtrate was adjusted to *p*H 6.0, 9.0, 10.0, or 11.0. Complete loss of activity was apparent at *p*H 1.0 and 12.0. A positive loop response was obtained with filtrate adjusted to *p*H 3.0 in only one of the seven different tests, and with filtrate adjusted to *p*H 5.0 in only two of seven different tests, indicating considerable, if not complete, loss of activity at these two *p*H values.

The effect of different enzymes on the activity of lyophilized culture filtrate and cell extract of strain NCTC 8798 is shown in Table 6.

The protein nature of the toxic factor is indicated by the fact that activity in both the filtrate and the cell extract was destroyed after treatment with Pronase, a proteolytic enzyme with a very broad substrate specificity. However, trypsin, a proteolytic enzyme of greater specificity, did not destroy the activity. Also, the activity was not affected by bacterial lipase, steapsin (pancreatic lipase), or α-amylase.

Although dialysis of culture filtrate and cell extract was routinely used to concentrate these preparations, the effect of extensive dialysis against distilled water was determined. A total of 20 ml of cell extract of NCTC 8798 was dialyzed against 200 ml or 2,000 ml of distilled water at 4 C for 40 hr. Both the dialysand (fluid within the dialyzing sac) and the dialysate were lyophilized and reconstituted to 20 ml with distilled water. The activity of the preparations in ileal loops was then determined. In all cases, activity was present in the dialysand but not in the dialysate, indicating that the active factor is nondialyzable.

TABLE 4. *Effect of heat on the ileal loop fluid-producing factor in cell extract and culture filtrate of Clostridium perfringens NCTC 8798*

Time heated at 55 C	Average loop fluid (volume/length ratio)[a]	
	Cell extract	Culture filtrate
min		
Unheated	1.4	1.6
5	1.5	Not tested
10	1.2	1.6
15	0.7	1.5
20	0.2	0.3
25	0.0	0.0

[a] Average ratio from three to four rabbits. Two milliliters of each test preparation was injected per ileal loop.

TABLE 5. *Effect of pH on the ileal loop fluid-producing factor in culture filtrate of Clostridium perfringens NCTC 8798*

*p*H level	Ileal loop fluid (volume/length ratio)[a]
1.0	0.0
3.0	0.08
5.0	0.14
6.0	0.95
9.0	1.0
10.0	0.5
11.0	0.7
12.0	0.0

[a] For *p*H 1.0, 3.0, and 5.0, the ratio shown is an average of the responses in seven different rabbits. All others are an average of the responses in three different rabbits.

DISCUSSION

The results presented in this paper indicate that a heat-labile toxic factor which has the ability to induce a diarrhea response in rabbits is present in the culture filtrate and cell extract of certain strains of *C. perfringens*. Previously, we had shown that good correlation existed between the ability of viable cells of strains of *C. perfringens* to produce fluid accumulation in rabbit ileal loops and overt diarrhea after injection of cells

TABLE 6. *Effect of selected enzymes on the ileal loop fluid-producing factor in culture filtrate and cell extract of Clostridium perfringens NCTC 8798*

Challenge prepn.	Ileal loop fluid (volume/length ratio)[a]				
	α-Amylase	Steapsin	Bacterial lipase	Trypsin	Pronase
Culture filtrate plus enzyme in buffer	1.2	1.0	1.5	1.1	0.0
Culture filtrate in buffer	1.2	0.9	1.2	1.1	1.1
Enzyme in buffer	0.0	0.0	0.0	0.0	0.0
Cell extract plus enzyme in buffer	0.9	1.2	0.9	0.7	0.0
Cell extract in buffer	0.9	1.4	1.3	0.7	1.1
Enzyme in buffer	0.0	0.0	0.0	0.0	0.0

[a] Average ratio from at least three different rabbits.

suspended in milk into the normal ileum of the rabbit (6). The present results show that reasonably good correlation also exists in the ability of not only viable cells but of cell extracts and concentrated culture filtrates of the same strain to produce both fluid accumulation in the ileal loop and diarrhea when injected into the normal ileum. As with the whole cells, the activity of cell-free preparations was dependent not only on the strain, but on the method of culturing the organism.

A summary of the response obtained in the rabbit after challenge with viable cells, cell extracts, or culture filtrates of various strains of *C. perfringens* is presented in Fig. 3. A total of 61.5% of the strains were active when 4-hr milk cultures of viable cells were injected into ileal loops. The 10 strains which were inactive when their viable cells were injected into the ileal loop were also inactive when tested for diarrhea production by viable cells. Likewise, cell extracts obtained from these strains were inactive in the ileal loop. A total of 87.5% of the strains whose viable cells were active in the loop also produced diarrhea after intraluminal challenge with viable cells. Of these same 16 strains, 87.5% produced cell extracts that were active in the loop. The loop active cell extracts of 70.0% of these strains that were tested also produced diarrhea after intraluminal challenge. Loop active culture filtrates were produced by 78.6% of the strains with loop active cell extracts; of this group, 66.7% of the filtrates tested also produced overt diarrhea.

The ability to obtain active preparations from cultures grown only in sporulation medium and not in Fluid Thioglycollate Medium, an asporogenic medium, may indicate that production of the toxic factor is associated with sporulation, or it may be a reflection only of nutritional differences in the two media. Studies are under way to determine whether production of the factor is related to sporulation of the organism.

The intracellular and extracellular diarrhea-producing factors are probably the same. Both crude preparations have comparable heat lability and are inactivated by Pronase but not by trypsin, lipase, or amylase. Although intracellular activity was detected as early as 3 hr after inoculation, extracellular activity was present at 24 hr but not 5 hr after inoculation. We are at present attempting to establish the time between 5 and 24 hr at which the factor may be detected in the filtrate. The factor may be released by lysis of the cells and may reach a detectable level only after considerable lysis has occurred. A dilution of the 48-hr 33× concentrated culture filtrate of NCTC 8798 by a factor of 1:6 is usually inactive in the ileal loop, indicating the low concentration of the toxic factor (Duncan and Strong, *unpublished data*).

Although the lecithinase of *C. perfringens* is an exoenzyme, both intracellular and extracellular lecithinase activity have been demonstrated (2). Strains of *C. perfringens* that produced large quantities of lecithinase excreted most of it into the surrounding medium, whereas strains that produced less lecithinase retained approximately 50% of the enzyme in the cytoplasm. Although a quantitative comparison of the diarrhea-producing factor in cell extracts and culture filtrates of our strains has not been made, the same sort of relationship that exists with lecithinase may exist with the diarrhea-producing factor. The fact that active cell extracts were obtained from strains NCTC 9851, NCTC 8449, and 68900, but active culture filtrates were not, may be the expression of such a relationship.

If the diarrhea-producing factor active in rabbits is associated with diarrhea production by *C. perfringens* in cases of human food poisoning, it is evident that some of the strains previously associated with food poisoning outbreaks have on repeated subculture lost their ability to produce the diarrhea factor, since it could be detected in cell extracts of only 14 of the 24 food poisoning-

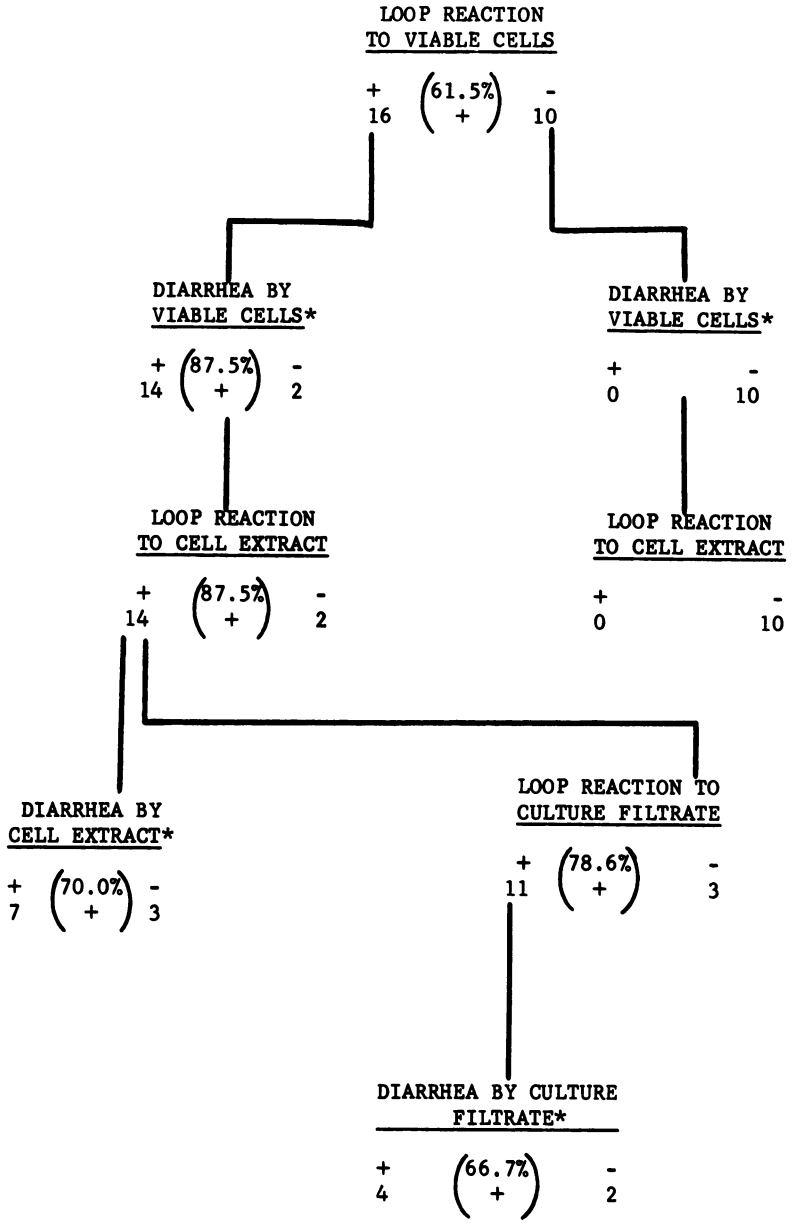

FIG. 3. *Summary of responses obtained in the rabbit after challenge with viable cells, cell extracts, or culture filtrates of various strains of C. perfringens. Summary based on data presented in Tables 2 and 3. Asterisk(*) indicates intraluminal injection.*

associated strains. Also, if the agent active in both humans and rabbits is the same, the failure to obtain food poisoning symptoms in humans fed culture filtrates of *C. perfringens* (3, 4) may have been caused by the low concentration of the active factor present in culture filtrates which were fed, or inactivation of the factor in the acidic environment of the stomach may have occurred. Our results indicated complete or partial loss of activity of the factor at *p*H 1.0, 3.0, 5.0, and 12.0.

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