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Rabbit Ileal Loop Response to Strains of *Clostridium perfringens*[1]

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The ligated loop of the rabbit intestine was investigated as a possible experimental model for the study of *Clostridium perfringens* food poisoning. The method of preparation of the challenge inoculum was important in determining whether a given strain would provoke a response. When cultures were grown for 4 hr at 37 C in Skim Milk (Difco), 14 of 29 type A strains isolated from food-poisoning outbreaks consistently produced exudation of fluid and consequent dilation of the ileal segments. In contrast, 15 of the 18 strains derived from other sources failed to elicit a response. By use of different inoculum preparations, nearly all strains could be made to give at least an occasional positive loop reaction. Diarrhea was not obtained in rabbits by intraluminal injection into the normal ileum or by per os administration of the cultures. Lecithinase, purified and in concentrated culture supernatant fractions, failed to produce a response in the isolated ileal loops.

Study of the pathogenesis of human food poisoning caused by *Clostridium perfringens* has been hindered by the lack of a readily available laboratory animal in which the food-poisoning syndrome can be reproduced. Oral administration of cultures of *C. perfringens* to monkeys resulted in an occasional loose stool only, and no unusual response was observed in guinea pigs or frogs challenged intragastrically or intraduodenally (8). Lambs developed diarrhea, the principal symptom of *C. perfringens* food poisoning in man, after administration of cultures either per os or intraduodenally via a fistula (A. H. W. Hauschild et al., Bacteriol. Proc., p. 6, 1967).

Phosphorylcholine, a product formed from lecithin by the α-toxin of *C. perfringens*, was suggested as the agent responsible for this food poisoning because the intestinal passage time of food in the mouse was significantly reduced (11). However, this observation could not be confirmed in mice or monkeys (15), and a human volunteer had no ill effects after ingesting 500 mg of the pure compound (2).

The ligated loop of the rabbit's small intestine has been useful in the study of bacterial diseases characterized by gastrointestinal symptoms. The loop responds to the injection of cultures (3), culture supernatant fluids (1), and cell lysates (4) of *Vibrio cholerae*, with gross dilation caused by fluid accumulation. The enteropathogenicity of *Escherichia coli* strains (10, 13) and of *Salmonella* and *Shigella* (12) has also been studied in this test system.

This report considers the possibility of using the ligated loop of the rabbit ileum as a model for the study of *C. perfringens* food poisoning.

MATERIALS AND METHODS

Cultures. Of the 46 *C. perfringens* type A strains used, 29 were isolates from feces of food-poisoning cases or from incriminated foods. Both the "classical" and "food-poisoning" strains (6, 7) were represented. The remaining 17 were isolates from random sources, such as raw vegetables, beef liver, ground beef, spiced luncheon meats, and flies. One type D strain was also tested. The known association of these strains to food poisoning is given in Table 3.

Stock cultures maintained frozen in Cooked Meat Medium (Difco) were subcultured in Fluid Thioglycollate Medium (BBL) for 16 to 20 hr at 37 C. Media for preparation of the animal challenge materials were inoculated with these "activated" cultures (1% by volume) and were always incubated at 37 C. Vigorously growing cultures were usually obtained after 4 hr by this procedure.

Spores were developed in a sporulation medium (5), harvested and washed three times with distilled water by centrifugation, and stored in water at 4 C.

Surgical procedure. New Zealand white rabbits of both sexes, acclimatized to the laboratory for at least 1 week, were 7 to 10 weeks old and weighed 1.4 to 2.2 kg at the time of testing. The animals were main-

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tained on the same pelleted food used by the animal supplier. Water was supplied ad libitum at all times, but the rabbits were fasted at least 24 hr and usually 48 hr immediately prior to testing.

The operative procedure was essentially that used by others (9). The animal was anesthetized with ether; the ileum was externalized and ligated approximately 90 cm anterior to the mesoappendix. Intestinal contents were washed down with 10 ml of physiological saline injected just below the tie, and the site of injection was isolated by a second ligature 5 cm below the first. Segments of 10 cm were tied off caudally from this ligature. Saline packs were employed to keep the exposed intestine moist during the operative procedure.

Intraluminal injections of 2 ml were made into four alternate loops so that a blank loop separated the test segments. A control loop injected with sterile culture medium was included in each rabbit. After returning the intestine into the abdominal cavity, the incisions were closed. After holding for 20 to 24 hr with only water available, the rabbit was sacrificed by the intravenous injection of Diabutal (Diamond Laboratories, Des Moines, Iowa).

Postmortem examination. The animal was opened immediately. Each ligated loop was slit lengthwise for examination of the contents. In later experiments, the length and fluid volume of the loops were measured to calculate the loop volume (ml)/length (cm) ratio, which was used to compensate for the unavoidable variability in the lengths of the loops (1). The control and uninjected (blank) loops of an occasional rabbit were all filled with fluid; the results in these rabbits were considered invalid.

C. perfringens was enumerated by transferring the slit intestinal segment and its content to a test tube and adjusting the fluid volume to 7 ml with physiological saline. No saline was added when the loop had more than this amount of fluid. After mixing thoroughly for 1.5 min with a Vortex mixer (Scientific Industries, Inc., New York, N.Y.), the contents of the tubes were divided. One portion was heated at 75 C for 20 min to kill the vegetative cells and simultaneously heat-activate the spores. Dilutions of the unheated and heated replicates were prepared in 0.1% peptone-water and plated in sulfite-polymyxin-sulfadiazine-agar (BBL), a selective medium for *C. perfringens*, to obtain total cell and spore counts, respectively. Incubation was at 37 C for 48 hr under a 90% N_2 and 10% CO_2 mixture.

Selected intestinal loops were preserved in 4% neutral buffered formaldehyde for histological examination. Paraffin sections were stained with hematoxylin and eosin.

RESULTS

Gross appearance of positive loops. Most of the exploratory work was done with two strains: NCTC 8238, a Hobbs' type 2 "food-poisoning" strain, and ATCC 3624, a "classical" type A having no known association with food poisoning. The strains were grown at 37 C for 4 hr in Veal Broth (15). ATCC 3624 never produced a reaction in the ileal loops, but the other strain gave variable results in different animals.

A positive loop response consisted of gross swelling of the loop due to the accumulation of 8 to 44 ml of light brown to bloody fluid of *p*H 7.6 to 7.9. Gas was present in many of the positive loops and in some of those not considered positive; the volume was much less in the latter. The distinction between a positive and negative response was based on the presence of fluid; merely observing the intact loop was misleading, for gas alone could result in slight dilation of a negative loop. Figure 1 shows the gross appearance of ligated loops with different responses.

Effect of culture medium on loop responses. Strains of *E. coli* produced variable responses in the ligated loop when injected in peptone-water, but they consistently evoked a positive reaction when injected in milk (13). The possibility that the response of the ligated loop to *C. perfringens* was influenced similarly by the culture medium was studied with eight strains.

The cultures for animal tests were grown at 37 C for 4 hr in Veal Broth, Fluid Thioglycollate Medium, Nutrient Broth (Difco), Skim Milk (Difco), and 5% tryptone (Difco). Seven strains produced a positive loop response when grown in milk. The same strains grown in the other media were ineffective, except for one cultured in Veal Broth.

Effect of age of rabbit. Animals of 3, 6, 7, 9, 10, and 12 weeks were tested. The isolated loops were injected with 4-hr milk cultures of NCTC

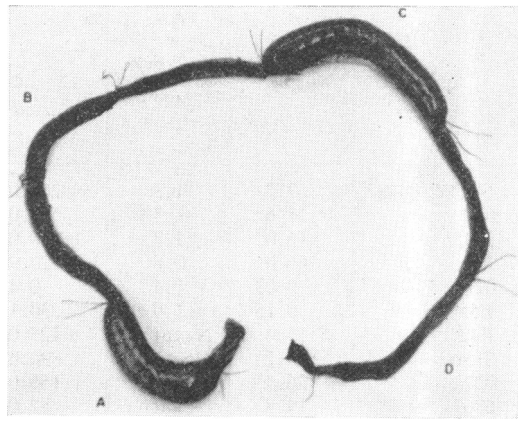

FIG. 1. *Gross appearance of the ligated rabbit ileum 24 hr after injection of 2 ml of cultures of Clostridium perfringens grown 4 hr at 37 C in Skim Milk. Loop A, strain NCTC 8798, 8 ml of fluid in loop; loop B, 2 ml of sterile milk, negative loop; loop C, strain T-65, 10 ml of fluid; loop D, strain 6867, negative loop.*

8238 and ATCC 3624; challenge inocula used were expected to produce consistently positive and negative responses, respectively. Results were identical to those obtained previously; this indicated that age of the animal was not the critical factor in determining whether a given strain would provoke a reaction in the isolated intestinal segment.

Correlation of number of organisms with loop response. Absence of a response was not due to failure of the organism to survive. In every instance where the ligated loop did not respond to challenge, the total cell count of *C. perfringens* in the loop at necropsy was much higher than the number of organisms administered in the inoculum (Table 1). The count of *C. perfringens* in the intestinal segment injected with sterile milk was higher than in the uninjected loop of the same animal, but multiplication in the presence of milk of *C. perfringens* indigenous to the intestine could account for only a small fraction of the final numbers in the challenged loops. The indication that a greater number of spores developed in the positive than in the negative loops (Table 1) was not statistically significant at the 95% confidence level.

The quantitative response of the loop (volume/length ratio) indicated a relationship to the number of cells in the challenge dose (Table 2); with a given strain, lower numbers produced less reaction. This was true whether spores or vegetative inocula were used. Final total counts per loop were approximately the same, although the number of organisms in the inoculum and the volume of fluid present in the segment at necropsy differed.

Time-course of a positive response. The development of a positive loop reaction after injection of *C. perfringens* 4-hr milk cultures was followed in respect to time (Fig. 2). An approximately linear increase in the loop volume/length ratio occurred through 24 hr with NCTC 8238 and 8798, but the plot of the response indices of strain 214a was erratic. However, the choice of a 20- to 24-hr holding period is supported by the data.

Loop response to food-poisoning and nonfood-poisoning associated strains. Responses of the ligated intestinal segments to all the *C. perfringens* strains tested are given in Table 3. The different methods of preparing the challenge inocula were used mainly with strains whose actively growing 4-hr milk cultures did not elicit a reaction.

The food-poisoning associated strains were most likely and the strains of other origins were least likely to produce a reaction in the ligated gut when 4-hr milk cultures were used for challenge. Several strains, not provoking a response when 4-hr milk cultures were used for injection, did so when the challenge inoculum was prepared by one of the other procedures.

TABLE 1. *Total cell and spore counts of Clostridium perfringens in positive and negative rabbit ileal loops injected with different strains*

Strain	Challenge[a,b]	Loop fluid volume/length ratio[c]	Final counts/loop					
			Challenged		Uninjected		Injected with 2 ml of sterile milk	
			Total	Spores	Total	Spores	Total	Spores
NCTC 8449	7.2	1.5	280.0	4.25	0.448	0.0	0.091	0.0
65	11.6	1.3	569.0	2.42	0.448	0.0	0.991	0.0
79955	16.0	1.7	1,060.0	49.5	0.042	0.00007	0.077	0.00021
127d	66.0	0.8	20.9	16.8	0.00035	0.00007	0.0028	0.00007
021	0.03	1.4	0.456	0.00475	0.00035	0.00007	0.0028	0.00007
F5a	0.15	1.0	30.1	69.3	0.00035	0.00007	0.0028	0.00007
F42	14.4	Negative	125.0	0.00016	1.23	0.00027	28.5	0.14
215b	14.2	Negative	890.0	0.002	1.23	0.00027	28.5	0.14
027	26.4	Negative	1,156.0	0.00012	0.042	0.00007	0.077	0.00021
6867	19.8	Negative	82.0	0.21	0.042	0.00007	0.077	0.00021
ATCC 3624	58.0	Negative	1,760.0	1.28	2.46	0.088	2.56	0.148
093	28.7	Negative	1,110.0	0.182	1.34	0.00001	2.0	0.00047
214d	18.0	Negative	1,800.0	45.0	1.34	0.00001	2.0	0.00047
ATCC 3629	14.0	Negative	2,740.0	0.0001	1.34	0.00001	2.0	0.00047

[a] A 2-ml amount of 4-hr-old milk cultures grown at 37 C injected per loop.
[b] All values $\times 10^6$.
[c] Positive ileal loop response when ratio is given.

TABLE 2. *Rabbit ileal loop response to the injection of graded amounts of spores or vegetative cells of Clostridium perfringens*[a]

Strain injected	Total no. injected	Loop vol/length ratio[b]	Final counts/loop	
			Total	Spore
NCTC 8238 spores	2 ml of sterile milk	Negative	3.01×10^5	7.14×10^3
	Blank	Negative	1.55×10^6	2.73×10^4
	2.58×10^6	0.6	4.13×10^9	1.11×10^8
	3.02×10^4	0.3	1.97×10^9	1.74×10^8
	2.80×10^2	0.3	2.70×10^9	4.90×10^7
NCTC 8238 vegetative cells	2 ml of sterile milk	Negative	1.0×10^2	1.40×10^2
	Blank	Negative	7.0×10^1	2.80×10^2
	3.20×10^6	1.4	2.0×10^9	1.06×10^8
	2.12×10^4	1.2	2.02×10^9	2.17×10^8
	9.80×10^2	0.7	1.09×10^9	4.07×10^8
NCTC 8798 vegetative cells	2 ml of sterile milk	Negative	6.40×10^4	1.03×10^3
	Blank	Negative	9.60×10^4	1.80×10^3
	5.0×10^5	1.5	1.32×10^8	1.40×10^5
	3.04×10^4	0.9	1.30×10^9	1.29×10^5
	2.20×10^3	0.1	4.10×10^8	2.0×10^5

[a] Spores and vegetative cells injected in a final volume of 2 ml of milk. Vegetative cells obtained from 16-hr cultures in Fluid Thioglycollate Medium.

[b] Positive response when loop fluid volume/length ratio is given.

Histological findings. The gross appearance of the challenged ligated loop was indicative of the presence or absence of tissue damage (Fig. 3). Normal bowel structure, with a minimal number of inflammatory cells in the mesentery, was found in intestinal segments not showing a macroscopically detectable reaction (fluid accumulation) to injection of two food-poisoning-associated and three nonassociated strains. Similar nonspecific histological changes were seen in two uninjected segments and five loops injected with sterile milk. The pathological picture of macroscopically positive loops ranged from complete destruction of the intestinal villi to only separation and slight effacing of the villi.

Attempts to induce diarrhea in normal rabbits. An amount of 10 ml of 4-hr-old milk cultures of strains NCTC 8238 and T-65 injected into the ileum of both fasted and unfasted 7-week-old rabbits. The animals were anesthetized with ether, the intestine was externalized, and the inoculum was injected intraluminally, approximately 80 cm anterior to where the intestine shares the mesentery with the appendix. The intestine of the rabbit was not ligated. Food and water were made available after the operation. Diarrhea did not occur in any of the rabbits.

Cultures of strains NCTC 8238 and NCTC 8798 grown for 16 hr in 20 ml of Fluid Thioglycollate Medium at 37 C were centrifuged, and the harvested cells were resuspended in 5 ml of

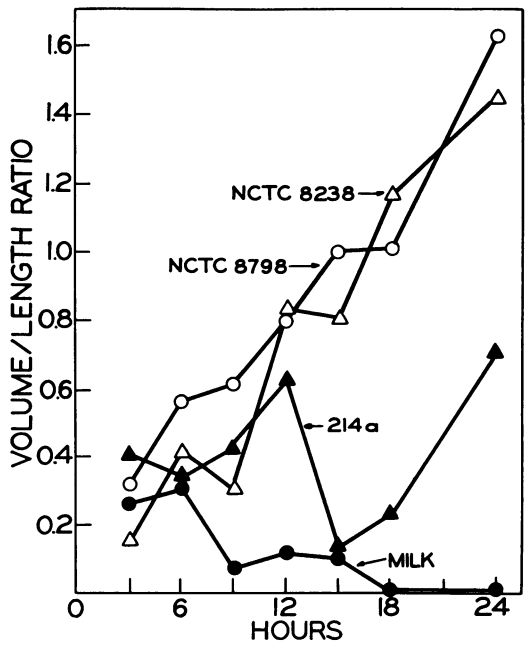

FIG. 2. *Time-course of appearance of fluid in rabbit ileal loops injected with Clostridium perfringens. Cultures incubated 16 hr at 37 C in 10 ml of Fluid Thioglycollate Medium; cells were collected by centrifugation and resuspended in 5 ml of sterile milk. Each suspension (2 ml) was injected into a loop. Each point is the average of results from two different rabbits.*

TABLE 3. *Responses of ligated ileal loops to injections of different strains of Clostridium perfringens*

Strains from food-poisoning outbreaks	Preparation of challenge inoculum[a]				Strains not from food-poisoning outbreaks	Preparation of challenge inoculum[a]			
	I	II	III	IV		I	II	III	IV
NCTC 8238	+++++[b]	+			ATCC 3624	−−	−+	+	+
NCTC 8247	−−	−	−	−+	F5a	−−	−−	++	
NCTC 8678	−−+	−		+	F42	−−	−	−−	−
NCTC 8679	++	+			F12	−−−+	+	−	+
NCTC 8449	+++	−−	++		027	−−	−	−	−
NCTC 8235	++	+			021	−−	−−	++	
NCTC 8798	++	+			030	−−	−+		
NCTC 8799	−−−	−			215[b]	−−	−	−	+
NCTC 9851	++	+			75	−−	−		+
NCTC 10240	++	−			65	−−+	−−	+	
77455	++	+			127d	−−	−+	+	
77516	++	−			164	−−−	−		+
79393	+−−	+			L2	−−	−	−	+
79394	−−	−	++		L83	−−	−	+	−
79955	−−	−+	++		L100	−−	−		+
S45	−−	−		+	108	−−	−	+	−
S34	−−	−		−	093	−−++	−		
S40	−−	−		+					
A48	−−	−		+	ATCC 3629 (type D)	−−	−	−	−
T65	++−	+							
214a	+++	−							
214d	−−	−	+	−					
6867	−−	−	+						
IU1168	+−−	−	+	−					
E13	++	+	+						
68900	++−−								
67944	++								
67945	++								
68227	++								

[a] I: Fluid Thioglycollate Medium culture incubated for 16 hr; 1 ml inoculated into 10 ml of Skim Milk and incubated for 4 hr. II: Same as I, except milk culture incubated for 16 hr. III: Five successive daily transfers in milk with last culture incubated for 16 hr. IV: Culture grown for 16 hr in 10 ml of Fluid Thioglycollate Medium; cells collected by centrifugation and resuspended in 5 ml of milk. All incubations at 37 C. Loops injected with 2 ml.

[b] Symbols: + or −, fluid or no fluid accumulation in ligated loop. Each + or − indicates the result obtained in different rabbits.

sterile milk. The cell suspensions were administered by stomach catheter to both 7-week-old rabbits fasted for 48 hr and 8-day-old suckling rabbits fasted overnight. Diarrhea was not induced by the procedure.

Effect of lecithinase C in ileal loops. A low (NCTC 8238) and a high (ATCC 3624) lecithinase-producing strain were grown in Veal Broth; a 30-fold concentration of the supernatant fluids was obtained by successive dialysis against 50% (w/v) Carbowax 20,000 and distilled water, followed by lyophilization and resuspension in *p*H 7.0, 0.02 M phosphate-buffered saline. Lecithinase activity was determined on a microgram basis (14).

Injections of 1,000 and 200 μg of these preparations into isolated loops produced no response. Similar challenges with 400 μg of purified lecithinase C (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline or with 1,000 μg dissolved in 2 ml of skim milk were also ineffective.

Effect of sterile culture supernatant fluids in ileal loops. If *C. perfringens* cultures which produced positive loop responses were producing a toxic material which was released into the growth medium, the same response should be obtained with cell-free culture supernatant fluids. Strains NCTC 8238 and NCTC 8798 were grown at 37 C for 4, 8, 12, 16, or 24 hr in milk, and culture supernatant fluids were passed through a Seitz filter under positive pressure. A 2-ml amount was injected into ileal loops. In all cases, negative results were obtained.

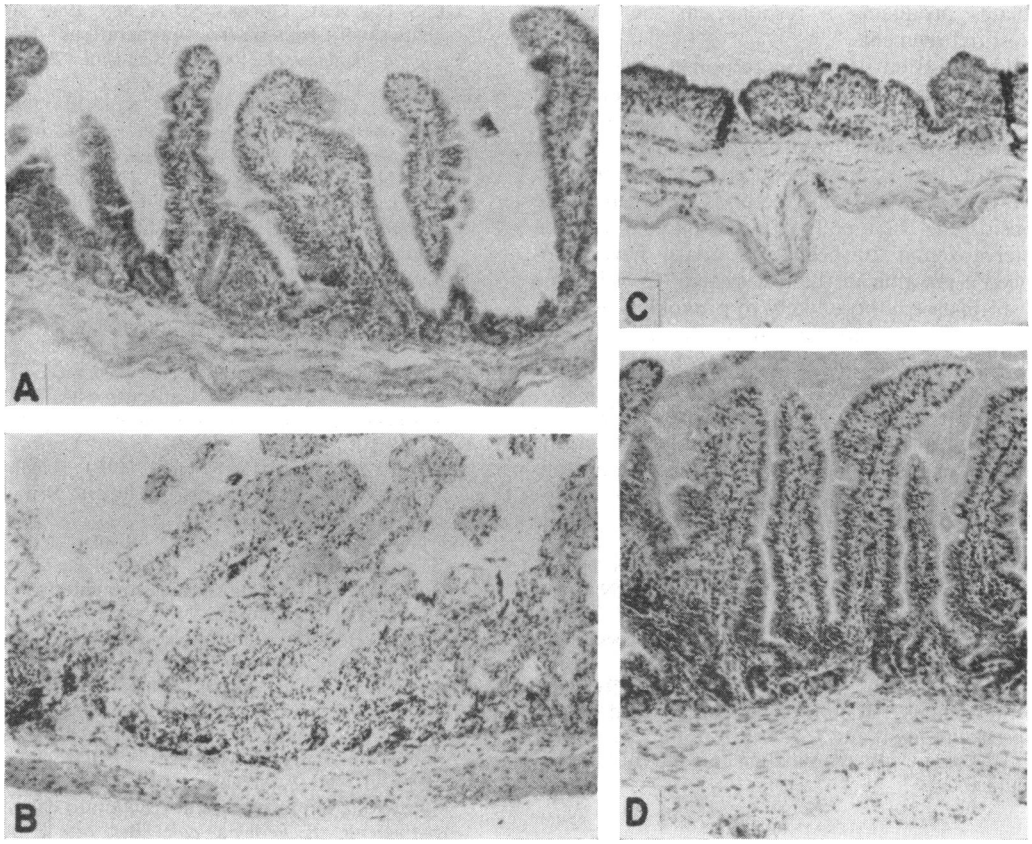

FIG. 3. *Histological sections of rabbit ileal loops injected with strains of Clostridium perfringens. Cultures grown in milk for 4 hr at 37 C were injected (2 ml) into each loop. All sections were stained with hematoxylin and eosin. Original magnification, × 41. (A) Strain 214a: positive loop with 14 ml of fluid. Dilated gut; villi separated and effaced but otherwise intact; edema with a few inflammatory cells in the mesentery. (B) Strain NCTC 8238: positive loop with 8 ml of fluid. Dilated gut; engorged vessels; hemorrhage in mucosa; almost all of mucosa digested; inflammatory cells and edema in mesentery. (C) Strain NCTC 10240: positive loop with 44 ml of fluid. Markedly dilated gut; mucosa almost effaced; inflammatory cells and hemorrhage. (D) Strain 65: negative loop. Normal gut; appears the same as loops uninjected or injected with sterile milk.*

The possibility existed that a toxic substance was produced in vivo. Positive ileal loops were obtained by injection of 4-hr milk cultures of strains NCTC 8238 and NCTC 8798; the fluids collected from the segments were centrifuged and sterilized by passage through a Seitz filter. An amount of 2 ml was then injected into ileal loops of another rabbit. The loops were negative in both cases.

DISCUSSION

The accumulation of fluid in the ligated ileal loop of the rabbit after intraluminal injection of *C. perfringens* type A cultures was influenced markedly by the method of preparation of the challenge inoculum. The culture medium in which the strains were grown was important; of five different media tested, cultures grown in skim milk gave the most frequent positive responses. However, cells developed in other substrates and suspended in milk for animal injection were also effective in provoking a reaction (Table 3).

The clearest differentiation between isolates from food-poisoning outbreaks and those from other sources was obtained by use of cultures grown in Skim Milk for 4 hr at 37 C. Of the 29 strains associated with outbreaks, 14 consistently induced a positive loop response as contrasted to none among the other 18 strains (Table 3). Since *C. perfringens* food poisoning is probably caused by both the so called "food-poisoning" and "classical" type A strains (6), it is interesting that both categories of type A were among the

isolates producing a response in the isolated intestinal segments.

If the ligated ileal loop response is related to the diarrhea of *C. perfringens* food poisoning in humans, the majority of the type A strains are potential causative agents of this illness; 42 out of 46 of these strains produced at least one positive response when all methods of testing were considered. The type D strain, capable of causing enterotoxemia in sheep and goats, never produced a reaction in the test system. Certain type A strains seem more likely to provoke a reaction in the isolated intestinal loop; it may be that these are the ones likely to be involved in human food-poisoning outbreaks.

A better understanding of the variables involved may make the ligated ileal loop of the rabbit a useful tool for the study of food poisoning caused by *C. perfringens*.

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LITERATURE CITED

1. BURROWS, W., AND G. M. MUSTEIKIS. 1966. Cholera infection and toxin in the rabbit ileal loop. J. Infect. Diseases **116**:183–190.
2. DACK, G. M. 1964. Microbial food poisoning. Food Technol. **18**:1904–1906.
3. DE, S. N., AND D. N. CHATTERJE. 1953. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Pathol. Bacteriol. **66**:559–562.
4. DE, S. N., M. L. GHOSE, AND A. SEN. 1960. Activities of bacteria-free preparations from *Vibrio cholerae*. J. Pathol. Bacteriol. **79**:373–380.
5. DUNCAN, C. L., AND D. H. STRONG. 1968. Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. **16**:82–89.
6. HALL, H. E., R. ANGELOTTI, K. H. LEWIS, AND M. J. FOTER. 1963. Characteristics of *Clostridium perfringens* strains associated with food and food-borne disease. J. Bacteriol. **85**:1094–1103.
7. HOBBS, B. C. 1965. *Clostridium welchii* as a food poisoning organism. J. Appl. Bacteriol. **28**: 74–82.
8. HOBBS, B. C., M. E. SMITH, C. L. OAKLEY, G. H. WARRACK, AND J. C. CRUICKSHANK. 1953. *Clostridium welchii* food poisoning. J. Hyg. **51**:75–101.
9. JENKIN, C. R., AND D. ROWLEY. 1959. Possible factors in the pathogenesis of cholera. Brit. J. Exptl. Pathol. **40**:474–481.
10. MCNAUGHT, W., AND G. B. S. ROBERTS. 1958. Enteropathogenic effects of strains of *Bacterium coli* isolated from cases of gastro-enteritis. J. Pathol. Bacteriol. **76**:155–158.
11. NYGREN, B. 1962. Phospholipase C-producing bacteria and food poisoning. Acta Pathol. Microbiol. Scand. (Suppl.) **160**:1–88.
12. TAYLOR, J., AND M. P. WILKINS. 1961. The effect of *Salmonella* and *Shigella* on ligated loops of rabbit gut. Indian J. Med. Res. **49**:544–549.
13. TAYLOR, J., M. P. WILKINS, AND J. M. PAYNE. 1961. Relation of rabbit gut reaction to enteropathogenic *Escherichia coli*. Brit. J. Exptl. Pathol. **42**:43–52.
14. Weiss, K. F., AND D. H. STRONG. 1967. Some properties of heat-resistant and heat-sensitive strains of *Clostridium perfringens* J. Bacteriol. **93**:21–26.
15. WEISS, K. F., D. H. STRONG, AND R. A. GROOM. 1966. Mice and monkeys as assay animals for *Clostridium perfringens* food poisoning. Appl. Microbiol. **14**:479–485.