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A SEVERE NECROTIC ENTEROTOXIN PRODUCED BY CERTAIN FOOD, FOOD POISONING AND OTHER CLINICAL ISOLATES OF *BACILLUS CEREUS*

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Summary.—The ability of certain strains of *Bacillus cereus* consistently to elaborate a filterable non-dialysable toxin capable of causing severe disruption and necrosis of the intestinal mucosa and submucosa is confirmed. This property is not universal to all *B. cereus* strains and different degrees of production of this toxin are exhibited by the different strains which produce it. The necrotic effect is produced by whole-cell cultures of the toxin producing strains in broth and in boiled rice. Some characteristics of this necrotic toxin are described and its relationship with the diarrhoeal and other known *B. cereus* toxins is discussed.

As a result of the investigation into the production of enterotoxins by isolates of *Bacillus cereus* from routine foods and from food poisoning cases, evidence was provided that strains could be classified into diarrhoeal, vomiting and non-enterotoxigenic types (Melling *et al.*, 1976; Turnbull, 1976). In addition, concentrated cell-free filtrates of broth cultures of a strain isolated from a brain abscess in an infant were observed to cause disruption of the normal villous architecture and a stripping away of the mucosa in ligated rabbit ileal ("rabbit loop") tests. This strain also caused fluid accumulation as did the diarrhoeal strains. The existence of a third enterotoxin was thus proposed which, it was suggested, may have been the pyogenic agent of the brain abscess from which the strain was originally isolated.

Further studies indicated that a strain isolated from a case of food poisoning was similarly able to disrupt the mucosa.

This investigation was undertaken to determine whether these strains consistently produced this effect in the rabbit intestine and under what conditions detectable levels of the causative toxin were elaborated. Tests were also carried out using other *B. cereus* strains from various sources in search of other strains consistently producing this type of toxin.

Guinea pig skin tests were included in an attempt to relate this toxin to the necrotic toxin described by Glatz and Goepfert (1973).

MATERIALS AND METHODS

Organisms.—In direct continuation of previous studies (Turnbull, 1976), tests were initially conducted using concentrated cell-free filtrates of cultures of 11 *B. cereus* strains. Further tests involving unconcentrated cell-free filtrates (UCCF), whole cell cultures and spore preparations of several of these strains were also carried out. The histories of the 11 strains are summarized in Table I. Five of the strains (4433, 4810, 2532, 4096, 2141) had been used in the previous studies and, apart from Strain 2141, were from foods or food poisoning cases. The remaining 6 strains (836–841) were all non-food-related clinical isolates kindly supplied by Mr A. J. Taylor, St. Mary's Hospital, London. The case history on Strain 936 has been described (Barnham and Taylor, 1977).

For the majority of tests, the organisms were grown in brain–heart infusion broth with

TABLE I.—*Histories of the* Bacillus cereus *Strains Used*

Strain	Serotype	Type and source
2141/74	11	Pyogenic—brain abscess
2532/74	UT	Routine isolate—raw rice
4096/73	4	Vomiting—cooked rice
4433/73	2	Diarrhoeal—meat loaf
4810/73	1	Vomiting—cooked rice
836/76	UT	Blood culture and wound site
837/76	17	Wound side after prostatectomy
838/76	UT	Antral washouts
839/76	13	Wound site of pinned leg
840/76	12	Site of total hip replacement
841/76	11	Baby—umbilical wound site

UT untypable

glucose added to 0·1% (BHIG; Difco) shaken for 18–20 h at 36°.

Preparation of toxins.—Tests using concentrated cell-free filtrates of the BHIG culture were carried out according to the method of Turnbull (1976). The BHIG culture was centrifuged (2000 *g* for 40 min) and the supernatant filtered through a 0·45-μm Millipore filter. Remaining deposits were plated out on blood agar and checked after overnight incubation at 36° for purity; periodic serotype checks were also made, by the method of Taylor and Gilbert (1975). Concentration of the filtrates was initially done by dialysis at 4° against 7 parts of polyethylene glycol ("Carbowax"; Union Carbide) in 10 parts (w/v) of water. In later tests, ammonium sulphate precipitation was used as an alternative concentration procedure. Preliminary tests on the value of Minicon-B15 ultra-filters (Amicon Corporation—exclusion mol. wt. 15,000) for concentrating the toxin were also carried out.

Animal assays.—The majority of tests carried out were by the ligated rabbit ileal tests as described by Turnbull (1976). Freshly weaned rabbits (750–1500 g) were used on all but one occasion when Strain 4096 was tested in an adult rabbit. Ileal sections were generally 8–12 cm long and adjacent loops were separated by interloops of approximately 2 cm. Two ml of the crude toxin preparations were injected into each loop. If less than 2 ml was available, then the length of the loop was reduced accordingly. The number of loops tied in a single rabbit was usually 5, never >6, and inocula from the different strains were administered in random order to allow for possible differences in sensitivity along the length of small intestine used.

The young rabbits were held for 7–8 h and the adult rabbit for 20 h between inoculation and killing and examination of the loops. The length (L) of the loop and the volume (V) of the accumulated fluid were recorded; a final V/L ratio of >0·3 was regarded as evidence of net fluid secretion into the lumen. Specimens of gut from each loop were transferred to ice-cold 10% formalin in 0·85% saline and prepared for histological examination. Sections were stained in haematoxylin and eosin and mucosal damage was assessed on a 0-to-4+ scale (Fig. 1).

Cell-free filtrates of heat-stable enterotoxin-producing *Escherichia coli* 0148 (Rowe, Taylor and Bettelheim, 1970) and 027 (Hobbs *et al.*, 1976), known to produce V/L ratios similar to those obtained with the toxigenic *B. cereus* strains in the same rabbit model, were prepared and used for comparisons and controls.

Other procedures.—Tests were also carried out with the rabbit loop technique using inocula of whole-cell cultures grown in BHIG or in rice. Two methods were used in the case of whole-cell cultures in BHIG. In the first, 2 ml of overnight culture (36° with shaking) were injected directly into the loops. In the second method, 0·2–0·5 ml of culture were made up to 2 ml with fresh BHIG just prior to injection into the loop with a view to obtaining growth of the *B. cereus in situ*. Rice cultures were prepared by the method of Melling *et al.* (1976) and spore suspensions were prepared according to the method of Gilbert, Stringer and Peace (1974).

Guinea pig necrotic skin tests were carried out in parallel with rabbit loop tests using a number of the concentrated cell-free filtrates; 0·05-ml volumes of the respective concentrates were injected intradermally in duplicate.

RESULTS

Cell-free culture filtrates

V/L ratios and semi-quantitative assessments of histopathological damage to the rabbit intestinal wall and of the necrotic reactions in guinea pig skin are shown in Tables II and III.

Using the polyethylene glycol concentration method by which the necrotic toxin was originally detected, a high degree of consistency was found among the strains (Table II). When freshly prepared, Strains 2141, 4096 and 4433 consistently produced moderate to severe mucosal damage while Strain 837 always produced extremely severe (4+) damage. Loss of efficacy resulted from a procedure in which concentrated filtrates of cultures of Strains 2141 and 4096 were prepared in batches, held at −10°, and thawed and refrozen for each of 6 successive

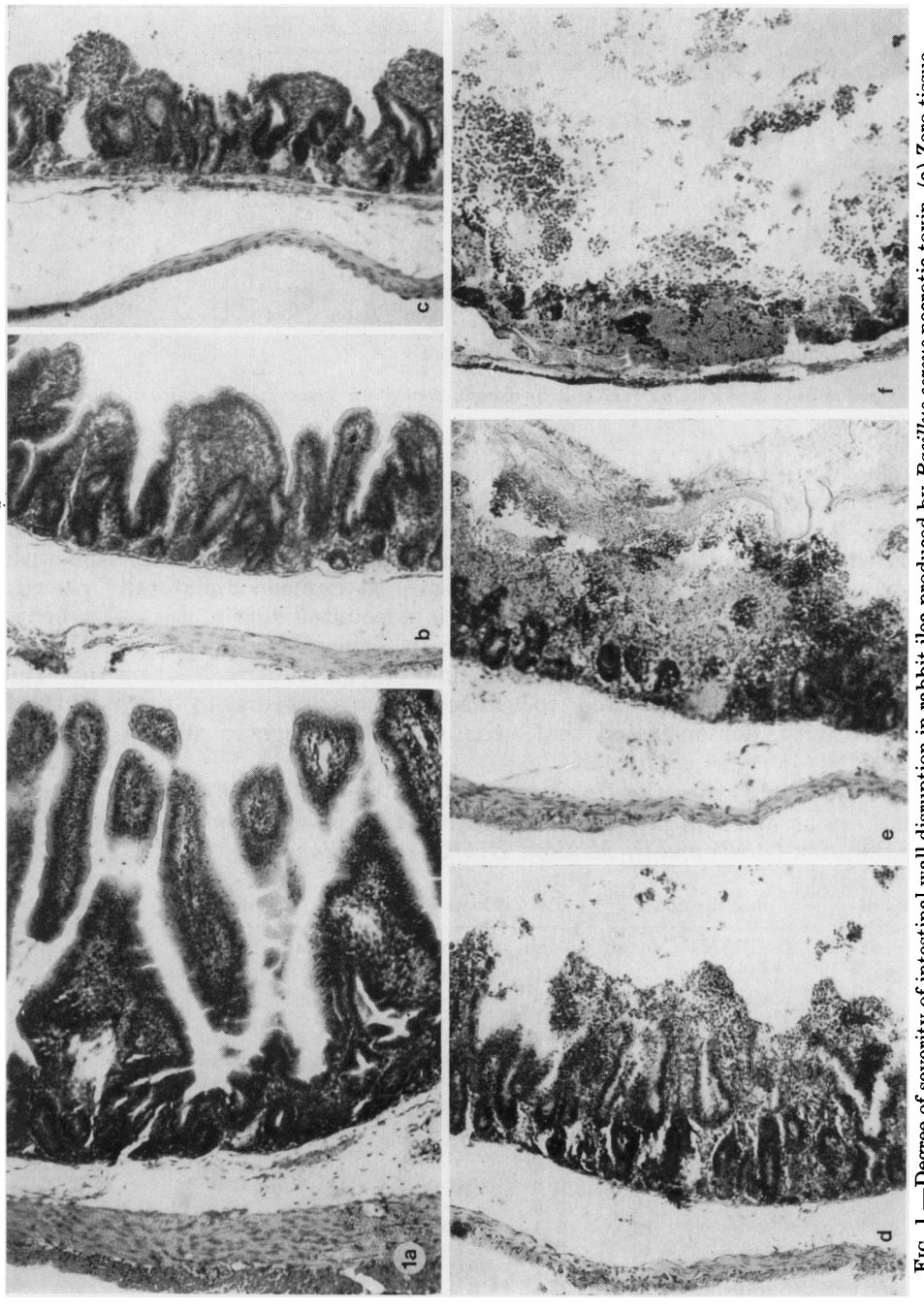


FIG. 1.—Degree of severity of intestinal wall disruption in rabbit ilea produced by *Bacillus cereus* necrotic toxin. (a) Zero tissue damage and no fluid accumulation (V/L $< 0{\cdot}3$). (b) Zero damage but pressure of accumulated fluid has shortened and thickened the villi and stretched the muscularis and submucosa. (c) $\pm$ to 1 + damage; foci of epithelial disruption apparent. (d) 2 +; upper villus disruption but crypts intact. (e) 3 +; general mucosal disruption, but some crypts remain. (f) 4 +; complete mucosal and submucosal destruction with muscularis also involved. These loops ruptured readily at necropsy. All photographs H. and E. $\times$ 30.

TABLE II.—*Results of Tests using Cell-free Filtrates of Cultures of* Bacillus cereus *Concentrated by Dialysis against Polyethylene Glycol*

Strain	No. of times tested	Mean conc.	Rabbit loop V/L*		Histological damage†		Guinea pig skin test†‡
			Mean	Range	Mean	Range	
2141	7	10	1·0	0·5–1·5	2+	1+ to 4+	2+
4096	8	8	1·1	0·5–1·4	2+	1+ to 4+	±
4433	2	6	1·3	1·2 and 1·4	3+	3+ and 4+	2+
4810	2	7	0·5	0·3 and 0·7	0	0 and 0	0
2532	2	6	0·8	1·1 and 0·5	2+	3+ and 1+	2+
836	4	10	0·4	0–0·7	±	0 to ±	3+
837	5	9	1·1	0·6–1·4	4+	All 4+	2+
838	4	10	0·8	0·2–1·7	±	0 to 1+	NT
839	3	7	0·5	0–0·7	1+	0 to 3+	NT
840	3	6	0·7	0–1·3	0	0 to ±	NT
841	3	6	0·7	0–1·2	0	0 to ±	NT

* V/L = ratio of volume of accumulated fluid : loop length.
† Measured on a 4+ scale (see Figs. 1, 3).
‡ Test performed once in duplicate for each culture except 4096 which was measured twice in duplicate.
NT = not tested.

tests. By the fourth thawing, at approximately 2 weeks, V/L readings were <0·3 and no histological damage was produced by these preparations.

With only a single exception, all loops that showed histological damage also showed fluid accumulation. *Vice versa*, results with Strains 4810, 836 and 838 to 841, which exhibited undetected to low necrotic activity, demonstrated that fluid accumulation could occur without associated histological damage. There was frequently a range of histopathological effect within one histological section with the severest damage unilaterally placed; this is accounted for in the assessments given in Tables II to IV. Epithelial and lamina propria cells exfoliating into the lumen after exposure to the toxins of the strongly toxigenic strains had nu-

TABLE III.—*Results of Tests using Unconcentrated Cell-free (UCCF) Filtrates of Cultures of* Bacillus cereus *and Filtrates Concentrated by Ammonium Sulphate Precipitation at 60% Saturation (ASP) and by Minicon B15 Ultrafiltration (Mi)*

Strain	No. of times tested	Concentration		Rabbit loop V/L*		Histological damage†		Guinea pig skin test†‡
		Method/UCCF	Mean	Mean	Range	Mean	Range	
2141	2	UCCF	1	1·1	1·3 and 0·9	1+	1+ to 2+	NT
2141	3	ASP	13	0·4	0·1–0·8	±	0 to 1+	±
4096	3	UCCF	1	1·1	0·8–1·3	2+	± to 3+	NT
4096	4	ASP	12	0·7	0·3–1·0	1+	0 to 4+	0
4096	3	Mi	11	0·5	0–0·8	1+	0 to 3+	4+
4433	3	UCCF	1	1·2	1·2–1·3	2+	1+ to 3+	NT
4433	2	ASP	13	0·7	0 and 1·4	0	0 and 0	2+
4810	3	UCCF	1	0·3	0–0·8	0	All 0	NT
4810	3	ASP	12	0·2	0–0·5	0	All 0	0
4810	1	Mi	13	0·8	—	±	—	0
2532	3	UCCF	1	0·8	0·2–1·3	0	All 0	NT
2532	5	ASP	13	0·5	0–1·1	1+	0 to 1+	2+
836	2	ASP	13	0·6	0·3 and 1·0	1+	0 and 2+	1+
836	1	Mi	13	0	—	0	—	1+
837	3	UCCF	1	1·1	0·7–1·7	3+	1+ to 4+	NT
837	3	ASP	10	0·8	0·4–1·2	3+	0 to 4+	2+
837	1	Mi	13	0·8	—	3+	—	4+

* V/L = ratio of volume of accumulated fluid : loop length.
† Measured on a 4+ scale (see Figs. 1, 3).
‡ Test performed once in duplicate for each culture tested.
NT = not tested.

merous pyknotic, karyorrhectic or distorted nuclei. In gross appearance, severely damaged loops were thin-walled, occasionally rupturing on handling at necropsy, and frequently exhibiting numerous petechiae in the serosa; the sloughed mucosal cells appeared as thick white matter in the accumulated luminal fluid.

Of additional interest was the histological damage caused by concentrated filtrates of cultures of Strains 4433 and 2532 (Table II) not noticed in the previous studies (Turnbull, 1976).

Using UCCF and ammonium sulphate precipitation and Minicon B15 ultrafiltration methods of concentrating the crude toxin preparations, generally, though not exclusively, weaker fluid accumulation responses and histopathological damage were exhibited (Table III) but the pattern among the different strains remained the same as with the polyethylene glycol concentration method. The toxin appeared to be precipitated at 60% saturation of the culture filtrate with ammonium sulphate but not at 80% or 100%.

Inspection of loops exhibiting V/L ratios $>1{\cdot}0$ in response to the toxins of *E. coli* 0148 and 027 confirmed the absence of accompanying pathological changes apart from shortening and thickening of the villi.

Whole-cell preparations

The results of tests with overnight rice and BHIG cultures of 4 and 6 of the *B. cereus* strains respectively are given in Table IV. In the case of the broth cultures, the pattern among the different strains virtually duplicated that found with UCCF. In the broth method in which *in situ* growth was attempted, all strains gave negative V/L and histological results. Counts of *B. cereus* in the loop contents were not attempted, however, and so it is not known whether bacterial multiplication had occurred.

The observation of up to 3+ levels of histological damage from rice cultures of Strain 4096 suggests that food sufficiently contaminated with this type of strain could be injurious to the intestine.

Of special interest was the finding that with both rice and broth cultures, the bacterial cells of Strain 4096 were clearly visible without special straining in large numbers in association with the damaged tissues (Fig. 2) while, in the case of the other strains, regardless of the extent of damage, the organisms could only be found in small numbers with the aid of special staining.

The presence of the organisms stimulated an acute inflammatory response not seen with the cell-free preparations and the degree of which correlated closely

TABLE IV.—*Results of Tests using Whole Cell Inocula from Brain–Heart Infusion Broth Cultures Incubated* 18 *h at* 36° (*BHIG*) *and Rice Cultures Incubated* 18 *h at* 32° (*Rice*)

Strain	No. of times tested	Method	Rabbit loop V/L*		Histological damage†	
			Mean	Range	Mean	Range
2141	3	BHIG	0·7	0–1·2	±	0 to 1+
4096	4	BHIG	0·8	0·2–1·3	2+	0 to 4+
4096	3	Rice	0·4	0–0·8	2+	0 to 3+
4433	3	BHIG	0·9	0·8–1·0	1+	0 to 2+
4433	5	Rice	0·2	0–0·6	0	All 0
4810	4	BHIG	0·5	0·1–0·8	0	0 to ±
4810	5	Rice	0·3	0–0·5	0	0 to ±
2532	5	BHIG	0·7	0·3–1·0	±	0 to 2+
2532	2	Rice	0·1	0 and 0·3	—	0 and 0
837	3	BHIG	0·6	0·4–0·7	2+	0 to 3+

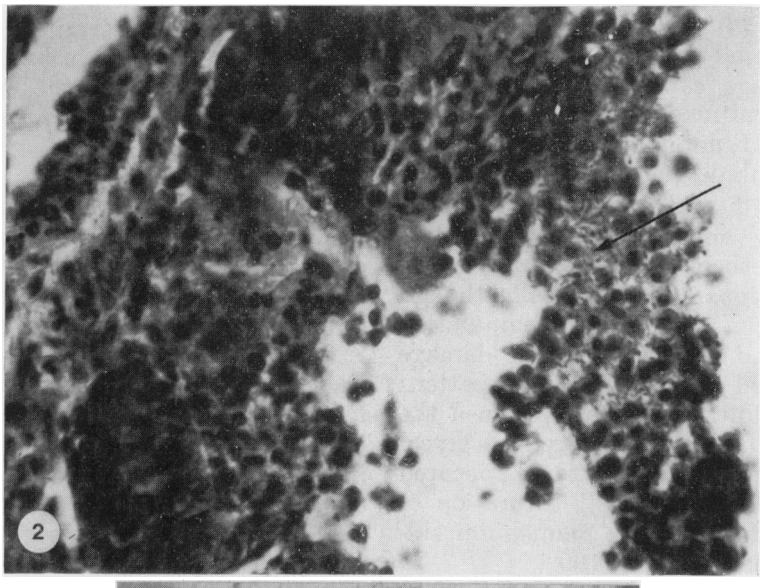
* V/L = ratio of volume of accumulated fluid : loop length.
† Measured on a 4+ scale (see Fig. 1).

with the extent of histological damage produced.

Spore preparations produced no effects in preliminary trials and further studies were postponed pending investigations into the various conditions under which spores are produced.

Further observations

Correlation of guinea pig necrotic skin



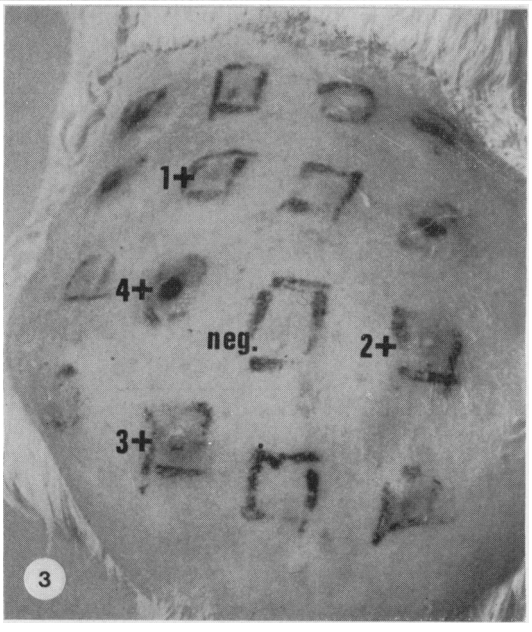


FIG. 2.— Large numbers of *Bacillus cereus* Strain 4096 evident in association with regions of villus disruption (arrow) following inoculation with rice culture. H. and E. × 400.

FIG. 3.—Guinea pig necrotic skin test.

test results with the damage in the rabbit intestine was at least partial (Tables II, III). Differences in the intensity of guinea pig necrotic skin reaction (Fig. 3) may have resulted from unequal intradermal inoculation from site to site in the guinea pig skin.

The lethal potential of the necrotic enterotoxin was demonstrated by the death of 6 of the rabbits $3\frac{1}{2}$–$5\frac{1}{2}$ h after inoculation of the loops despite attempts to revive with adrenalin; the deaths were always in association with one or more extremely strong responses to Strains 837, 2141 or 4096. Infant mice fed intragastrically with concentrated filtrates of cultures of Strains 2141 and 4096 (837 was not done) died within 1 h.

Liver and kidney sections were taken on all occasions to assess systemic uptake of the toxins and histological damage caused thereby. No histological damage was noted in the liver; in the kidney, occasional slight increases in inflammatory cells in the glomerular region and mild congestion of the capillaries were the only changes.

DISCUSSION

The results presented confirm and extend the hypothesis of Turnbull (1976) that certain strains of *B. cereus* can elaborate a toxin capable of severely disrupting the intestinal wall. Freshly prepared concentrated cell-free filtrates of the infant brain abscess isolate with which the phenomenon was first noted were consistently able to produce this effect but, in fact, to a lesser degree than 3 of 10 other strains similarly examined.

Strains producing the necrotic toxin clearly do so to different degrees under given conditions but the results with rice cultures and BHIG whole cell cultures *in situ* indicate that the opportunity for adequate multiplication by the organism is a prerequisite to production of sufficient toxin to produce tissue damage. The strain exhibiting the greatest toxin-producing ability (837) had readily responded to antibiotic therapy in the 79-year-old patient from whose inflamed surgical wound site it had been isolated and it was not at that time associated with extensive tissue damage.

The significance of the fact that mucosal disruption was, in all but one instance, associated with fluid accumulation is undetermined; passage of fluid from blood to lumen may result as much from physical effects of mucosal breakdown as from physiological effects of the toxin, although Kinsey *et al.* (1976) report that transmucosal permeability remains unchanged in the severe alternations in intestinal histology produced by a salmonella infection. That fluid accumulation was not, *vice versa*, necessarily associated with the necrotizing effect was evident from the results obtained with Strains 4810, 836 and 838 to 841. This supports the earlier proposal (Turnbull, 1976) that the behaviour of 2 separate factors is being observed.

The results do not rule out the possibility that the intestinal necrotic toxin is identical to the guinea pig necrotic skin toxin of Glatz and Goepfert (1973). On the other hand, differences in degree of reaction in the intestinal mucosa as compared with the guinea pig skin make it difficult to be certain they are identical. The *B. cereus* 4ac strain used by these workers was not included in this study.

Spira and Goepfert (1975) proposed that the guinea pig necrotic skin factor may be identical to the diarrhoeagenic factor; no evidence is provided here strongly to support or refute this hypothesis. However, where these authors report that repeated freezing and thawing of the concentrated filtrate had little effect on the diarrhoeagenic activity, it was found in the present study that the intestinal necrotic activity of Strains 2141 and 4096 was lost after the fourth thawing 2 weeks after preparation. In agreement with Spira and Goepfert's results were the present findings that polyethylene glycol concentration gave a better recovery of the enterotoxin

than ammonium sulphate precipitation on the basis of simple concentration factor. By approximate assessment, a six-fold concentration with polyethylene glycol was equivalent to fifteen- and twenty-fold factors with Minicon B15 ultrafiltration and ammonium sulphate respectively. The ultrafiltration results with Minicon B15 imply that the necrotic toxin is a compound with a mol. wt. >15,000.

Glatz and Goepfert (1973) were unable to separate lethal and guinea pig necrotic skin toxin. Preliminary evidence of the lethal potentials of strains producing intestinal necrotic toxin has been apparent in this study.

Reports of histopathological damage or lack of it in the intestine following introduction of enterotoxins of various Gram-positive and Gram-negative bacteria have been reviewed (Turnbull, 1976). Histological damage in guinea pig ileum in response to fairly high concentrations (100 μg/ml) of *Staphylococcus aureus* delta toxin has now also been reported (O'Brien and Kapral, 1976). Results with concentrated cell-free filtrates of *Vibrio cholerae* NCTC 7254 in the investigation of Turnbull (1976), and with cell-free preparations of loop positive *E. coli* in this study, confirmed the usual finding of an absence of mucosal degeneration in the 7-h rabbit model. However, that *E. coli* also may, on occasion, be associated with necrotizing enterocolitis is illustrated in the report of Speer *et al.* (1976).

Steps towards purification of the necrotic toxin and closer analysis of its relationship with diarrhoeal, vomiting and other rabbit loop active factors are now planned, although the obvious instability of the former can be expected to impede progress.

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