

# Effect of *Escherichia coli* on Fluid Transport across Canine Small Bowel

## MECHANISM AND TIME-COURSE WITH ENTEROTOXIN AND WHOLE BACTERIAL CELLS

R. L. GUERRANT, U. GANGULY, A. G. T. CASPER, E. J. MOORE, N. F. PIERCE,  
and C. C. J. CARPENTER

*From the Departments of Medicine, Baltimore City Hospitals, Baltimore 21224,  
and The Johns Hopkins University, Baltimore, Maryland 21205, and the  
National Institutes of Health, Bethesda, Maryland 20014*

**ABSTRACT** An *Escherichia coli* strain isolated from a patient with severe cholera-like diarrhea elaborates a partly heat-labile enterotoxin shown to cause prompt adenylyl cyclase stimulation and isotonic fluid secretion by canine jejunum. Both responses disappear upon removal of the enterotoxin. The duration of action of a submaximal dose of this *E. coli* enterotoxin was brief, despite sustained exposure to the jejunum, suggesting inactivation of the enterotoxin by its interaction with the mucosa.

Inoculation of whole bacterial cultures of this *E. coli* strain into canine duodenum was followed by bacterial survival and induction of net secretion after 4–7 h. The onset of fluid production was associated with increasing gut mucosal adenylyl cyclase activity. Washed bacterial cells could also produce fluid secretion. In vivo multiplication of this enterotoxin-producing *E. coli* was demonstrated 6–12 h after intraduodenal inoculation of approximately  $10^8$  organisms. This was associated with fluid secretion. Intestinal fluid production occurred without microscopic pathology in the mucosa.

### INTRODUCTION

Recent studies have suggested that noninvasive, enterotoxigenic *E. coli* strains cause a major portion of severe acute diarrheal disease, both in developing nations (1–3) and in the United States (4). In recent studies about

Dr. Guerrant is a Research Physician with the National Institutes of Health. Dr. Pierce is the recipient of a Research Career Development Award from the National Institutes of Health.

Received for publication 19 July 1972 and in revised form 15 February 1973.

50% of the adults hospitalized in Calcutta with acute “undifferentiated” cholera-like diarrhea had small bowel colonization by homogeneous isolates of *E. coli* which were not of previously recognized enteropathogenic serotypes. Cell-free culture filtrates of these strains contain a partly heat-labile enterotoxin which causes fluid accumulation in ligated segments of rabbit small bowel (2, 3). In canine jejunum, crude *E. coli* enterotoxin (ECT)<sup>1</sup> induces outpouring of isotonic fluid similar in composition to that induced by cholera enterotoxin (CT), but following a very different time-course (5). Earlier studies have suggested that the mechanism of action of ECT may be similar to that of CT, in that ECT does not alter the rate of jejunal fluid accumulation after a maximum secretory rate has been induced by CT (5). Further studies have indicated that ECT has an effect qualitatively similar to that of CT in stimulating adenylyl cyclase in rabbit ileal mucosa and in rat lipocytes (6).

It was the purpose of this study to further explore the mechanism of diarrhea induced by ECT by answering the following questions: (a) Is canine gut mucosal adenylyl cyclase activity consistently increased after intraluminal ECT administration, and if so, is the increased adenylyl cyclase activity correlated with net secretion of isotonic fluid by the canine small bowel? (b) Does challenge of the canine small bowel with viable enterotoxin-producing *E. coli* cause net fluid secretion, and if so, is this fluid secretion associated with enterotoxin production and with increased mucosal adenylyl cyclase activity?

<sup>1</sup>Abbreviations used in this paper: CT, cholera enterotoxin; ECT, *E. coli* enterotoxin; PSP, phenolsulfonylphthalin.

(c) Can the brief duration of action of ECT be due, at least in part, to deactivation of the enterotoxin within the bowel?

## METHODS

*Preparation of enterotoxins and viable cultures for intestinal challenge.* ECT was prepared from strain 334 (serotype O 15:H 11) which was isolated from the jejunum of a patient with severe cholera-like diarrhea in Calcutta (patient 924 in a previous publication [1]). A control preparation ("ECT" 10405) was made from *E. coli* strain 10405, which was isolated from the stool of a convalescing cholera patient at the Cholera Research Laboratory in Dacca, Bangladesh, and provided by Dr. Doyle J. Evans, Jr. Culture filtrates of *E. coli* 10405 have demonstrated no enterotoxin activity when tested in ligated segments of canine (5) or rabbit small bowel (6), nor do they enhance lipolysis in viable isolated rat epididymal lipocytes. Cultures were maintained on sealed nutrient agar slants at room temperature. Enterotoxin was prepared as described elsewhere (5). Briefly, Syncase broth (7) containing 0.1% sucrose was inoculated with 0.01 ml of an overnight Syncase culture and then shaken at 30°C for 18 h. Cells were removed by centrifugation and filtration. After dialysis and lyophilization, the dry powder was pooled into a single lot for this study and stored at 4°C. Purified CT was prepared by Finkelstein and LoSpalluto, (8) and provided as NIH lot 1071 by Dr. John Seal (National Institutes of Health).

For intestinal challenge with viable organisms, *E. coli* 334 and 10405 were grown at 37°C for 18 h in 5 ml peptone water (0.5% NaCl, 1% peptone [Difco Laboratories, Detroit, Mich.]) to approximately  $10^8$  viable organisms/ml. 100 ml Syncase broth (0.5% sucrose) in a high surface-to-volume ratio (2 cm<sup>2</sup>/ml) Roux flash was inoculated with 0.01 ml of this culture and incubated for 18 h at 37°C. A washed preparation of *E. coli* 334 was prepared from the above culture by twice centrifuging at 16,300 *g* for 30 min at 4°C, discarding the supernatant, and resuspending the cells in 100 ml of fresh Syncase. Duodenal challenge was performed promptly with 30 ml of either the washed or whole cultures (about  $10^8$  viable bacteria/ml) or with whole cultures diluted 200-fold in sterile Syncase (about  $10^6$  viable bacteria/ml), the latter used to detect *in vivo* bacterial multiplication. Counts of viable bacteria in the inocula were determined by the drop-plate technique (9).

*Preparation of dogs for challenge with enterotoxin or whole cells.* The dogs were mongrels of either sex weighing 10–20 kg. Dogs to be challenged intrajejunally with enterotoxin were starved overnight, anesthetized with pentobarbital, and the jejunum was exposed through a midline incision. Beginning 10 cm distal to the Treitz ligament, seven adjacent 10-cm jejunal segments were tied off *in situ* with umbilical tape, while care was taken to preserve normal blood supply. Each segment was cannulated with a multiperforated polyethylene tube (ID = 0.066 inches; OD = 0.098 inches) and separated from adjacent segments by two umbilical tape ligatures. Each segment was then washed by syringe aspiration with 10-ml volumes of physiologic saline until it yielded a clear effluent. All solutions employed were warmed to 37°C before use.

For duodenal challenge with viable organisms, dogs were similarly prepared except that a single 25-cm duodenal segment was isolated. Care was taken to preserve intact blood supply and exclude the common bile duct. Each end

of the segment was ligated with umbilical tape around a no. 24 Foley catheter without inflation of the balloon. The segment was washed with 200 ml of isotonic saline, flushed with 200 ml of air, and allowed to drain freely from both ends to a single collection flask. Loop effluents were measured hourly. Only loops secreting less than 2 ml/h during the two control periods preceding challenge were used. No attempt was made to perfuse duodenal segments or to flush them between collection periods, since this might interfere with bacterial multiplication and enterotoxin production. The duodenum, rather than the jejunum or ileum, was chosen because normal canine jejunum and ileum consistently absorb isotonic fluid, and relatively small alterations in fluid transport by these segments can be appreciated only by studies employing continuous intestinal perfusion (10).

*Determination of net water and electrolyte fluxes in small bowel.* In jejunal segments exposed to enterotoxin-containing electrolyte solutions, net water and electrolyte fluxes were measured during consecutive 10-min periods. For control studies, 12 ml of a solution containing Na (145 meq/liter), K (6 meq/liter), Cl (126 meq/liter), HCO<sub>3</sub> (25 meq/liter), and phenolsulfonphthalein (PSP, 50 mg/liter) was instilled into each segment. 2 ml was mixed by syringe aspiration and withdrawn as the initial sample. 10 min later the loop was emptied and a portion of the contents held as the final sample. During the 5 min between study periods, each segment was washed twice with 10 ml of saline. Enterotoxin challenge was accomplished by adding 500 µg/ml ECT 334 or ECT 10405, or 1 µg/ml purified CT to the test solution. Osmolarity of the test solution with 500 µg/ml ECT 334 was 308 mosmol/ml. This dose of ECT-334 represented a maximal dose, preliminary studies having shown that similar changes in net fluxes were induced by doses of 250 and 2500 µg/ml. In each dog two segments were studied before enterotoxin challenge, five segments were used to study the effects of ECT 334 and ECT 10405 challenge, and one segment was used to study the effect of CT. The position of these segments was systematically varied to compensate for any differences in function between proximal and midjejunum. To study the duration of effect of ECT 334 during prolonged contact with jejunal mucosa, ECT concentrations of 50 and 2500 µg/ml in the above solution were employed.

The original test solution and the initial and final samples were analyzed colorimetrically for PSP concentrations with a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) (11). To determine the net water flux for each study period, initial and final segment volumes were calculated as follows:

$$V_i = \frac{[\text{PSP}]_o \cdot V_o}{[\text{PSP}]_i},$$
$$V_f = \frac{[\text{PSP}]_i \cdot V_i}{[\text{PSP}]_f}.$$

Net water flux =  $V_f - V_i$  (µl/cm jejunum/min) where,  $V_o$  = original volume of test solution instilled,  $V_i$  = calculated initial volume, and  $V_f$  = calculated final volume. Sodium concentrations were determined with a flame photometer with internal lithium standard (Instrumentation Laboratory, Inc., Lexington, Mass.). Net sodium flux was expressed as sodium concentration in calculated net fluid added to or removed from the bowel lumen.

Duodenal segments were inoculated with 30 ml of one

of the culture preparations described above, and the catheters clamped for 1 h. Thereafter fluid was allowed to drain freely from the segment and measured at hourly intervals. Hematocrits were determined hourly, and saline was infused intravenously at a rate adequate to maintain a stable hematocrit during study. Plasma protein determinations (12) immediately before bacterial inoculation, and at 3 and 6 h after, confirmed that adequate hydration was maintained.

**Enterotoxin assay.** Duodenal fluid produced in the 6th h after challenge with whole cultures of *E. coli* 334 was assayed for enterotoxin in rabbit small bowel segments by a modification of the method of Kasai and Burrows (13). The fluid was sterilized by centrifugation for 45 min at 12,100 *g* at 4°C and passage through a 220-nm filter (Millipore Corporation, Bedford, Mass.). A series of 4-cm segments of small bowel were prepared in 8–10 wk-old New Zealand white rabbits as previously described (5). In each rabbit, segments were injected with 1 ml of sterile filtrate, normal saline (two segments), or a known potency of ECT 334 (two segments). After 6 h the animals were sacrificed, the volume of fluid in each segment measured, and the segment length determined. Values from three rabbits with negative saline control segments and positive ECT 334 segments were used to assay each sample.

**Adenyl cyclase assay in gut mucosa.** Biopsies of duodenum and jejunum for adenyl cyclase assay and histologic examination were obtained as described elsewhere (14). In jejunal segments they were taken immediately after enterotoxin challenge and immediately after the subsequent flux study periods. No more than two biopsies were performed in any segment. In the duodenum biopsies were obtained immediately before bacterial inoculation, and 3, and 6 h after.

Biopsy tissue was cooled to 0°C in isotonic saline, cold-washed with a solution of 75 mM Tris and 25 mM MgCl<sub>2</sub> at pH 8, and mucosal cells were immediately separated by scraping with glass slides. Within 1 min of biopsy, mucosal cells were homogenized with 10 strokes of a TenBroeck hand homogenizer in the same solution, and 20 μl of the homogenate was added to the reaction mixture. Immediately after being shaken on a vortex mixer, the reaction mixture was incubated for 10 min at 37°C. Activity of adenyl cyclase was assayed essentially by the method of Krishna, Weiss, and Brodie (15). The final composition of the reaction mixture (each assay = 50 μl) was 30 mM Tris (pH 8), 10 mM HgCl<sub>2</sub>, 10 mM theophylline, 5 mM phosphoenolpyruvate, 50 mg/ml pyruvate kinase, 20 mg/ml myokinase, and 1.5 mM ATP with 1 μCi [ $\alpha$ -<sup>32</sup>P]ATP. After a 10-min incubation, the reaction was stopped by adding of 0.5 ml of recovery mixture containing 50 μg adenosine 3',5'-cyclic monophosphate (cAMP), 100 μg ATP, and 0.01 μCi [<sup>3</sup>H]cAMP, shaking on a vortex mixer, and placing in a boiling water bath for 3 min. Reaction blanks for each set of experiments were incubated before adding protein homogenate and stopping. Cooled to room temperature, the content of each assay tube was placed on a 0.6-by-4-cm chromatographic column of Dowex AG 50 W-X2 (200–400 mesh) cation exchange resin (Dowex Ion Exchange Resins, Dow Chemical Co., Midland, Mich.). After elution of 99.2% of the ATP with the initial 2 ml of distilled water, the second 2 ml was collected. It contained about 55% of the newly formed [<sup>32</sup>P]cAMP as determined by [<sup>3</sup>H]cAMP recovery. The remaining ATP was largely removed from this eluent by twice precipitating and centrifuging at 700 *g* for 10 min with 0.2 ml 0.15 M Ba(OH)<sub>2</sub> and 0.2 ml 5% ZnSO<sub>4</sub> at pH 7.5–8.0. After the

second centrifugation, 2 ml of the supernate was placed in 15 ml of a scintillation mixture made with 2 liters of toluene, 1 liter of Triton-X (Packard Instrument Co., Inc., Downers Grove, Ill.), 16.5 g of 2,5-diphenyloxazole, and 0.5 g of 1,4-bis-(2-[4-methyl-5-phenoxazoly])-benzene. Double-isotope counting of <sup>3</sup>H and <sup>32</sup>P in each sample was done in a Packard Tri-Carb Liquid Scintillation Spectrometer (model 3375).

The protein content of the homogenate was determined by the method of Lowry, Rosebrough, Farr, and Randall (12); the homogenate had been diluted to an estimated range of 30–90 μg/20 μl (i.e., per assay) before the start of the assay. The adenyl cyclase activity was linear over this range of protein concentration. The results, expressed as picomoles of cAMP formed per milligram of homogenate protein per 10 min incubation, were then calculated from the specific activity of [<sup>32</sup>P]cAMP formed minus the reaction blank. The total amount of cAMP formed was corrected for recovery of cAMP as determined by [<sup>3</sup>H]-cAMP.

Curves were obtained which determined that 10 min was the optimal incubation time and that pH 8 was optimal for measurement of adenyl cyclase activity in canine jejunal mucosa stimulated by ECT 334. Direct addition of ECT 334 (500 μg/ml) to the homogenate had no effect upon conversion of ATP to cAMP.

**Histopathologic studies.** An unscraped portion of each biopsy was fixed in 10%-buffered formaldehyde and stained with routine hematoxylin and eosin, and with combined Alcian Blue periodic acid Schiff for mucin (16). Specimen pairs taken before *E. coli* inoculation, and 6 h after were examined by a pathologist who was unaware of the treatment of each tissue. The degrees of inflammation, tissue invasion by bacteria, goblet-cell prominence, and dilatation of villous lacteals or crypts were noted in each specimen.

**Statistics.** Except where specified, all *P* values were obtained by paired analysis using Student's *t* test.

**Materials.** [ $\alpha$ -<sup>32</sup>P]ATP [<sup>3</sup>H(G)] 2–6 Ci/mmol) and adenosine 3',5'-cyclic phosphate, ammonium salt ([<sup>3</sup>H]cAMP, 13–17 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. Cation exchange resin AG 50 W-X2 (200–400 mesh) was purchased from Bio-Rad Laboratories, Richmond, Calif. ATP, sodium salt, cAMP, phosphoenolpyruvate, and pyruvate kinase and myokinase from rabbit muscle were obtained from Sigma Chemical Co., St. Louis, Mo. All other listed and unlisted chemicals were standard commercial preparations and were used without further purification.

## RESULTS

**Jejunal challenge with preformed enterotoxin.** The effect of ECT on net jejunal movement and mucosal adenyl cyclase activity was studied in nine dogs. Net fluid secretion occurred only during the 10-min study period in which ECT 334 was in contact with jejunal mucosa (Fig. 1). During that period, fluid secretion differed significantly from initial control absorption (*P* < 0.01), and recovery of normal absorption occurred by the time the segments had been washed twice and restudied for 10 min (*P* < 0.01). The onset of and recovery from fluid secretion corresponded with simultaneous changes in activation of mucosal adenyl cyclase. The immediate increase and the prompt fall in adenyl cyclase activity

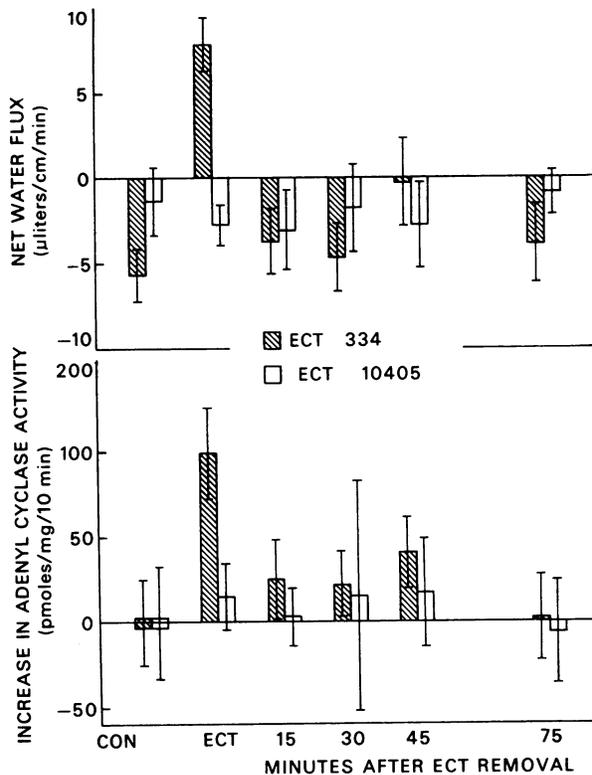


FIGURE 1 Time-course of net water flux and change in mucosal adenylyl cyclase activity in canine jejunal segments studied *in situ*. ECT indicates period during which ECT 334 or ECT 10405 was placed in gut segment. Statistical analysis and control adenylyl cyclase values are indicated in Results section. Net water absorption is indicated by negative values. Change in adenylyl cyclase activity is compared to control values before ECT exposure. Vertical bars indicate SEM.

within adjacent study periods were significant ( $P < 0.01$  and  $P < 0.05$ , respectively).

The control-culture filtrate, ECT 10405, produced no significant change in net water flux or in adenylyl cyclase activity. There were significant differences in paired observations ( $n = 6$ ) of net water flux (difference =  $11.8 \mu\text{l}/\text{cm}/\text{min}$ ,  $P < 0.02$ ) and mucosal adenylyl cyclase activity (difference =  $89.4 \text{ pmol cyclic AMP}/\text{mg protein}/10 \text{ min}$ ,  $P < 0.05$ ) during mucosal exposure to ECT 334 or ECT 10405. The mean control adenylyl cyclase values were  $137 \pm 25$  (SEM) and  $158 \pm 34$  (SEM) pmol cAMP formed per mg protein per 10-min incubation for all nine dogs and for the six dogs with paired ECT 10405 controls, respectively.

These data were also paired with maximal CT responses 3 h after challenge. Maximum observed fluid secretion and adenylyl cyclase responses to ECT 334 were 46% and 30% of the CT effects, respectively. The differences between the maximum observed responses to ECT

334 and to CT were highly significant ( $P < 0.001$ ,  $n = 9$ ) for both adenylyl cyclase activity and intestinal fluid secretion.

The calculated mean sodium concentration of the net fluid absorbed from the lumen in control studies was  $147 \text{ meq}/\text{liter} \pm 10$  (SEM,  $n = 8$  dogs). The net fluid added to the lumen in the same segments in the presence of ECT 334 had a mean sodium concentration of  $159 \text{ meq}/\text{liter} \pm 6$  (SEM).

The duration of effect of ECT 334 when left in contact with jejunal mucosa was studied by injecting segments with 12 ml containing 50 or 2500  $\mu\text{g}/\text{ml}$  ECT 334. At 0, 15, 30, 45, and 60 min, 0.5-ml samples were obtained for PSP analysis to determine net water flux. After 90 min all fluid was removed, a 0.5 ml portion was taken for PSP analysis, and the remainder was placed in a previously unused control segment for 15 min to detect any residual enterotoxin effect. To confirm the validity of this method, two control segments were similarly studied in each dog through the 90-min period without addition of enterotoxin.

The effect of the higher dose of ECT 334 persisted throughout the 90-min period (Fig. 2). Furthermore, persisting enterotoxic activity was evident when the residual content of the segment was placed into a control

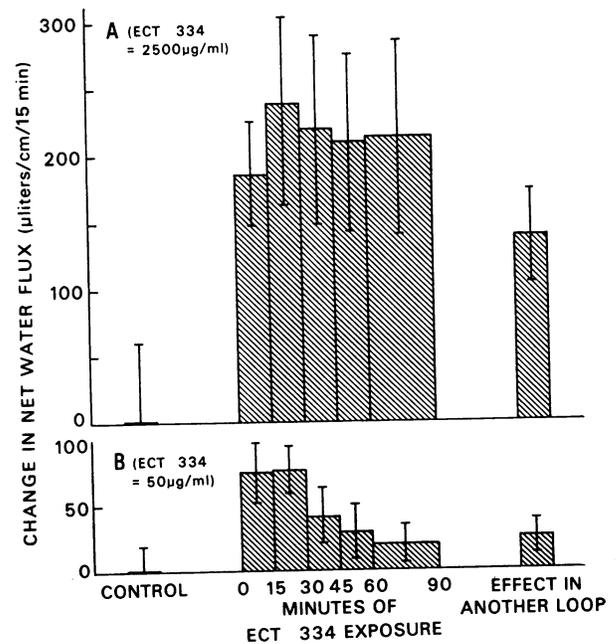


FIGURE 2 Duration of the effects of supramaximal and submaximal doses of ECT 334 upon net water flux in canine jejunum. Statistical analysis is given in Results section. Control net water flux showed absorption of  $163 \mu\text{l}/\text{cm}/15 \text{ min}$  in graph A, and  $143 \mu\text{l}/\text{cm}/15 \text{ min}$  in graph B. All changes represented either decreased absorption or secretion. Vertical bars indicate SEM.

segment ( $P < 0.01$ ,  $n = 7$ ). In contrast, the effect of a submaximal dose of ECT 334 (50  $\mu\text{g}/\text{ml}$ ) was transient, even though left in contact with the small bowel. Injection of the remaining material into a control segment did not produce a significant change in water flux ( $P > 0.1$ ,  $n = 6$ ). Control segments maintained normal absorption throughout the 90-min period.

**Duodenal challenge with viable *E. coli*.** Inoculation with whole cultures of *E. coli* 334 produced a rapid but transient net fluid secretion in the 19 animals studied, after correcting for the initial 30-ml inoculum (Fig. 3). This was done by subtracting 30 ml from the effluent collected at the end of the 1 h bacterial inoculation period. Net secretion decreased slightly during the 2nd h, then increased steadily to the maximal observed rate of fluid secretion and mucosal adenyl cyclase stimulation 6 h after bacterial inoculation (Table I). The transient net secretion observed in the 1st h in segments inoculated with whole cultures of *E. coli* 334 was thought to be due to preformed enterotoxin in the inoculum. Transient initial fluid secretion was not seen after inoculation with washed cells of strain 334; however, the total 7-h fluid output was comparable in magnitude to that induced by whole cultures. Control strain 10405 produced slight net secretion 2–7 h after inoculation; however, the secretory rate 7 h after inoculation was significantly less than that induced by strain 334 ( $P < 0.02$ ). The pH of duodenal fluid obtained during control periods varied from 6.8 to

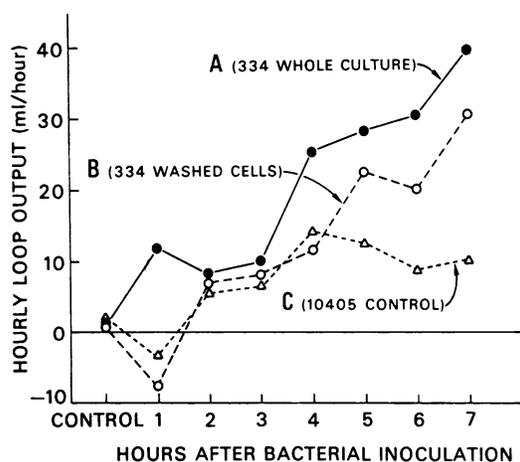


FIGURE 3 Volume of loop output after inoculation with whole cultures of *E. coli* 334 (curve A,  $n = 19$ ), washed cells of *E. coli* 334 (curve B,  $n = 4$ ) and whole cultures of *E. coli* 10405 (curve C,  $n = 10$ ). Data at 1 h represent the net output after subtracting the initial 30-ml inoculum from each value. Studies beyond 7 h were complicated by altered function of controls associated with histopathologic changes of peritonitis, interstitial edema, crypt dilatation, and focal atrophy in the bowel mucosa. Using grouped analysis: at 1 h  $A - B = 20$  ml/h,  $P < 0.2$ ; at 7 h  $A - C = 29$  ml/h,  $P < 0.01$ ; at 7 h  $B - C = 20$  ml/h,  $P < 0.02$ .

TABLE I  
Increase in Adenyl Cyclase Activity of Jejunal Mucosa after Whole Culture Inoculation

Time after inoculation	Mean increase in adenyl cyclase activity over paired controls	
	<i>E. coli</i> 334 ( $n = 19$ )	<i>E. coli</i> 10405 ( $n = 7$ )
h	pmol/mg protein/10 min ( $\pm$ SEM)	
3	+37 ( $\pm 22$ ) NS	+28 ( $\pm 18$ ) NS
6	+57 ( $\pm 19$ ) $P < 0.01$	-10 ( $\pm 18$ ) NS

7.4 and did not detectably affect the subsequent response to bacterial inoculation.

Mucosal adenyl cyclase activity increased after inoculation of *E. coli*, and by 6 h the increase was statistically significant ( $P < 0.01$ , Table I). No increase in adenyl cyclase activity was demonstrable in segments inoculated with equal numbers of strain 10405.

Enterotoxic activity was demonstrable in only 3 of 12 duodenal effluents collected 6 h after *E. coli* 334 inoculation, during near-maximal secretory and enzyme responses. The average volume in ligated rabbit small bowel segments with these three samples was 0.65 ml/cm. Enterotoxin had been demonstrable in cell-free supernates of the original challenge material (average response = 0.80 ml/cm).

The mean inoculum in 19 dogs challenged with whole cultures of *E. coli* 334 was  $6.7 \times 10^8$  viable bacteria/ml. Approximately 6 times the total initial inoculum was recovered in fluid secreted during the first 6 h, and the fluid at the 6th h still had a bacterial concentration of  $2.2 \times 10^7$  viable organisms/ml. Viable bacterial counts at 6 h were virtually identical ( $1.8 \times 10^7/\text{ml}$ ) in duodenal fluid of animals inoculated with strain 10405. Table II shows the multiplication of *E. coli* 334 in vivo up to 12 h after challenge of dogs with whole cultures previously diluted 1:200 in sterile Syncase. Bacterial multiplication was associated with increasing fluid secretion.

**Histopathologic studies.** There were no histopathologic changes demonstrable in tissue examined immediately after ECT 334 or ECT 10405 challenge when compared with paired control specimens. Likewise, tissue taken 6 h after *E. coli* 334 or *E. coli* 10405 inoculation was not detectably different from paired preinoculation control specimens (Figs. 4 and 5). Mucin staining revealed no appreciable differences in goblet-cell morphol-

TABLE II  
*In vivo* Multiplication of *E. coli* 334 and Fluid Secretion in Eight Duodenal Segments

	Hours after inoculation			
	3	6	10	12
Total viable bacterial counts recovered from loop, $\times 10^6 (\pm \text{SEM})$	2.3 ( $\pm 1.0$ )	23.5 ( $\pm 8.3$ )	196 ( $\pm 79$ )	2,890 ( $\pm 1,680$ )
Hourly fluid output, milliliters ( $\pm \text{SEM}$ )	2.6 ( $\pm 0.7$ )	7.0 ( $\pm 2.7$ )	10.3 ( $\pm 3.7$ )	15.4 ( $\pm 7.9$ )

Initial inoculum was 30 ml containing  $2.8 \pm 0.4 (\times 10^6)$  *E. coli* 334/ml. Duodenal output before inoculation averaged  $0.8 \pm 0.3$  ml/h.

ogy or intracellular distribution of mucus between control segments and those exposed to ECT 334.

### DISCUSSION

The pathogenesis of acute diarrhea caused by enterotoxin-producing strains of *E. coli* bears a number of striking similarities with cholera: the organism colonizes, but does not invade, the small bowel (1, 17); sterile dialyzed culture filtrates contain an enterotoxin which stimulates mucosal secretion of an isotonic, protein-poor electrolyte solution (5); the same culture filtrates inhibit net sodium absorption and induce net chloride secretion by isolated viable rabbit ileal mucosa (18); finally, these culture fil-

trates stimulate adenylyl cyclase activity in rabbit small bowel mucosa and in isolated viable rat epididymal lipocytes (6). The two differ, however, in at least one important respect, the time-course of enterotoxin action. Maximal alteration of mucosal adenylyl cyclase and fluid secretion does not occur until 150 min after CT challenge and is sustained for more than 24 h thereafter (14). This effect ensues despite attempts to wash CT from the intestine after a 10-min exposure. By contrast, earlier studies showed that the secretory response of canine jejunum to ECT 334 was fully developed during a 90-min enterotoxin perfusion period and absorption was largely

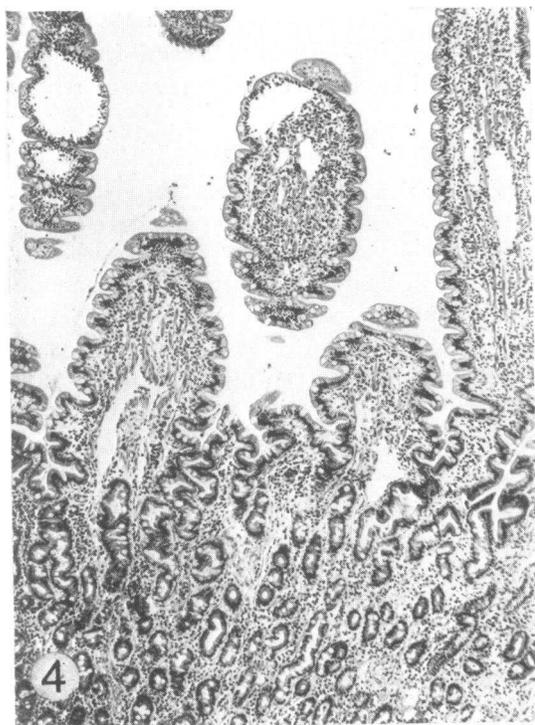


FIGURE 4 Preinoculation control canine duodenum illustrating normal microscopic structure. H and E stain.  $\times 65$ .

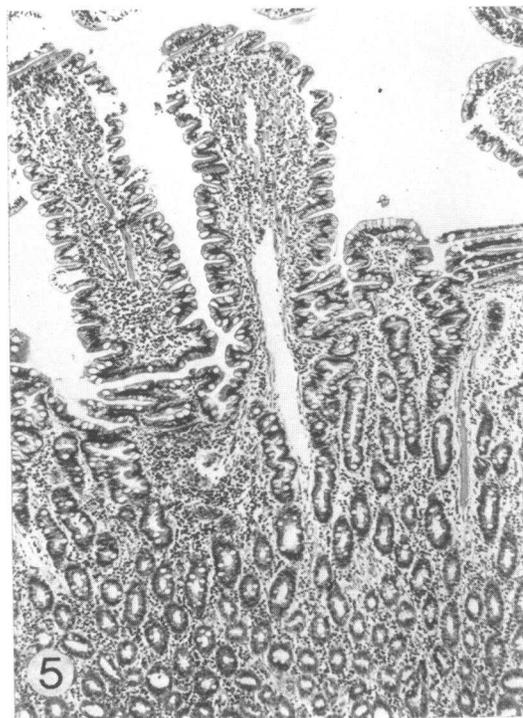


FIGURE 5 Canine duodenum 6 h after inoculation of approximately  $10^9$  *E. coli* 334. No significant change is noted from the paired control in Fig. 4. H and E stain.  $\times 65$ .

restored during the first 90 min after enterotoxin perfusion (5). In the present study the time-course of the secretory effect of a maximal dose of ECT 334 was more precisely defined, being demonstrable during the first 10 min of jejunal exposure and full recovery occurring by 5 min after enterotoxin removal. The shorter duration of ECT effect may explain, at least in part, the shorter duration of diarrhea associated with enterotoxin-producing *E. coli* (19).

Earlier studies have shown differences in the mucosal binding sites of ECT and CT, and have suggested that these differences may contribute to differences in their time-course of action (20). This study shows that ECT 334 loses enterotoxic activity after exposure to jejunal mucosa. This loss could result from enterotoxin absorption or from enterotoxin inactivation within the lumen or as a result of enterotoxin interaction with the mucosa. In any case this inactivating process may explain, at least partly, the brief duration of ECT effect. A sustained secretory response to ECT of several h duration is possible but appears to require either continuous perfusion with fresh ECT or injection of a large amount of ECT into a ligated gut segment<sup>a</sup> (5). ECT is frequently assayed by measuring the cumulative secretory response in in vivo ligated segments of rabbit or mouse small bowel<sup>a</sup> (3, 21). The above observations suggest measurement of this secretory response must be made earlier when studying smaller ECT doses than when studying larger ones, to avoid obliteration of the secretory effect by early recovery of intestinal absorptive function.

Previous studies have established a clear temporal relation between adenylyl cyclase activation and jejunal secretion induced by CT (14). This study also establishes a consistent temporal relation between the secretory response to jejunal inoculation with preformed ECT 334 or to duodenal inoculation with viable *E. coli* 334 and increased adenylyl cyclase activity in the affected mucosa. Adenylyl cyclase stimulation appeared to be related to enterotoxic activity, since it was observed only in the presence of secretion induced by a viable enterotoxin-producing *E. coli* or its culture filtrate. The control-culture filtrate produced neither jejunal secretion nor adenylyl cyclase activation. Duodenal segments challenged with viable *E. coli* of the control strain showed slight net fluid secretion during the 7-h observation without adenylyl cyclase activation, raising the possibility that pathways other than adenylyl cyclase activation by enterotoxin may be involved in intestinal secretion caused by viable bacteria.

Finally, this study shows that the secretory effects of preformed enterotoxin, including adenylyl cyclase activation, can be reproduced by duodenal inoculation with

<sup>a</sup>Evans, D. G., D. J. Evans, Jr., and N. F. Pierce. Unpublished observations.

viable enterotoxin-producing *E. coli*. When the inoculum size was reduced, these *E. coli* were demonstrated to undergo appreciable in vivo multiplication. Preformed enterotoxin in the whole culture challenge probably accounted for the transient secretory response during the 1st h but exerted no effect thereafter, since the same secretory course was observed in segments challenged with previously washed bacteria and was presumably caused by enterotoxin production in vivo. Our inability to detect enterotoxic activity in most of the duodenal effluents suggests that the enterotoxin was produced in small amounts and largely mucosa bound, or that it was inactivated, or both. The absence of demonstrable histopathologic change or tissue invasion is consistent with the concept that the secretory process is enterotoxin-mediated and does not involve structural damage visible on light microscopy. The demonstration of intestinal secretion induced by bacterial inoculation suggests that the dog may be employed for further studies of the mechanism of diarrheal disease induced by viable *E. coli* and for study of the immune response to intestinal colonization with *E. coli*.

#### ACKNOWLEDGMENTS

The authors wish to express their appreciation to Dr. Raphael Garcia-Bunuel and his staff for preparation and study of the tissue biopsies. We are also grateful to Mr. Kenneth Rent and Mr. Thomas Wainwright for their technical assistance, and to Mrs. Julie Yang for preparation of the enterotoxins. We thank the staff of the Gerontology Research Center, National Institutes of Child Health and Human Development for the use of facilities provided under the Guest Scientist Program.

This work was supported by grant no. AI-07625 from the United States-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

#### REFERENCES

1. Gorbach, S. L., J. G. Banwell, B. D. Chatterjee, B. Jacobs, and R. B. Sack. 1971. Acute undifferentiated human diarrhea in the tropics. I. Alterations in intestinal microflora. *J. Clin. Invest.* 50: 881.
2. Gorbach, S. L. 1970. Acute diarrhea—a toxin disease? *N. Engl. J. Med.* 283: 44.
3. Sack, R. B., S. L. Gorbach, J. G. Banwell, B. Jacobs, B. D. Chatterjee, and R. C. Mitra. 1971. Enterotoxigenic *Escherichia coli* isolated from patients with severe cholera-like disease. *J. Infect. Dis.* 123: 378.
4. Gorbach, S. L., and C. M. Khurana. 1972. Toxigenic *Escherichia coli*: a cause of infantile diarrhea in Chicago. *N. Engl. J. Med.* 287: 791.
5. Pierce, N. F., and C. K. Wallace. 1972. Stimulation of jejunal secretion by crude *Escherichia coli* enterotoxin. *Gastroenterology.* 63: 439.
6. Evans, D. J., Jr., L. C. Chen, G. T. Curlin, and D. G. Evans. 1972. Stimulation of adenylyl cyclase by *Escherichia coli* enterotoxin. *Nature (Lond.)* 236: 137.
7. Finkelstein, R. A., P. Atthasampunna, M. Chulasmaaya, and P. Charunnethee. 1966. Pathogenesis of ex-

- perimental cholera: biologic activities of purified pro-cholera A. *J. Immunol.* **96**: 440.
8. Finkelstein, R. A., and J. J. LoSpalluto. 1970. Session III: Production, purification, and assay of cholera toxin. Production of highly purified cholera and cholera-genoid. *J. Infect. Dis.* **121**(Suppl.): S63.
  9. Miles, A. A., and S. S. Misra. 1938. The estimation of the bacteriocidal power of the blood. *J. Hyg.* **38**: 732.
  10. Carpenter, C. C. J., R. B. Sack, J. C. Feeley, and R. W. Steenburg. 1968. Site and characteristics of electrolyte loss and effect of intraluminal glucose in experimental canine cholera. *J. Clin. Invest.* **47**: 1210.
  11. Schedl, H. P., and J. A. Clifton. 1961. Small intestinal absorption of steroid. *Gastroenterology.* **41**: 491.
  12. 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.
  13. Kasai, G. J., and W. Burrows. 1966. The titration of cholera toxin and antitoxin in the rabbit ileal loop. *J. Infect. Dis.* **116**: 606.
  14. Guerrant, R. L., L. C. Chen, and G. W. G. Sharp. 1972. Intestinal adenyl cyclase activity in canine cholera: correlation with fluid accumulation. *J. Infect. Dis.* **125**: 377.
  15. Krishna, G., B. Weiss, and B. B. Brodie. 1968. A simple, sensitive method for the assay of adenyl cyclase. *J. Pharmacol. Exp. Ther.* **163**: 379.
  16. Mowry, R. W. 1963. The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. *Ann. N. Y. Acad. Sci.* **106**: 402.
  17. DuPont, H. L., S. B. Formal, R. B. Hornick, M. J. Snyder, J. P. Libonati, D. G. Sheahan, E. H. LaBrec, and J. P. Kalas. 1971. Pathogenesis of *Escherichia coli* diarrhea. *N. Engl. J. Med.* **285**: 1.
  18. Al-Awqati, Q., C. K. Wallace, W. B. Greenough, III. 1972. Stimulation of intestinal secretion in vitro by culture filtrates of *Escherichia coli*. *J. Infect. Dis.* **125**: 300.
  19. Banwell, J. G., S. L. Gorbach, N. F. Pierce, R. Mitra, and A. Mondal. 1971. Acute undifferentiated human diarrhea in the tropics. II. Alterations in intestinal fluid and electrolyte movement. *J. Clin. Invest.* **50**: 890.
  20. Pierce, N. F. 1973. Differential inhibitory effects of cholera toxoids and ganglioside on the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. *J. Exp. Med.* **137**: 1009.
  21. Punyashthiti, K., and R. A. Finkelstein. 1971. Enteropathogenicity of *Escherichia coli*. I. Evaluation of mouse intestinal loops. *Infect. Immun.* **4**: 473.