

Differences in the Response of Rabbit Small Intestine to Heat-Labile and Heat-Stable Enterotoxins of *Escherichia coli*

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The response of adult rabbit small intestine to the heat-stable (ST) and heat-labile (LT) enterotoxins of *Escherichia coli* has been investigated by employing the ligated loop technique. Fluid accumulation was determined in relation to enterotoxin dose and duration of gut exposure. The individual responses to ST and LT differed in a characteristic manner. Onset of net fluid accumulation in response to ST appeared to be immediate even at the lowest dose tested. Onset of net fluid accumulation in response to LT was rapid at high doses but delayed at low doses. Maximum volume per length ratios elicited by ST occurred between 4 and 6 h after injection of loops over the entire range of doses tested. However, maximum ratios elicited by LT occurred no less than 10 h after injection even at low doses. Fluid accumulation elicited by LT increased in duration with increasing dose; high doses of LT producing a response which was sustained for at least 18 h. The net effect of these differences in reaction characteristics is a sharp increase in the proportion of the cumulative net secretory response attributable to LT with time. Therefore, a 6-h assay time is appropriate for the titration of ST, whereas an 18-h assay is not. The 18-h assay was found more appropriate for toxin-antitoxin neutralization studies since only LT was neutralized by anti-enterotoxin serum. LT of *E. coli* (swine) strain P-263 and (human) strain 334 was neutralized by antibody stimulated by enterotoxin from *E. coli* (human) strain H-10407. LT was labile to mild acid conditions, whereas ST was not.

It is increasingly evident that enterotoxin-producing strains of *Escherichia coli* play an important role in human acute diarrheal illnesses (5, 10, 11). In 1967, Sakazaki et al. (19) observed that many strains of *E. coli* associated clinically with acute diarrhea are noninvasive. This was followed by observations that noninvasive strains of *E. coli* associated with acute diarrhea in both man and animals produce an enterotoxin(s) which elicits a cholera-like secretory response in the small intestine (1, 8, 9, 13, 16, 18, 20). Various models developed for the study of the pathophysiological effects of *Vibrio cholerae* enterotoxin have also been successfully employed for characterizing the biological activities of *E. coli* enterotoxin (6, 7, 15-17, 18). The adult rabbit ligated intestinal loop technique of De (4), modified for the titration of cholera enterotoxin and antitoxin activities (2,

14) has proven especially useful for investigating the diarrheagenic effect of *E. coli* enterotoxin (6, 15, 18).

Various investigators have employed a variety of modifications of the ligated loop method, different strains of *E. coli*, and differing methods of enterotoxin preparation in their investigations of enterotoxin. This plus the fact that *E. coli* produces both heat-stable (ST) and heat-labile (LT) enterotoxins has caused several conflicts in reports describing the degree of heat lability and the relative potency of *E. coli* enterotoxin activities. The primary goal of the present study was to describe the kinetics of fluid accumulation in rabbit small intestine induced by enterotoxin preparations containing both ST and LT activities. This paper demonstrates that the relative contributions of the two (ST and LT) activities to the total observed response can vary greatly with dose and especially with assay time. It shows that these

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differences can be important when assaying ST, LT, or antibody to LT in rabbit small bowel loops. Finally, this report presents new data comparing the effects of the enterotoxins of *E. coli* and *V. cholerae* on the rabbit small intestine.

MATERIALS AND METHODS

Bacterial strains. Two enterotoxigenic strains of *E. coli* were studied. Strain H-10407 (serotype 078:H11) was isolated from the liquid stool of a patient with severe cholera-like *Vibrio*-negative diarrhea in Dacca, Bangladesh; the subculture employed in this work was derived from the original stock culture in Dacca by one of the authors (D.J.E.) and employed in previous studies (7). *E. coli* strain 334 (serotype 015:H11) was isolated from jejunal fluid of a similar case of severe "undifferentiated diarrhea" in Calcutta, India (11). Stock cultures were maintained on slants composed of 2.0% peptone (Difco), 0.5% NaCl, and 2.0% agar.

Enterotoxin preparations. The medium employed for the production of enterotoxin was composed as follows: 20 g of Casamino Acids (Difco); 1.5 g of yeast extract (Difco); 2.5 g of NaCl; 8.71 g of K_2HPO_4 (0.05 M); and 1.0 ml of a trace salts solution, added to distilled water in that order, adjusted to pH 8.5 with 0.1 N NaOH, and brought to a final volume of 1 liter. The trace salts mixture consisted of 5% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$ dissolved in 0.001 N H_2SO_4 . Shake cultures (500 ml per 2.5 liter in a low-form flask) were loop-inoculated from stock slants and agitated at 140 shakes per min for 18 h in a 37 C shaking water-bath. Cells were removed by centrifugation at $12,000 \times g$ for 45 min followed by membrane (0.45- μ pore size; Millipore Corp.) filtration of the culture supernatant extract. Enterotoxin was precipitated by slowly adding solid $(NH_4)_2SO_4$ to 90% saturation at 4 C with stirring followed by 20 min without stirring. After centrifugation at $12,000 \times g$ for 45 min at 4 C, the pellet was dissolved in 0.02 M tris(hydroxymethyl)aminomethane-chloride buffer (pH 7.8; 25 ml per liter of culture supernatant extract) and dialyzed for 18 h against 650 volumes of the same buffer at 4 C. An inactive residue was removed by centrifugation of the dialysand at $20,000 \times g$ for 15 min at 4 C. The final supernatant extract (approximately 30-times concentrated with respect to the original volume) was sterilized by membrane (0.45- μ pore size; Millipore Corp.) filtration. Essentially all of the enterotoxin activity (both ST and LT) was recovered in the dialysand. Subsequent storage at 4 C resulted in decreasing enterotoxin activity. However, dialysands immediately lyophilized showed no detectable loss of activity during long-term (at least 2 months) storage at -45 C. In this study, dialysands of three lots of enterotoxin prepared on consecutive days were pooled, dialyzed again as above, lyophilized, and stored at -45 C until needed.

A preparation of enterotoxin derived from *E. coli* strain P-263, serotype 08:K87, K88a, b:H19, associated with an enteric infection (coli-bacillosis) of swine) was kindly prepared and provided by H. W.

Moon and S. C. Whipp (15). This lot consisting of a 65.5-fold "Diaflo" XM-50 concentrate of culture filtrate was only employed in serum neutralization studies.

V. cholerae enterotoxin was a crude toxin preparation (National Institutes of Health lot 001) supplied by John Seal, National Institute of Allergy and Infectious Diseases, and referred to here as cholera toxin.

Rabbit ligated intestinal loop assay. The adult rabbit intestinal loop assay (14) was performed essentially according to the modifications of Pierce and Wallace (17) employing albino rabbits averaging 1.5 to 2.0 kg in weight. Animals were fasted for 48 h prior to use. Under local anesthesia, the small bowel was flushed with 10 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.4), and ligated segments, usually 20 averaging 4 to 5 cm long, were prepared by employing a single tie of surgical silk between segments. Each segment received a 1.0-ml intraluminal injection consisting of enterotoxin dissolved in PBS, or PBS (negative controls), 2.0 mg of isotonic cholera toxin (positive controls), or other samples as specified. All samples, including controls, were injected in duplicate in each animal and in random sequence employing a minimum of three rabbits per study. After injection of the loops the abdomen was closed. Animals were sacrificed at predetermined time intervals by intravenous injection of pentobarbital. The abdomen was opened, and the small intestine was excised. After the volume of fluid in each segment was measured (by withdrawal into a syringe of appropriate volume) the lengths of the empty segments were determined, and volume per length ratios (ml/cm) were recorded. Results were considered valid only if the positive and negative controls gave appropriate responses.

Definition of ST and LT. Enterotoxin activity detectable after boiling for 30 min is referred to as ST; activity destroyed by such treatment is referred to as LT. According to these criteria, the amount of fluid accumulation due to LT was derived by difference as follows: mean volume per length ratio elicited by untreated enterotoxin minus mean volume per length ratio elicited by boiled enterotoxin assayed in the same intestine. The abbreviations ST and LT are employed for brevity only and do not necessarily indicate that these enterotoxin activities are identical with ST and LT activities produced by *E. coli* enteropathogenic for swine or other domestic animals (12).

Preparation of antitoxin. Three adult albino rabbits received at 5-day intervals seven sequential doses (of approximately 200 μ g each) of *E. coli* strain H-10407 enterotoxin containing both ST and LT activities. For the first intramuscular injection the enterotoxin was emulsified in incomplete Freund adjuvant. The remaining doses of enterotoxin dissolved in physiological saline were injected by the same route at multiple sites. Fourteen days after the last injection, the rabbits were bled, and sera were collected and pooled, distributed in 2.0-ml samples, and stored at -45 C until needed. Control serum was a pool derived from the same rabbits prior to immunization.

Antitoxin neutralization. Rabbit serum containing anti-(H-10407)-enterotoxin was obtained as described. *E. coli* enterotoxin in PBS was mixed with an equal volume of rabbit immune serum (10-fold serial dilutions starting at 1:5) in PBS containing 0.1% bovine serum albumin. Mixtures were incubated at 37 C for 30 min prior to injection of 1.0-ml samples into rabbit intestinal segments. Control mixtures consisted of a 1:10 final dilution of control serum, enterotoxin without serum, boiled enterotoxin without serum, and PBS, respectively. Final concentrations of the enterotoxins in the injection mixtures were as follows: *E. coli* strain H-10407, 150 $\mu\text{g/ml}$; strain 334, 350 $\mu\text{g/ml}$; and strain P-263, 1:6 dilution of the fluid preparation described above.

Effect of pH on enterotoxin. Lyophilized *E. coli* strain H-10407 enterotoxin was dissolved in unbuffered saline at a concentration of 400 $\mu\text{g/ml}$. A series of incubation mixtures was prepared by combining samples of the enterotoxin solution with equal volumes of 0.05 M PBS solutions (pH 5.0, 6.0, and 7.0, respectively) and with 0.05 M tris(hydroxymethyl)-aminomethane-chloride-buffered saline solution (pH 8.0 and 9.0). Mixtures were incubated at 37 C for 4 h and then neutralized to pH 7.0 to 7.2 by the addition of equal volumes of isotonic buffers preadjusted to appropriate pH values. The experimental control containing equal volumes of enterotoxin in unbuffered saline and 0.05 M PBS (pH 7.0) was kept at 4 C for 4 h before dilution to final concentration by the addition of an equal volume of the same buffer. One-milliliter samples of test and control mixtures were injected into rabbit intestinal segments according to the standard procedure described above.

RESULTS

Kinetics of fluid accumulation. Lyophilized *E. coli* strain 334 enterotoxin was employed to investigate the response of the rabbit small bowel to ST and LT as a function of dose and assay time. Two doses, 81 and 729 $\mu\text{g/ml}$, respectively, were chosen on the basis of preliminary titration data. Figure 1 illustrates the results of an experiment in which rabbits were sacrificed at various intervals after injection of loops with the larger dose of enterotoxin. Data taken from a comparable series of tests employing cholera toxin are shown for comparison. The onset of net fluid accumulation in response to both LT and ST is rapid and perhaps immediate. The initial rate of net fluid accumulation from LT appears to be slower than that from ST. This type of response is in sharp contrast to that elicited by cholera toxin which is characterized by an easily demonstrable time lag between exposure and detectable fluid accumulation (Fig. 1). In loops treated with ST alone, maximum volume per length ratios occur 6 h after injection and decline thereafter. In contrast, fluid accumulation due to LT continues to increase for at least 18 h. Therefore, the net

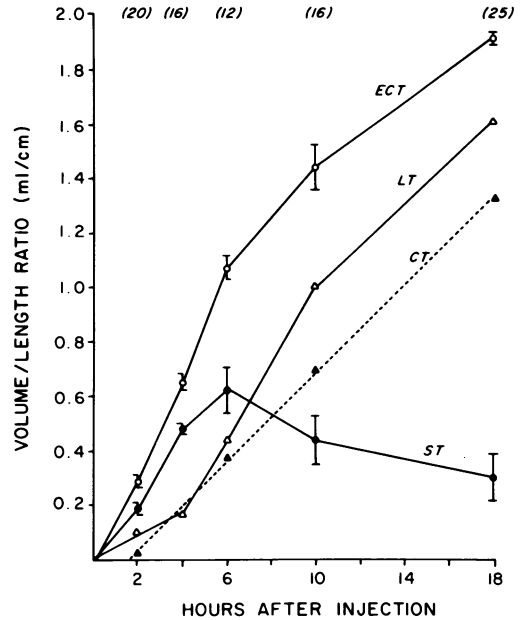


FIG. 1. Time-course of fluid accumulation in intestinal loops in response to large doses of enterotoxins. Symbols: (O) *E. coli* strain 334 enterotoxin (729 $\mu\text{g/ml}$); (●) ST activity; (Δ) LT activity; (\blacktriangle) crude cholera toxin (0.5 mg/ml, $n = 6 - 12$). Numbers in parentheses: loops per data point for *E. coli* enterotoxins. Vertical bars: ± 1 standard error of the mean (SEM).

observed response prior to 7 h (early response) is predominantly a function of ST, whereas that after 7 h (late response) is largely a function of LT.

Figure 2 illustrates the results of the second series of determinations in which the lower dose was employed. A lower dose of cholera toxin (0.125 mg/ml) was also employed here for comparison. At this dose, the response to LT is characterized by a 4-h lag before net fluid accumulation occurs, and it is not sustained. Induction of net fluid accumulation by ST remains rapid in onset and is maximal by 4-h postinjection.

Effect of time-course on titration of enterotoxin. Figure 3 shows the results of a series of experiments in which the response of the rabbit intestine to *E. coli* strain 334 ST and LT was determined as a function of dose. Assay times of 6 and 18 h (Fig. 3) were chosen for comparison because these times are relevant to those previously reported by various investigators (6, 7, 17, 18). At 6-h postinjection ST activity predominates and is detectable at a level of 9 $\mu\text{g/ml}$. This is in sharp contrast to the results obtained at 18-h postinjection in which ST is detectable only at the highest dose tested

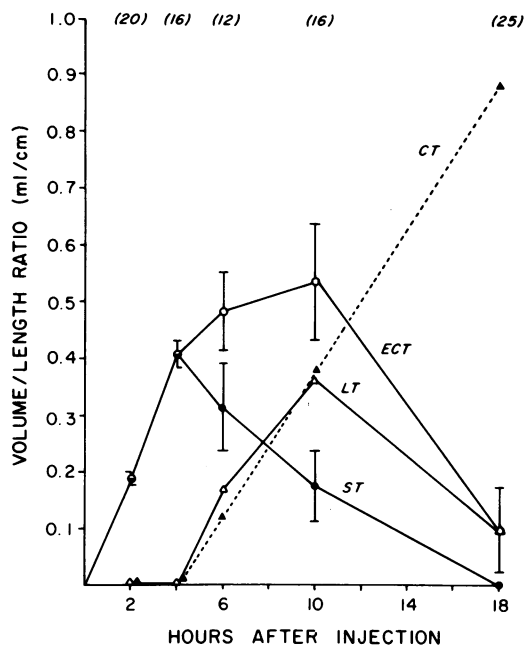


FIG. 2. Time-course of fluid accumulation in intestinal loops in response to small doses of enterotoxins. Symbols: (O.) *E. coli* strain 334 enterotoxin (81 μ g/ml); (●) ST activity; (Δ) LT activity; (\blacktriangle) crude cholera toxin (0.125 mg/ml, $n = 6 - 12$). Numbers in parentheses: loops per data point for *E. coli* enterotoxins. Vertical bars: ± 1 SEM.

and accounts for only a small portion of the observed secretory response. Differences between the slopes of 6- and 18-h titration curves and in the portion of the cumulative net secretion caused by ST resulted from the observed dose-dependent differences in duration of the individual responses to ST and LT (Fig. 1 and 2). In contrast, the response to cholera toxin is long-lasting irrespective of dose, as indicated by the parallel character of the 6- and 18-h titration curves (Fig. 3, inset).

Cross-neutralization of *E. coli* enterotoxins. Neutralization was determined by preincubation of *E. coli* enterotoxin with various serum dilutions prior to injection into rabbit intestinal segments. Figure 4 illustrates neutralization obtained with the homologous (H-10407) toxin-antitoxin system using an assay time of 6 h. Figure 5 shows the results of neutralization experiments in which the same immune serum was employed in concert with heterologous enterotoxins derived from *E. coli* strain 334 and the swine strain P-263. Although the enterotoxin derived from the swine strain was neutralized to an extent comparable to that observed with the homologous enterotoxin (Fig.

4), that derived from strain 334 was not. A large portion of strain 334 enterotoxin activity was not neutralizable by the anti-(H-10407)-enterotoxin serum. This unneutralizable portion of the activity is comparable in magnitude to that of the ST component (boiled control, Fig. 5). Other experiments (unpublished data) have demonstrated that ST of both strains, H-10407 and 334, is not neutralized by antibody preparations which we employed.

Figure 6 shows results of similar neutralization experiments employing strain 334 and H-10407 enterotoxins but assayed at 18 h. In contrast to the 6-h assay results, complete neutralization of both strain 334 and H-10407 enterotoxins was obtained. Control segments injected with boiled enterotoxin of both strains were negative, although the respective enterotoxin doses were not altered from those employed in the 6-h assays. Thus, complete inhibition of net secretory response to enterotoxins containing both ST and LT is obtainable by employing an assay time at which ST activity is negligible.

Effect of pH on *E. coli* enterotoxin. Investigations into the effect of initial pH of various growth media on the yield of enterotoxin(s) from *E. coli* strain 334 and H-10407 demonstrated that the yield of LT, but not of ST, was reduced in media with initial pH values at or below 6.5 and also in weakly buffered media. These results prompted us to extend our investigations into the effects of pH on the stability of ST and LT under mild conditions such as those encountered during bacterial growth. Figure 7 illustrates the results of an experiment in which enterotoxin derived from strain H-10407 was preincubated in buffers at various pH values for 4 h at 37 C prior to neutralization of pH and injection into standard intestinal loops. Injected mixtures were isotonic with plasma. An assay time of 18 h was employed to avoid detection of ST. These results demonstrate that LT is labile to mild acid, but not to mild alkaline, conditions. Similar experiments were carried out with boiled preparations of enterotoxin employing a 6-h assay time to test the effect of pH on ST. ST activity was not diminished by such treatment at either pH 5.0 or 9.0 conditions, nor was it diminished by boiling samples of the same preparation for 30 min at pH 5.0.

DISCUSSION

In this study, we employed potent enterotoxigenic strains of *E. coli* (334 and H-10407) isolated from cases of acute diarrhea in Calcutta, India, and Dacca, Bangladesh, respectively.

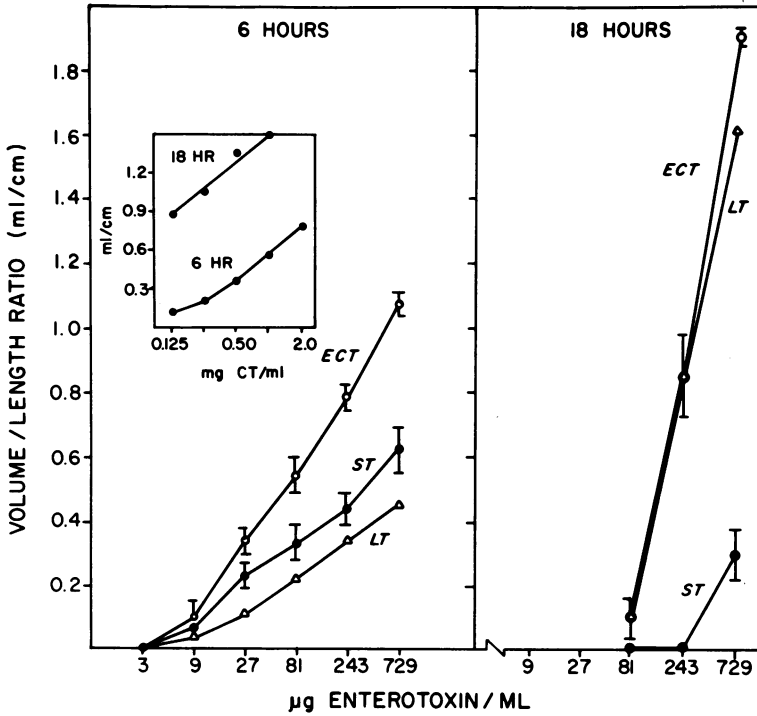


FIG. 3. Effect of assay time on titration of *E. coli* strain 334 enterotoxin. Symbols: (O) unboiled enterotoxin; (●) ST activity; (Δ) LT activity. Loops per point: 12 to 25. Vertical bars: ±1 SEM. Inset: paired titrations of crude cholera toxin.

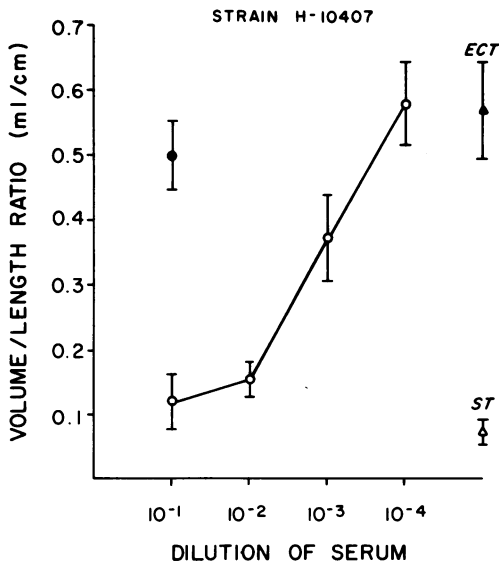


FIG. 4. Neutralization of *E. coli* strain H-10407 enterotoxin by homologous immune serum. Symbols: (▲) enterotoxin (ECT) alone; (○) enterotoxin plus immune serum; (●) enterotoxin plus control serum; (Δ) boiled enterotoxin control. Assay time: 6 h. Loops per point: 12. Vertical bars: ±1 SEM.

Each strain elaborates ST and LT enterotoxins, although this is not consistently apparent from earlier reports describing the enterotoxic activities of these strains (R. L. Guerrant et al., *J. Clin. Invest.*, vol. 52, 1973, in press; references 7, 17, 18). These strains do appear to differ in their relative production of ST and LT under the conditions employed; a higher portion of total enterotoxic activity from strain 334 was more ST than from strain H-10407. A third enterotoxin derived from an *E. coli* strain pathogenic for swine was employed in studies of enterotoxin neutralization by antitoxin. Neutralization of LT from all three strains by a single antitoxin serum supports the possibility that LT from both human and animal pathogens is a single antigenic moiety (21).

Both ST and LT elicit fluid accumulation in the rabbit small intestine, but the time-course of the net secretory response to ST differs markedly from that of LT. The onset of a net secretory response to ST was very rapid but was not sustained beyond approximately 6 h, even at the highest dose employed. By contrast, the rate of net fluid accumulation in response to LT was initially less rapid and there was a demonstrable delay in onset of net secretion with the

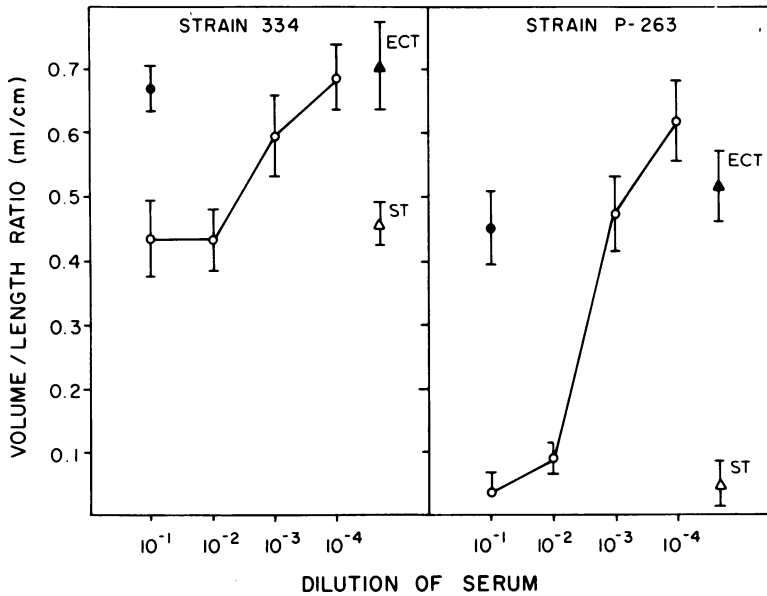


FIG. 5. Neutralization of heterologous *E. coli* enterotoxin by anti-(H-10407)-enterotoxin rabbit immune serum. Symbols: (▲) enterotoxin (ECT) alone; (○) enterotoxin plus immune serum; (●) enterotoxin plus control serum; (△) boiled enterotoxin control. Assay time: 6 h. Loops per point: Strain 334 toxin, 12; Strain P-263 toxin, 14. Vertical bars: ± 1 SEM.

lowest doses of LT employed. Furthermore, although the cumulative response to low doses of LT did not increase beyond 10 h, the cumulative secretory response increased in both magnitude and duration with increasing dose. Both ST and LT of *E. coli* elicit secretory responses which differ from that of cholera enterotoxin since the latter elicits a delayed, but steadily increasing, net secretory response at very low as well as at high doses.

The magnitude and duration of the net secretory response to LT, and to a lesser extent ST, is clearly dose dependent. The precise duration of these responses is difficult to determine in ligated intestinal segments. Nevertheless, the shortened duration of the net secretory response to lower doses of both ST and LT does indicate that the secretory response to either form of *E. coli* enterotoxin is much shorter than the response to cholera toxin. Studies on the kinetics of fluid accumulation in the canine model in response to *E. coli* enterotoxin by Pierce and Wallace (17) and by Guerrant et al. (*J. Clin. Invest.*, vol. 52, 1973, in press) employing Thirty-Vella jejunal loops are in close agreement with our observations. These workers demonstrated that the effect of *E. coli* strain 334 enterotoxin on the dog intestine is very rapid in onset and disappears soon after removal of enterotoxin. Again, this is in contrast to the delayed, but sustained, response elicited by cholera toxin in the

same model system (3). The following explanation is offered as one interpretation of these observations: (i) Binding and activation of mucosal secretion by *E. coli* enterotoxin, both ST and LT, is rapid. (ii) The secretory response to both ST and LT is of brief duration. In the case of LT, at least, this may be due to deactivation of the enterotoxin after mucosal binding, as indicated by the work of Guerrant et al. (manuscript in preparation). (iii) Binding sites can be repeatedly reactivated by subsequent binding of unreacted LT, thus producing a sustained secretory response in the presence of high doses of LT. (iv) There may be little or no reactivation of mucosal binding sites by ST, or there may be rapid inactivation of ST in the lumen of the intestine. Studies reported elsewhere indicate the existence of different binding sites for cholera enterotoxin and either ST or LT of *E. coli*, probably accounting for, at least in part, the differences in the time-courses of the secretory responses observed in his study (17a).

The increase in the portion of the cumulative secretory response attributable to LT with increased assay time is of practical importance in studies of *E. coli* enterotoxin which employ ligated segments of adult rabbit small intestine as the assay system. Preparations containing both ST and LT activities may appear to be predominately heat stable when assayed after 6 h and wholly heat labile when assayed after 18

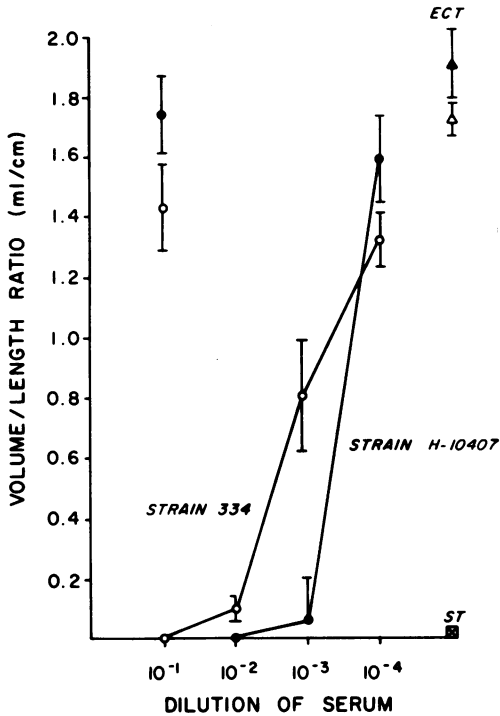


FIG. 6. Neutralization of *E. coli* enterotoxins by anti-(H-10407)-enterotoxin rabbit immune serum. Symbols: (●—●) *E. coli* strain H-10407 enterotoxin (150 µg/ml) plus immune serum; (●) same as (●—●) plus control serum; (▲) H-10407 enterotoxin (ECT) alone; (○—○) *E. coli* strain 334 enterotoxin (350 µg/ml) plus immune serum; (○) same as (○—○) plus control serum; (Δ) strain 334 enterotoxin (ECT) alone; (×, □) boiled enterotoxin controls. Assay time: 18 h. Loops per point: 14 to 16. Vertical bars: ±1 SEM.

h. For example, Evans et al. (7) and Sack et al. (18) reported the enterotoxins of *E. coli* strain H-10407 and strain 334, respectively, to be heat labile. Both groups employed an 18-h assay period. By contrast, the present studies employing both strains and other studies with strain 334 (17), employing a 6-h assay period, demonstrated appreciable levels of ST in culture filtrate preparations of both strains.

The observed differences between the kinetics of fluid accumulation elicited by ST and LT, the sensitivity of LT but not ST to acid pH, and the nonantigenic nature of ST give further support to the concept that ST and LT are distinctly different enterotoxins. This has been previously suggested by Gyles (12) on the basis of differences in size of the two enterotoxins. Also, as previously observed with ST of *E. coli* isolated from swine (12), we found that relatively large amounts of ST are required to

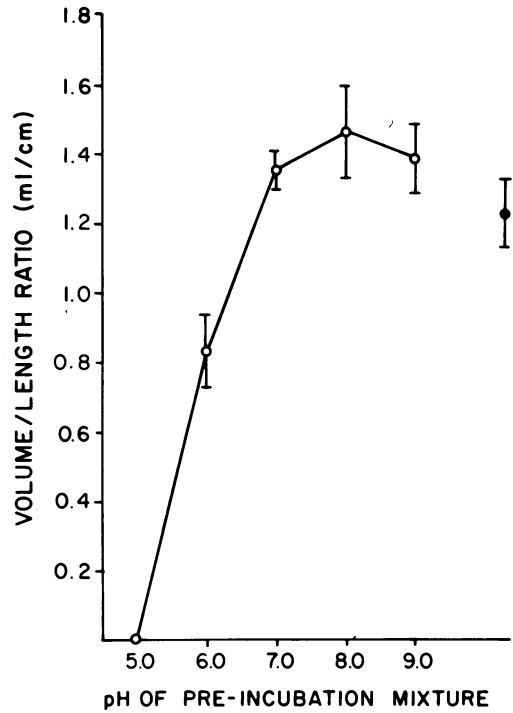


FIG. 7. Effect of pH on stability of *E. coli* strain H-10407 enterotoxin. Preincubation was at 37 C for 4 h prior to neutralization and injection as described in Materials and Methods. Symbols: (○) experimental: (●) pH 7.0, 4 C control. Assay time: 18 h. Loops per point: 12. Vertical bars: ±1 SEM.

elicit appreciable fluid accumulation in rabbit gut segments.

Finally, it would seem important that efforts be made to standardize the rabbit intestinal model system if the intention of the investigator is to describe properties of *E. coli* enterotoxin(s). On the basis of our experience during this investigation, we suggest that: (i) a 6-h assay period be employed in the titration of ST in the rabbit loop system and, (ii) a longer in vivo incubation time (16–18 h) be employed in the titration of LT activity and in studies involving neutralization of LT by antibody. Also, we suggest that it would be profitable to employ standardized LT and ST preparations in conjunction with pertinent experiments, as adult rabbits exhibit much individual variation in their response to *E. coli* enterotoxin(s), particularly to ST.

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