

Heat-stable enterotoxin of *Escherichia coli*: *In vitro* effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine

(cyclic AMP/secretion/absorption)

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Communicated by Kurt J. Isselbacher, March 13, 1978

ABSTRACT A partially purified preparation of the heat-stable enterotoxin of *Escherichia coli* caused a rapid and persistent increase in electric potential difference and short-circuit current when added *in vitro* to the luminal surface of isolated rabbit ileal mucosa. As little as 1 ng/ml produced an easily detectable response. Under short-circuit condition, the enterotoxin abolished net Cl⁻ absorption; this change was half that produced by theophylline, which stimulated net secretion. The enterotoxin did not change cyclic AMP concentration but caused large and persistent increases in cyclic GMP concentration. The electrical and nucleotide responses exhibited similar and unusually broad concentration-dependences and maximal effects could not be demonstrated. Theophylline elevated cyclic GMP concentration 3-fold both in the presence and absence of the enterotoxin, suggesting no effect of the toxin on cyclic GMP phosphodiesterase. Guanylate cyclase [GTP pyrophosphatase (cyclizing); EC 4.6.1.2] activity in a crude membrane fraction from intestinal epithelial cells was stimulated 7-fold by the enterotoxin. These results suggest that guanylate cyclase stimulation is the basis for the toxin's diarrheagenic effect.

Two enterotoxins have been identified among the extracellular products of *Escherichia coli* isolated from humans and other mammals with diarrheal disease—one heat-labile (1, 2) and the other heat-stable (1, 3, 4). The former is immunologically crossreactive with cholera toxin (5) and, like cholera toxin, stimulates adenylate cyclase (6). The latter acts more rapidly (7) and has a lower molecular weight [5000 or less (8)]. Heretofore there has been little insight into its mode of action. Recent advances in the purification of the heat-stable enterotoxin (ref. 8 and W. J. Laird and D. M. Gill, unpublished data) have made it possible to study its effects on intestine *in vitro*. We report here that the heat-stable enterotoxin inhibits active Cl absorption and stimulates guanylate cyclase [GTP pyrophosphatase (cyclizing); EC 4.6.1.2] in rabbit ileal mucosa. The data suggest that cyclic GMP (cGMP) is the intracellular mediator of the toxin's effect on ion transport.

METHODS

Preparation and Assay of Enterotoxin. *E. coli* C600 (B14), which produces heat-stable enterotoxin only, was grown in broth culture (pH 8.5; no glucose) for 18 hr at 37°. After centrifugation the supernate was chromatographed on Amberlite XAD-2. The column was first washed with 20% methanol/1% acetic acid in water and toxin was then eluted with 99% methanol/1% acetic acid. The eluate was concentrated by air-drying and then gel filtered on Sephadex LH-20 that had

been equilibrated with 99% methanol/1% acetic acid. A broad peak of toxin activity appeared just behind the void volume. The eluate was reconcentrated and again filtered on Sephadex LH-20 that had been equilibrated with water. Toxin was then eluted with water and the eluate was stored in a refrigerator with preservatives. Even when stored for 6 wk at room temperature, no loss of activity could be detected. The final material was 200- to 1000-fold purified but still gave several peaks on silica gel chromatography. A detailed description of this procedure will be published elsewhere (W. J. Laird and D. M. Gill, unpublished data).

Enterotoxin activity was assayed in suckling mice (9). Fifty-microliter aliquots of toxin in water were introduced by transabdominal injection into the stomachs of 2 to 4-day-old suckling mice (CD-1 Swiss white from Charles River Laboratories, Boston, MA). After 60 min the mice were killed with CHCl₃ and ratios of total intestinal weight to total body weight were determined. Control ratios were about 0.06 and maximal ratios were about 0.13. A mouse unit was defined as the amount of toxin producing a half-maximal increase in ratio. Serial 1:1 dilutions of toxin were assayed; three mice were used for each dilution.

Three separately prepared batches of toxin were used for various phases of the present study. An aliquot from one of these batches was lyophilized and weighed: one mouse unit was found to represent about 10 ng of this material. Since we determined the specific activity of only one of these batches of toxin, we have chosen to express enterotoxin concentration in mouse units/ml rather than in ng/ml.

Electrical and Cl⁻ Flux Measurements. Pieces of distal ileum from New Zealand white, male rabbits (2-3 kg) were stripped of muscle and mounted in Ussing chambers (usually four per animal) as described (10). The bathing medium (HCO₃/Ringer's solution bubbled with 5% CO₂ in O₂ and maintained at 37°) was the same as that previously used (10) except that half the Cl⁻ was replaced with equimolar amounts of SO₄²⁻ and mannitol. This was done in order to reduce the passive unidirectional Cl⁻ fluxes, thereby permitting changes

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Abbreviations: cGMP, cyclic GMP; cAMP, cyclic AMP; PD, electric potential difference; *I*_{sc}, short circuit current; EGTA, [ethylenedis-(oxyethylenenitrilo)]tetraacetic acid.

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in net fluxes to be more accurately determined. Decreasing Cl^- concentration did not lessen the change in net Cl^- flux produced by theophylline (compare Table 1 with data in ref. 11). Electric potential difference (PD), conductance (G), short-circuit current (I_{sc} , the amount of current needed to nullify the PD) and unidirectional mucosa-to-serosa (m-to-s) and s-to-m fluxes were determined as described (10).

Cyclic Nucleotide Measurements. Mucosal scrapings (20–40 mg) from rabbit ileum were incubated at 37° in low Cl^- /Ringer's solution (see above) as described (11). After 20 min, varying amounts of heat-stable enterotoxin were added. At various times thereafter tissues (usually three pieces) were quickly transferred into 3–5 ml of cold 5% trichloroacetic acid in 0.1 M HCl containing either [^3H]cAMP (0.03 pmol) or [^3H]cGMP (0.2 pmol) added as a recovery marker. Samples were prepared for assay as described (11) with one exception: for cGMP assay the pH of the acetate buffer used to redissolve the dried residues was increased to 6.2. Fifty-microliter aliquots were assayed in duplicate for cyclic AMP (cAMP) with the protein kinase binding procedure of Gilman (12). One hundred microliters were assayed in duplicate for cGMP by radioimmunoassay of the acetylated derivative as described by Harper and Brooker (13). The kit provided by New England Nuclear (Boston, MA) was used. This assay provided quantitative recovery of two known amounts of unlabeled cGMP (0.8 and 2.5 pmol/mg of protein) which were added to trichloroacetic acid along with tissue.

To test the validity of cGMP measurements, dried residues of tissue extracts [prepared as described (11)] were dissolved in 0.3 M perchloric acid and applied to Dowex 1-X2, 200–400 mesh, 0.7×10 cm anion exchange columns (Cl^- form) that had been equilibrated with water. After the column was washed with 15 ml of 0.01 M HCl to remove cAMP, cGMP was eluted with 8 ml of 0.1 M HCl (J. Blumberg, personal communication). After lyophilizing and redissolving in acetate buffer (pH 6.2), a portion of the cGMP was directly assayed and a portion was incubated with bovine heart cyclic nucleotide phosphodiesterase (Sigma) as described by Butcher (14). Four milliuunits of enzyme were added per estimated pmol of cGMP. After 30 min of incubation at 30° , 4 ml of 5% trichloroacetic acid was added and the trichloroacetic acid supernate was processed for cGMP assay as described above.

Guanylate Cyclase Determinations. A 50- to 60-cm segment of ileum was resected, rinsed with cold 7 mM phosphate/0.15 M NaCl (pH 7.4), filled with the same buffer containing 0.4 mM dithiothreitol, kept on ice for 10 min, and finally emptied and rinsed again with phosphate-buffered saline. It was then everted and divided into 15-cm segments, one or more of which was (were) stretched over the shaft of an E_1 Vibromixer (Chempec Inc., Woodbury, NY) and vibrated at low speed into Ca^{2+} -free HCO_3^- /Ringer's solution (10) containing 0.4 mM [ethylenedis(oxyethylenetriolo)]tetraacetic acid (EGTA) and 0.1% bovine serum albumin. The medium, which was bubbled with 5% CO_2 in O_2 and maintained at 37° , was changed at 5-min intervals for a total of 15 min. Each 5-min collection was immediately sedimented in the cold at $50 \times g$ for 5 min. Histological examination of gut remaining at 15 min revealed that most crypts were still present. After the cell pellets were resuspended in fresh medium, the three fractions were combined and sedimented again. This cell pellet was resuspended in 12 mM Tris-HCl (pH 7.4) containing 3 mM MgCl_2 , 6 mM KCl, 0.2% bovine serum albumin, and 1 mM dithiothreitol and disrupted in the cold with 70 strokes of a tightly fitting Dounce pestle. Microscopic examination revealed membrane fragments, nuclei, and cell ghosts which took up

trypan blue. After sedimentation in the cold at $13,000 \times g$ for 10 min, the pellet was resuspended in the hypotonic Tris buffer to which 20% vol/vol of glycerol had been added. Aliquots of the suspension were then placed into multiple small glass vials and stored frozen in liquid N_2 . Guanylate cyclase activity proved stable in the frozen state for at least 2 months. After thawing, the membranes were sedimented at $13,000 \times g$ for 10 min, resuspended in 10 vol of 50 mM Tris-HCl (pH 7.5) containing 5 mM MgCl_2 , 1 mM dithiothreitol, and 0.05% bovine serum albumin, sedimented again, and then resuspended in the same buffer to provide a protein concentration of about 5 mg/ml.

Guanylate cyclase activity in 30–100 μg of membrane protein was determined by measuring the conversion of [α - ^{32}P]GTP (1 μCi ; 0.4 mM) to [^{32}P]cGMP in the presence of 5 mM MgCl_2 , 0.1 mM MnCl_2 , 0.46 mg of Lubrol PX per ml; 3 mM phosphoenolpyruvate, 7 μg of pyruvate kinase, 5 mM KCl, 1 mM 1-methyl-3-isobutylxanthine, and 0.05% bovine serum albumin, all dissolved in 50 mM Tris-HCl (pH 7.5). Membrane suspension (12 μl), heat-stable enterotoxin in 50 mM Tris buffer or buffer alone (10 μl), and the reaction mixture (48 μl) were added individually to the assay tubes, adhering to the walls as three separate drops. Reactions were then initiated by mixing. Incubations were for 10 min at 35° and were terminated by addition of 0.5 ml of cold 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, and 0.03 μCi of [^3H]cGMP. Immediately thereafter 50 μl of 50% trichloroacetic acid was added. After sedimentation at $1200 \times g$ for 10 min, [^{32}P]cGMP was isolated from the supernate by ion-exchange and alumina column chromatography as described by Nesbitt *et al.* (15). Three milliliters of column eluate were dissolved in Bray's solution (16) and radioactivity was measured. Enzyme activities were determined in duplicate and were corrected for recovery of [^3H]cGMP and for an enzyme blank prepared by incubating heat-denatured membranes in the above reaction mixture. The blank accounted for about 25% of the total ^{32}P eluted under control conditions. In the presence of the enterotoxin, [^{32}P]cGMP generation was linear with the amount of membrane protein added up to at least 125 μg and continued to increase with time for at least 15 min.

Materials. *E. coli* C600 (B14) was kindly provided by Stanley Falkow (University of Washington, Seattle, WA). [^3H]cAMP (30–50 Ci/mmol) and [^3H]cGMP (5–10 Ci/mmol) were obtained from New England Nuclear, [α - ^{32}P]GTP (5–12 Ci/mmol) was from New England Nuclear, Amersham Dowex anion exchange resins were from Bio-Rad, neutral alumina was from Brinkmann, and 1-methyl-3-isobutylxanthine was from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were obtained from Sigma.

RESULTS

Effects of enterotoxin on ion transport

The basic characteristics of the electrical response to heat-stable enterotoxin are shown in Fig. 1. Addition of the enterotoxin to the mucosal (luminal) reservoir produced a rapid (onset with 30 sec) increase in PD, which became maximal within 5 min and usually persisted at that level (PD also increased after serosal addition, although more slowly). The increase in PD produced by mucosal addition could be reversed by replacing the mucosal bathing medium with fresh Ringer's solution. Addition of theophylline after 60 mouse units of enterotoxin per ml resulted in a further increase in PD which was, however, smaller than that produced by theophylline in the absence of the enterotoxin.

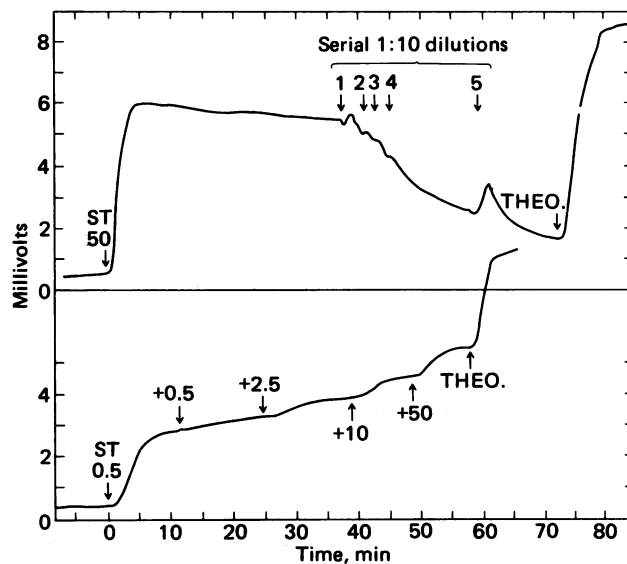


FIG. 1. Effect of heat-stable enterotoxin on transepithelial PD. The upper and lower panels give results for separate tissues from the same animal. Enterotoxin was added on the mucosal side; the specified increments in concentration are in mouse units per ml. Theophylline (THEO.) was added to the serosal side (5 μ mol/ml). Reversibility was examined by replacing the solution in the mucosal-side reservoir several times, each change representing a 1:10 dilution of the prior concentration.

As indicated in Figs. 1 and 2, we observed progressive increments in PD and I_{sc} over a surprisingly broad range of enterotoxin concentrations (0.1–266 mouse units/ml) and we were unable to demonstrate a maximal effect. Because these data were generated by sequential additions of toxin to the same tissues, we did four additional experiments to compare, in tissues

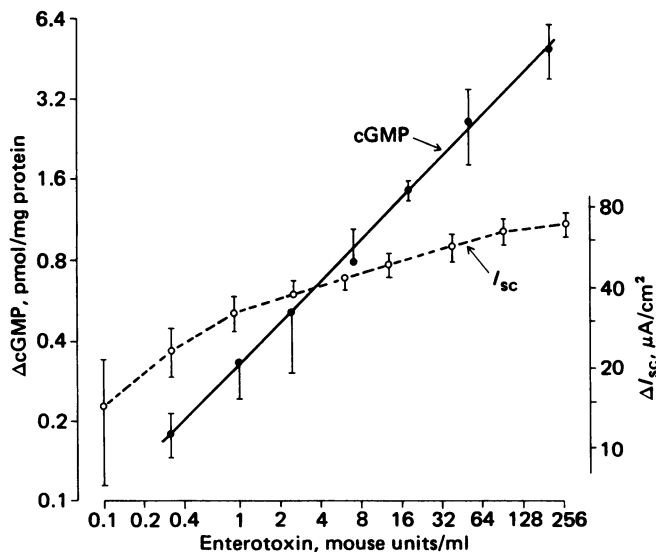


FIG. 2. Effects of various enterotoxin concentrations on cGMP concentration and I_{sc} . Each point represents the mean \pm 1 SEM (brackets) of cGMP determinations on tissues from four animals or of I_{sc} measurements on tissues from six animals. The concentration of cGMP in the absence of enterotoxin was 0.11 ± 0.03 pmol/mg of protein. The least squares regression line for cGMP concentration and the square root of enterotoxin concentration. I_{sc} responses were elicited by sequential additions (5–10 min apart) to the same tissues.

from the same animal, the changes in I_{sc} produced by sequential additions with the changes produced by single additions (0.1–100 mouse units/ml) to four separate tissues. Both methods gave the same dose-response relationship, indicating that rapid desensitization to the enterotoxin does not develop and therefore cannot account for the observed broad concentration-dependence of the electrical response.

In light of the previously observed stimulation of secretion by a Ca^{2+} ionophore (17), we looked for evidence of Ca^{2+} dependence. Omission of Ca^{2+} from the mucosal bathing medium did not diminish the electrical response to the enterotoxin (added to the mucosal side) and titration of the mucosal Ca^{2+} with EGTA did not reverse an already elicited response. Thus, heat-stable enterotoxin does not appear to significantly increase the Ca^{2+} permeability of the brush border membrane. Effects of the enterotoxin on unidirectional and net Cl^- fluxes across the short-circuited mucosa are compared in Table 1 to those of theophylline, which causes flux changes identical to those caused by cAMP (10). Under short-circuit condition, a net Cl^- flux ($J_{ms}^{Cl} - J_{sm}^{Cl} \neq 0$) suggests active transport of Cl^- . The enterotoxin abolished the net Cl^- absorption present under baseline conditions. Theophylline produced an appreciably larger effect, stimulating net secretion. Both agents decreased J_{ms}^{Cl} and increased J_{sm}^{Cl} , although the effect of theophylline on J_{sm}^{Cl} was larger than that of the enterotoxin. The combination of enterotoxin and theophylline did not have a greater effect than did theophylline alone. Na^+ fluxes were not measured. The enterotoxin also reduced tissue conductance by 15%, the same change in conductance previously observed with theophylline and cAMP (11) (theophylline had a smaller effect on conductance in the present experiments due probably to the later time of its addition).

Effects of enterotoxin on cyclic nucleotide concentrations

Heat-stable enterotoxin did not alter cAMP but produced a persistent increase in cGMP concentration (Table 2). That the radioimmunoassay was indeed measuring an increase in cGMP and not some other compound was confirmed as follows: (i) The same cGMP concentration was measured in different dilutions of the same extracts (after correcting for dilution). (ii) The same cGMP concentration was also measured in tissue extracts purified by anion exchange chromatography (see *Methods*). (iii) Finally, treatment with bovine heart cyclic nucleotide phosphodiesterase (see *Methods*) reduced the measured cGMP concentration to 5% of its former value.

As shown in Fig. 2, the rate of increase in cGMP concentration was constant over the entire range of enterotoxin concentrations tested, varying from $1.7 \times$ basal at 0.3 mouse units/ml to $46 \times$ basal at 266 mouse units/ml.

Theophylline produced about a 3-fold increase in cGMP concentration in both enterotoxin-treated and control mucosa (Table 2), suggesting that the toxin affects guanylate cyclase and not cGMP phosphodiesterase.

Effect of enterotoxin on guanylate cyclase activity

Heat-stable enterotoxin stimulated guanylate cyclase when added directly to a crude membrane fraction from isolated intestinal epithelial cells. In the presence of 5 mM Mn^{2+} , only 1.5- to 2-fold stimulation was achieved. When the Mn^{2+} concentration was reduced to 0.1 mM (5 mM Mg^{2+} and 0.05% Lubrol PX added[§]), however, 7-fold stimulation was obtained

[§] The reduction in Mn^{2+} concentration reduced basal enzyme activity to about 1/8 its original value.

Table 1. Effect of heat-stable enterotoxin on Cl⁻ transport

Tissues	J_{ms}^{Cl}	J_{sm}^{Cl}	J_{net}^{Cl}	I_{sc}	G
Control					
Baseline	6.8 ± 0.53	4.1 ± 0.26	2.7 ± 0.51	1.4 ± 0.34	25 ± 1.8
+ Theophylline	4.5 ± 0.30	6.9 ± 0.35	-2.5 ± 0.50	4.1 ± 0.42	23 ± 1.4
<i>P</i>	<0.002	<0.001	<0.001	<0.001	<0.05
Enterotoxin-treated					
Baseline	5.2 ± 0.38	4.8 ± 0.33	0.4 ± 0.22	2.3 ± 0.27	21 ± 1.0
+ Theophylline	4.0 ± 0.33	6.6 ± 0.61	-2.5 ± 0.64	3.5 ± 0.28	21 ± 1.1
<i>P</i>	<0.02	<0.025	<0.01	<0.001	NS
<i>P</i> (control vs. enterotoxin)					
Baseline	<0.02	<0.025	<0.01	<0.05	<0.025
+ Theophylline	NS	NS	NS	0.05	NS

Means ± 1 SEM for seven paired experiments. Tissues were short-circuited and unidirectional Cl⁻ fluxes from m-to-s (J_{ms}^{Cl}) and from s-to-m (J_{sm}^{Cl}) were measured. $J_{net}^{Cl} = J_{ms}^{Cl} - J_{sm}^{Cl}$. Fluxes and I_{sc} are in $\mu\text{Eq/hr}\cdot\text{cm}^2$; conductance (G) is in mmhos/cm². Time periods for baseline and post-theophylline flux measurements were 70–100 and 145–175 min after tissues were mounted, respectively. Enterotoxin (50 mouse units/ml) was added to the mucosal medium 30 min before the first flux period and theophylline (5 $\mu\text{mol/ml}$) was added to the serosal medium 30 min before the second flux period. NS, not significant.

(Fig. 3).^h Introduction of 1% Lubrol PX greatly increased basal activity but prevented stimulation by enterotoxin. These observations indicate a similarity between effects of the enterotoxin and free fatty acids on guanylate cyclase: high concentrations of Mn²⁺ and Lubrol prevent guanylate cyclase stimulation by free fatty acids in fibroblasts (19).

Higher concentrations of enterotoxin were required to stimulate guanylate cyclase activity in isolated membranes than to increase cGMP concentration in intact cells. Loss of sensitivity to agonists during membrane isolation procedures is not unusual, however. Such losses have been noted for adenylate cyclase stimulation by hormones, especially catecholamines (20).

DISCUSSION

A partially purified heat-stable *E. coli* enterotoxin has been shown to inhibit active Cl⁻ absorption and to stimulate particulate guanylate cyclase in intestinal epithelial cells. Remarkably small amounts (as little as 0.1 mouse unit or 1 ng/ml) were required to stimulate intact cells. The data suggest that cGMP is the intracellular mediator for the change in ion transport: increases in both cGMP concentration and I_{sc} were maximal within 5 min of adding toxin and both persisted for at least 30 min. At the low end of their dose-response curves (Fig. 2), electrical and cGMP responses showed similar sensitivities to toxin. At higher enterotoxin concentrations, the electrical response appeared to approach a maximum while the cGMP response continued to increase linearly, but it is not uncommon for the physiological response to a hormone to become maximal before the cyclic nucleotide response does. Finally, enterotoxin does not appear to act through an increase in either cAMP concentration or plasma membrane Ca²⁺ permeability, the two already established means for eliciting secretion in the small intestine (17).ⁱ

^h Since our assays contained 3 mM phosphoenolpyruvate, which inhibits guanylate cyclase activity (18), we compared the enzyme activities obtained with the present assay system with those obtained using 10 μg of creatine kinase (Sigma) per assay and 7 mM creatine phosphate: with the latter regenerating system, guanylate cyclase activities in both the presence and absence of enterotoxin were about twice those obtained with pyruvate kinase and phosphoenolpyruvate, but the relative stimulation by the enterotoxin was the same.

ⁱ There also is no increase in cGMP concentration in rabbit ileum when secretion is stimulated with a Ca²⁺ ionophore (unpublished data).

Earlier studies with isolated small intestinal mucosa have presented uncertain and potentially contradictory observations on the role of cGMP in intestinal electrolyte transport. A secretory PD can be elicited with relatively large amounts of cGMP or 8-bromo-cGMP (10, 21), but the possibility that this effect results from an increase in cAMP concentration due to inhibition of cyclic nucleotide phosphodiesterase has not been excluded. Cholinergic agonists also stimulate secretion (22) and cause a short-lived (5–10 min) increase in cGMP concentration. Unlike enterotoxin, however, they do not stimulate guanylate cyclase in isolated plasma membranes (23) and their stimulation of intestinal secretion is dependent on extracellular Ca²⁺ (17). Epinephrine, through α -adrenergic receptors, also produces a short-lived increase in cGMP concentration (21), but its effect on ion transport is essentially opposite to that of the enterotoxin and the cholinergic agonists (24). Insulin and cholecystokinin octapeptide also produce short-lived increases in cGMP (21), but neither stimulates secretion. Indeed, there is evidence that both have a weak, α -adrenergic-like effect on ion transport (25, 26). The increases in cGMP produced by these neurotransmitters and hormones may not all occur in the same cell type affected by enterotoxin and they may be compensatory responses to other overriding cellular changes. They do not, therefore, preclude a role for cGMP in the stimulation of secretion. Nonetheless, in view of these apparently contradictory

Table 2. Effects of heat-stable enterotoxin on cAMP and cGMP levels

Enterotoxin, mouse units/ml	Cyclic nucleotide, pmol/mg protein		
	5 min	30 min	35 min (+ theophylline)
A. cAMP (<i>n</i> = 4)			
0	12.2 ± 0.95	7.6 ± 2.2	27.2 ± 5.7
62.5	10.7 ± 2.5	9.6 ± 3.4	22.6 ± 5.3
B. cGMP (<i>n</i> = 4)			
0	0.15 ± 0.048	0.08 ± 0.024	0.27 ± 0.060
1.6	0.61 ± 0.017	0.68 ± 0.14	2.00 ± 0.40
78	2.62 ± 0.60	2.42 ± 0.31	8.55 ± 0.66

Means ± 1 SEM. Times refer to min after addition of enterotoxin. Theophylline, 5 $\mu\text{mol/ml}$, was added at 30 min.

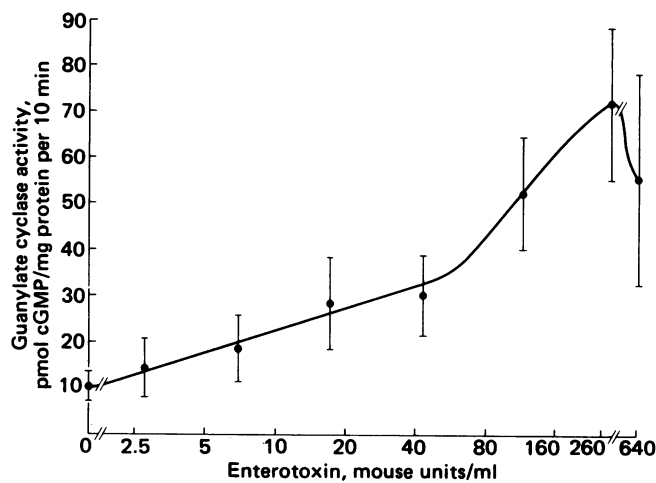


FIG. 3. Guanylate cyclase activity as a function of [enterotoxin]. Points and brackets shown are means \pm 1 SEM for five complete experiments on membranes pooled from four animals. The decline in activity at the highest concentration was observed in only two of the five animals and is not statistically significant.

findings, the possibility that the enterotoxin has another mode of action should not be prematurely dismissed.

cAMP has two separable effects on ion transport in mammalian small intestine: (i) inhibition of a coupled uptake process for NaCl in the brush border and (ii) stimulation of active anion secretion (27). It is likely that the former effect is exerted on villus cells and the latter on crypt cells. Since the enterotoxin inhibited net Cl⁻ absorption but, unlike theophylline, did not cause net secretion, it is tempting to postulate that cGMP exerts only one of the two effects produced by cAMP, probably the former. Of interest in this regard is the demonstration by DeJonge (28) of a brush border protein kinase which phosphorylates that membrane and which is more sensitive to cGMP than to cAMP. It may also be relevant that most of the guanylate cyclase activity in mammalian small intestine is found in villus cells (29, 30) and that a high specific activity of the enzyme has been found in isolated brush border membranes (23, 29, 30).

The structure of heat-stable enterotoxin and the mechanism for its stimulation of guanylate cyclase are unknown. There are basic differences between its action and that of cholera toxin: in intact cells, the enterotoxin acts far more rapidly and its action, unlike that of cholera toxin, is readily reversible. Thus, unlike cholera toxin, the enterotoxin appears to dissociate readily from the cell membrane, to rapidly transmit a transmembrane signal, and to produce a readily reversible change in the activity of the target nucleotide cyclase. In studies with cell lysates and isolated plasma membranes, the action of cholera toxin has been shown to be absolutely dependent on the presence of NAD and to be greatly enhanced by one or more cytosol macromolecules (31). The present experiments suggest that neither NAD nor cytosol macromolecules are needed for the action of heat-stable enterotoxin, although contributions from trace amounts still adherent to washed plasma membranes have not been excluded.

Note Added in Proof. Since this manuscript was submitted for publication, Hughes *et al.* (32) reported increases in mucosal cGMP concentration in segments of mouse intestine exposed for 20 min to culture

filtrates containing heat-stable *E. coli* enterotoxin. They also reported that the 8-Br analogue of cGMP stimulates intestinal secretion.

We are grateful to Stephanie Orellana for technical assistance. This work was supported by National Institutes of Health Grants AI 13083 and AI 09029. W.J.L. was recipient of National Research Service Award AI-05366 from the U.S. Public Health Service.

- Gyles, C. L. (1971) *Ann. N.Y. Acad. Sci.* **176**, 314–322.
- Sack, R. B., Jacobs, B. & Mitra, R. (1974) *J. Infect. Dis.* **129**, 330–335.
- Sack, D. A., Merson, M. H., Wills, J. G., Sack, R. B. & Morris, G. K. (1975) *Lancet* **ii**, 239–241.
- Ryder, R. W., Wachsmuth, I. K., Buxton, A. E., Evans, D. G., DuPont, H. L., Mason, E. & Barrett, F. F. (1976) *N. Engl. J. Med.* **295**, 849–853.
- Gyles, C. L. (1974) *J. Infect. Dis.* **129**, 277–283.
- Dorner, F. & Mayer, P. (1975) *Infect. Immun.* **11**, 329–335.
- Evans, D. G., Evans, D. J. & Pierce, N. F. (1973) *Infect. Immun.* **7**, 873–879.
- Alderete, J. F. & Robertson, D. C. (1978) *Infect. Immun.* **19**, 1021–1030.
- Dean, A. G., Ching, Y-C, Williams, R. G. & Harden, L. B. (1972) *J. Infect. Dis.* **125**, 407–411.
- Field, M., Fromm, D. & McColl, I. (1971) *Am. J. Physiol.* **220**, 1388–1396.
- Field, M., Sheerin, H. E., Henderson, A. & Smith, P. L. (1975) *Am. J. Physiol.* **229**, 86–92.
- Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 305–312.
- Harper, J. F. & Brooker, G. (1975) *J. Cyclic Nucleot. Res.* **1**, 207–218.
- Butcher, R. W. (1976) in *Methods in Enzymology*, eds Colowick, S. P. & Kaplan, N. O. (Academic, New York), Vol. 38 pp. 218–223.
- Nesbitt, J. A., Anderson, W. B., Miller, Z., Pastan, I., Russell, T. R. & Gospodarowicz, D. (1976) *J. Biol. Chem.* **251**, 2344–2352.
- Bray, G. (1960) *Anal. Biochem.* **1**, 279–285.
- Bolton, J. E. & Field, M. (1977) *J. Membr. Biol.* **35**, 159–173.
- Hardman, J. G., Davis, J. W. & Sutherland, E. W. (1969) *J. Biol. Chem.* **244**, 6354–6362.
- Wallach, D. & Pastan, I. (1976) *J. Biol. Chem.* **251**, 5802–5809.
- Field, M. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3299–3303.
- Brasitus, T. A., Field, M. & Kimberg, D. V. (1976) *Am. J. Physiol.* **321**, 285–292.
- Isaacs, P. E. T., Corbett, C. L., Riley, A. K., Hawker, P. C. & Turnberg, L. A. (1976) *J. Clin. Invest.* **58**, 535–542.
- Walling, M. W., Mircheff, A. K., Van Os, C. H. & Wright, E. M. (1978) *Am. J. Physiol.*, in press.
- Field, M. & McColl, I. (1973) *Am. J. Physiol.* **225**, 852–857.
- Fromm, D., Field, M. & Silen, W. (1969) *Surgery* **66**, 145–148.
- Fromm, D. & Silen, W. (1970) in *Current Topics in Surgical Research*, eds Zuidema, G. D. & Skinner, D. B. (Academic, New York) Vol. 1, pp. 249–251.
- Field, M. (1976) in *Acute Diarrhoea in Childhood*, Ciba Symposium 42, eds Elliott, K. & Knight, J. (Elsevier/Excerpta Medica/North Holland, Amsterdam, Netherlands), pp. 109–122.
- DeJonge, H. R. (1976) *Nature* **262**, 590–593.
- Quill, J. & Weiser, M. M. (1975) *Gastroenterology* **69**, 470–478.
- DeJonge, H. R. (1975) *FEBS Lett.* **53**, 237–242.
- Gill, D. M. (1976) *J. Infect. Dis.* **133**, s55–s63.
- Hughes, J. M., Murad, F., Chang, B. & Guerrant, R. L. (1978) *Nature* **271**, 755–756.