# Stimulation of Intestinal Mucosal Adenyl Cyclase by Cholera Enterotoxin and Prostaglandins

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ABSTRACT The effects of several prostaglandins (PG) and a highly purified preparation of cholera enterotoxin (CT) on intestinal mucosal adenyl cyclase activity and the effect of CT on intestinal mucosal cyclic 3′,5′-adenosine monophosphate concentration were determined in guinea pig and rabbit small intestine and were correlated with the effects of the same agents on ion transport. Adenyl cyclase activity, measured in a crude membrane fraction of the mucosa, was found at all levels of the small intestine with the highest activity per milligram protein in the duodenum. The prostaglandins, when added directly to the assay, increased adenyl cyclase activity; the greatest effect (2-fold increase) was obtained with $PGE_1$ (maximal effect at 0.03 mM) and $PGE_2$. The prostaglandins also increased short-circuit current (SCC) in isolated guinea pig ileal mucosa, with $PGE_1$ and $PGE_2$ again giving the greatest effects. The prior addition of theophylline (10 mM) reduced the subsequent SCC response to $PGE_1$ and vice versa. It was concluded, therefore, that the SCC response to $PGE_1$, like the response to theophylline, represented active Cl secretion. CT increased adenyl cyclase activity in guinea pig and rabbit ileal mucosa when preincubated with the mucosa from 1 to 2.5 hr in vitro or for 2.5 hr in vivo but not when added directly to the assay. The increments in activity caused by $PGE_1$ and NaF were the same in CT-treated and control mucosa. Cyclic 3′,5′-AMP concentration in rabbit ileal mucosa was increased 3.5-fold after a 2 hr preincubation with CT in vitro. Phosphodiesterase activity in the crude membrane fraction of the mucosa was unaffected by either CT or $PGE_1$. A variety of other agents including insulin, glucagon, parathormone, thyroid-stimulating hormone, L-thyroxine, thyrocalcitonin, vasopressin, and epinephrine all failed to change adenyl cyclase activity. It is concluded that CT and certain prostaglandins produce small intestinal fluid secretion by increasing mucosal adenyl cyclase activity, thereby stimulating an active secretory process.

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## INTRODUCTION

Previous studies by Field and his coworkers demonstrated that the addition of cyclic 3′,5′-adenosine monophosphate (cyclic 3′,5′-AMP),[1] or theophylline to isolated rabbit ileal mucosa stimulates an active secretion of chloride and inhibits the active absorption of sodium (1–3). This reversal in the direction of net solute transport was also observed when cholera enterotoxin (CT) was placed on the luminal side of isolated rabbit ileal mucosa (2). More recent experiments employing in vivo perfused loops in the dog as well as isolated rabbit ileal mucosa have demonstrated that certain prostaglandins (PG) induce fluid secretion in vivo and stimulate the chloride secretory process in vitro (4, 5).

The effects of cholera enterotoxin and prostaglandins on small intestinal ion transport are clearly similar to those of cyclic 3′,5′-AMP and theophylline, a known phosphodiesterase inhibitor (6). Furthermore, certain of the prostaglandins are known to affect cyclic 3′,5′-AMP levels in other tissues (7), and recently it has been demonstrated that cholera enterotoxin has cyclic AMP-like effects on isolated fat cells (8), liver, and platelets (9). It has been proposed that the cholera enterotoxin and prostaglandin-induced gut fluid losses may be mediated by means of an interaction with adenyl cyclase in the intestinal mucosa (2, 4, 5), but direct evidence for such an interaction has heretofore been lacking.

[1] Abbreviations used in this paper; AMP, adenosine monophosphate; CT, cholera entertoxin; PG, prostaglandins; SCC, short-circuit current.

The studies reported here demonstrate that purified cholera enterotoxin and certain prostaglandins stimulate adenyl cyclase activity in a broken cell preparation obtained from the small intestinal mucosa of experimental animals. Cholera enterotoxin and those prostaglandins which are effective in influencing adenyl cyclase activity also cause short-circuit current (SCC) changes in isolated ileal mucosa which are characteristic of the chloride secretory process. Finally, cyclic 3′,5′-AMP levels in the intestinal mucosa have been measured, and a striking increase following the incubation of tissue with cholera enterotoxin has been demonstrated. The results of these studies strengthen the hypothesis that the secretory effects of cholera enterotoxin and certain prostaglandins are mediated by an interaction with the adenyl cyclase system.

## METHODS

*Adenyl cyclase assay-tissue preparation.* Male long-haired quinea pigs (500-1100 g), male New Zealand white rabbits (2-3 kg), and albino male rats of the Sprague-Dawley strain (100-150 g) were used in the present study. After an overnight fast, the animals were sacrificed by a blow to the head followed by exsanguination. The small intestinal segment to be used was quickly removed, rinsed in ice-cold isotonic saline, and everted over a chilled glass rod. The everted gut was gently blotted, placed on a chilled glass plate, and the mucosa was scraped with a glass microscope slide. All subsequent operations were performed at 0°-4°C. The mucosal scrapings were homogenized (1 g/50 ml) in 0.25 M sucrose containing 0.02 M glycylglycine buffer, pH 7.8, and $10^{-3}$ M $MgSO_4$ with three passes of a motor-driven Teflon pestle using a glass homogenizer. The suspension was sedimented at $2000 \times g$ for 10 min in a Sorvall RC-2 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The resultant pellet was resuspended with a glass rod in 0.02 M glycylglycine buffer, pH 7.8, containing $10^{-3}$ M $MgSO_4$ (50 ml/g of original wet weight). This suspension was sedimented at $2000 \times g$ for 20 min. The resultant pellet was again resuspended with a glass rod and washed three more times in the same magnesium containing glycylglycine buffer (10 ml/g of original wet weight) employing 10 min of centrifugation at $2000 \times g$. After the fourth wash, the final pellet was resuspended with a glass rod in a sufficient volume of 0.02 M glycylglycine buffer at pH 7.8, containing $10^{-3}$ M $MgSO_4$, such that each milliliter contained between 6.0 and 15 mg of protein as determined by the method of Lowry, Rosenbrough, Farr, and Randall (10). Electron microscopic examination of the final pellet revealed the presence of numerous membranous structures including some which were recognizable as brush borders.

*Adenyl cyclase assay-incubation method.* Adenyl cyclase activity was measured by a modification of the procedure described by Krishna, Weiss, and Brodie (11). The reaction medium consisted of 50 mM Tris-HCl buffer at pH 7.4, 5 mM $MgCl_2$, 10 mM caffeine, 2 mM adenosine triphosphate (ATP), 2 μCi per ml of adenosine triphosphate-8-$^{14}$C, and an ATP-regenerating system consisting of 10 mM phosphoenolypyruvate and 250 μg/ml of pyruvate kinase. Including enzyme, test materials, or solvent controls whenever appropriate, the final volume of incubation was 0.5 ml. The usual enzyme concentration was between 1.2 and 3.0 mg of protein per assay. Whenever NaF was included in the assay, its concentration was 10 mM. Prostaglandins were kindly provided by Dr. John Pike of The Upjohn Co., Kalamazoo, Mich., and were prepared as stock solutions at a concentration of 1 mg per ml in 9.5% ethanol containing $1.7 \times 10^{-3}$ M $Na_2CO_3$ (final pH of 7.0). When not specified, the prostaglandin concentration was 50 μg per assay ($2.9 \times 10^{-4}$ M for $PGE_1$) in the results presented in the tables and figures. Crystalline beef insulin (Eli Lilly and Co., Indianapolis, Ind.) and porcine thyrocalcitonin, 17 U/mg (gift of Dr. L. Sherwood) were dissolved in 0.1 N acetic acid and diluted before use. Bovine parathyroid hormone (Wilson Laboratories, Chicago, Ill.), synthetic vasopressin, 102 IU/mg (Calbiochem), crystalline glucagon (Sigma Chemical Co., St. Louis, Mo.), and thyroid stimulating hormone (Thytropar, Armour Pharmaceutical Co., Chicago, Ill.) were dissolved in $H_2O$, and L-thyroxine (Smith, Kline and French Laboratories, Philadelphia, Pa.) was dissolved in 0.01 N NaOH. Whenever these test materials were included in the assay, appropriate solvent controls were employed.

Incubations were initiated by the addition of enzyme and were performed at 37°C for 10 min unless otherwise noted in the legends to the tables and figures. Determinations were routinely done in triplicate and for each set of incubations a reaction blank without enzyme was employed. Just before the termination of the reaction, 0.1 ml of a solution containing 0.06 mg of cyclic 3′,5′-AMP and 1 μCi of cyclic 3′,5′-AMP-$^3$H was added to each tube and the reaction was then terminated by immersing the tubes in a boiling water bath for 3 min. The cyclic 3′,5′-AMP-$^3$H served to determine the recovery of cyclic 3′,5′-AMP during the subsequent steps. After heating, the precipitate was removed by centrifugation at $1500 \times g$ for 15 min and the supernatant material was passed over a $0.5 \times 3.0$ cm column containing Dowex 50W-X4, hydrogen form, 200 to 400 mesh (Calbiochem). 1 ml fractions were collected; the cyclic 3′,5′-AMP was eluted with water and generally emerged between 4 and 6 ml. To each fraction, 0.1 ml of 0.25 M $ZnSO_4$ followed by 0.1 ml of 0.25 M $Ba(OH)_2$ was added. The mixture was agitated, centrifuged at 1500 *g* for 5 min, and the additions of $ZnSO_4$ and $Ba(OH)_2$ repeated without disturbing the first precipitate. After centrifugation at 1500 *g* for 5 min, the supernatant fluid was decanted and a third precipitation step was performed. After the final centrifugation, 1.0 ml portions of the supernate from each of the four fractions was added to 10 ml of the solution described by Bray (12). The $^{14}$C and $^3$H were measured simultaneously in an Intertechnique SL40 liquid scintillation spectrometer (Intertechnique Instruments, Inc., Dover, N. J.). The amount of cyclic AMP formed by the action of adenyl cyclase was calculated from the specific activity of the ATP-$^{14}$C and the recovery of cyclic 3′,5′-AMP-$^3$H by using data from the single sample containing the peak recovery of the added $^3$H marker (generally 40–50%). Results are expressed as millimicromoles of cyclic 3′,5′-AMP formed in excess of the reaction blank.[2] Statistical analyses were performed by Student's *t* test for paired variates.

Adenosine triphosphate-8-$^{14}$C (54.1 μCi/mole) and cyclic 3′,5′-AMP-$^3$H (16.3 Ci/μmole) were obtained from the New England Nuclear Corp., (Boston, Mass.) and Schwarz BioResearch Inc., (Van Nuys, Calif.), respectively. Other nu-

[2] Although the endogenous content of ATP was not measured, it was assumed to be relatively low in comparison to the amount of exogenous substrate added to the extensively washed membrane preparation.

cleotides, pyruvate kinase (type II), and phosphoenolpyruvate were purchased from the Sigma Chemical Co.

*Cholera enterotoxin experiments.* The mucosa from the distal 20-40 cm of the small intestine of the guinea pig or rabbit was scraped as described above, minced, and then randomized on a chilled glass plate with a spatula. Between 0.5 and 1.5 g of mucosal scrapings were added to a 50 ml Erlenmeyer flask containing 20 ml of a Ringer-bicarbonate buffer at pH 7.4, with 20 mM glucose, to which had been added either active cholera enterotoxin or enterotoxin which had been heat-inactivated in a boiling water bath for 15 min. The highly purified cholera enterotoxin (13) was kindly provided by Dr. R. A. Finkelstein. The Ringer-bicarbonate solution employed had the following ionic composition in millimoles per liter: Na, 141; K, 10; Ca, 1.25; Mg, 1.1; Cl, 127; $HCO_3$, 25; $H_2PO_4$, 0.3; and $HPO_4$, 1.65. The final concentration of enterotoxin in the flask was either 0.5 or 1.0 $\mu$g/ml as noted in the legends. The flasks were sealed with perforated rubber stoppers and placed in a 37°C metabolic incubator oscillating at a rate of 100 per min. The flasks were constantly gassed with 95% $O_2$-5% $CO_2$ which was first bubbled through water in order to minimize evaporative water loss. After varying periods of preincubation lasting up to 2.5 hr, the contents of the flasks were sedimented at 19,000 *g* for 10 min at 0°-4°C. The supernate was discarded and the pellet was homogenized in 0.25 M sucrose containing 0.02 M glycylglycine buffer at pH 7.8 and $10^{-3}$ M $MgSO_4$. Membranes were then prepared as described above, and adenyl cyclase activity was measured.

Experiments were also undertaken in which cholera enterotoxin was placed in isolated loops of rabbit ileum in vivo. After an overnight fast, rabbits weighing 2-3 kg were anesthetized with intravenous sodium pentobarbital (10 mg/kg). A 25 cm loop of distal ileum was isolated and polyethylene tubing was inserted and tied into each end. The loop was flushed with isotonic saline followed by air, and was then filled with 25 ml of the Ringer-bicarbonate buffer, pH 7.4, containing either 5 $\mu$g of active cholera enterotoxin or 5 $\mu$g of toxin which had been heat inactivated. The abdominal wall was closed and the polyethylene cannulae secured to the skin. Body temperature was maintained with a blanket and hydration was maintained by the intravenous infusion of isotonic sodium chloride (30 ml/hr). After 2.5 hr of preincubation in vivo, the animals were sacrificed with an overdose of barbiturate. The ileal loops were quickly removed, the fluid was drained, the loops everted and rinsed in fresh, cold Ringer-bicarbonate buffer, pH 7.4, and mucosal scrapings were prepared. The mucosa was homogenized and membranes prepared for the assay of adenyl cyclase activity as described previously.

*Phosphodiesterase assay.* Cyclic nucleotide phosphodiesterase activity was assayed by measuring the conversion of cyclic 3′,5′-AMP-$^3$H to adenosine 5′-monophosphate. The reaction mixture contained 50 mM Tris-HCl buffer at pH 7.4, 5 mM $MgCl_2$, 10 mM caffeine, 0.002 mM cyclic 3′,5′-AMP, and 0.4 $\mu$Ci/ml of cyclic 3′,5′-AMP-$^3$H. Including enzyme, solvent controls when appropriate, or $PGE_1$, the final volume of the incubation was 0.25 ml and the usual enzyme concentration was between 1.25 and 2.0 mg of protein per assay. The phosphodiesterase assay was performed in the presence and absence of $PGE_1$ and on membranes isolated from tissue which had been preincubated for 2.5 hr in vitro with either active or heat-inactivated cholera toxin as described above. Incubations were performed for 10 min at 37°C and were terminated by immersing the tubes in a boiling water bath for 3 min. Water was then added to bring the final volume to 1.0 ml. The tubes were mixed and the precipitate sedimented at 1500 *g* for 20 min. After the precipitation of adenosine 5′-monophosphate by the addition of $ZnSO_4$ and $Ba(OH)_2$, 0.5 ml of the supernate was assayed for $^3$H. The disappearance of cyclic 3′,5′-AMP-$^3$H from the supernatant fluid was a measure of the phosphodiesterase activity in the membrane preparation. Descending paper chromatography of the supernatant fluid in an isopropanol : ammonia : water (7 : 1 : 2) system, using cyclic 3′,5′-AMP-$^{14}$C as a marker, indicated that all of the tritium was present as cyclic AMP.

*Cyclic 3′,6′-AMP levels.* Rabbits which had been fed a normal rabbit chow *ad libitum* were killed by a blow to the neck. A 30 cm segment of distal ileum was excised, cut open along the mesenteric border, and rinsed with the Ringer-bicarbonate solution. The mucosa was separated from the underlying muscle by scraping with the edge of a glass microscope slide and was divided into 12–16 smaller sections which were randomized in cold Ringer-bicarbonate. Sections were removed, gently blotted, weighed, and placed in 10 ml of Ringer-bicarbonate solution containing 5 mM D-glucose. Four such incubation flasks with about 500 mg of mucosa in each were prepared from each animal. The flasks were individually gassed with 95% $O_2$-5% $CO_2$ and incubated with shaking at 37°C for 2 hr. The incubation was terminated by adding 0.5 ml of 11.6 N perchloric acid containing a small amount of cyclic 3′,5′-AMP-$^3$H as a recovery marker. A cyclic AMP–rich fraction was then obtained and assayed as outlined by Brooker, Thomas, and Appleman (14) with the following departures: After neutralization with KOH, each sample was added to 0.3 g of Norit "A" charcoal (Eastman Chemical Co., Rochester, N. Y.) which had been washed first in 1 N HCl and then in water. After shaking for 15 min, the charcoal was separated by centrifugation and washed twice with water. Nucleotides were then eluted from the charcoal by shaking for 15 min with an ethanol : ammonia : water solution (50 : 5 : 45 by volume). Samples were then centrifuged and the supernates evaporated under vacuum at 45°C. Subsequent purification was as outlined by Krishna, Weiss, and Brodie (11), except that the $Ba(OH)_2$ and $ZnSO_4$ precipitation step was performed before samples were applied to the columns. Eluted fractions containing most of the $^3$H were evaporated and redissolved in 0.5 ml of 100 mM Tris-HCl (pH 8.0). A 50 $\mu$l portion was assayed for $^3$H to determine the recovery of cyclic AMP originally present (range: 10–30%). Samples were assayed in quadruplicate and read against a simultaneously determined standard curve between 0 and 80 $\mu\mu$moles of cyclic AMP. Samples reading higher than 80 $\mu\mu$moles were reassayed at a higher dilution. In order to test the specificity and accuracy of the method, the following substances were added to 10 ml of Ringer-bicarbonate and put through the entire procedure: ATP, 0.1 mM; theophylline, 10 mM; and cyclic AMP, 0.2 $\mu$M (six samples). Assay of the ATP and theophylline samples yielded no apparent cyclic AMP. Assay of the six cyclic AMP samples indicated a concentration of 0.255 $\pm$0.055 $\mu$M (1 SD). As an additional test of specificity, portions of two extracts with elevated apparent cyclic AMP concentrations were preincubated with a 10-fold excess of phosphodiesterase for 10 min. After boiling and centrifugation, portions of the supernates were assayed in the usual manner. No detectable cyclic 3′,5′-AMP remained.

*Measurements of short-circuit current.* 20-30-cm segments of distal ileum from rabbits or guinea pigs were excised, cut open along the mesenteric border, rinsed clean, and placed in oxygenated Ringer-bicarbonate solution. 2- to 3-centimeter sections were stripped of muscularis and

mounted in chambers as previously described (15). Usually four tissues from the same animal were mounted. Tissues were bathed with Ringer-bicarbonate solution maintained at 37.5°C and gassed with 95% $O_2$-5% $CO_2$ (15). D-glucose (10 mM) was added to the serosal bathing solution and an equimolar amount of mannitol was added to the luminal solution. The pH of the solution remained constant at 7.4 for at least 3 hr in vitro.

The method for determining the short-circuit current (SCC) has been described previously (15, 16).

## RESULTS

*Measurement of adenyl cyclase activity in intestinal mucosa.* Adenyl cyclase activity was detectable in membrane fractions prepared from the small intestinal mucosa of the rat, rabbit, and guinea pig. Under basal conditions the amount of cyclic 3′,5′-AMP formed was quite small, but 5- to 10-fold increases in activity could regularly be obtained by the addition of $10^{-2}$ M NaF to the incubation mixture. Enzyme activity was a direct function of tissue protein concentration both in the presence and absence of $10^{-2}$ M NaF (Fig. 1). In the presence of NaF, linear kinetics were obtained during the first 5 min of incubation (Fig. 2). In the control tubes, although the formation of cyclic 3′,5′-AMP increased throughout the experiment, the rate of the reaction gradually decreased, despite the presence of both caffeine and an ATP-generating system. This decrease in reaction rate after the first few minutes has also been noted with many other tissues (17-19). The results with fluoride make it unlikely that this is due to substrate limitation. Enzyme concentration and time curves in the presence and absence of NaF, similar to those demonstrated in Figs. 1 and 2 with guinea pig ileal mucosa, were also obtained with enzyme prepared from rat and rabbit intestinal mucosa.

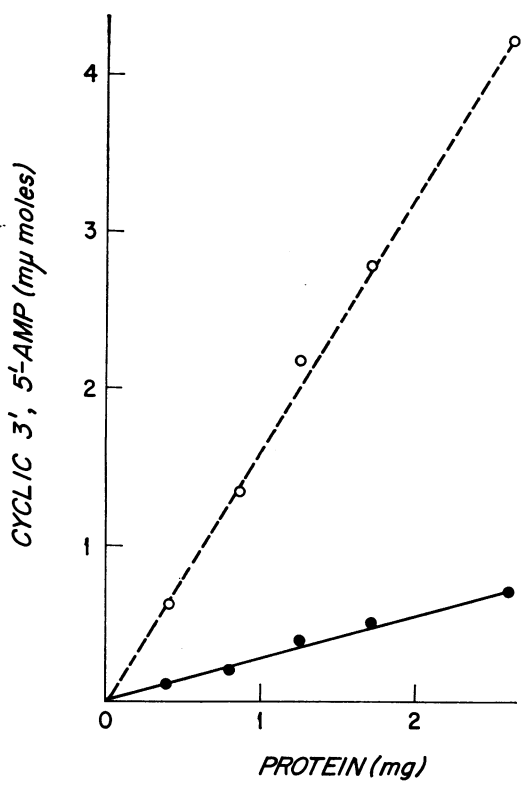


FIGURE 1 Effect of NaF on adenyl cyclase activity in membranes from guinea pig ileal mucosa, expressed as a function of mg membrane protein in the incubation mixture (0.5 ml). ●——●, no additions; ○---○, + NaF ($1.0 \times 10^{-2}$ M).

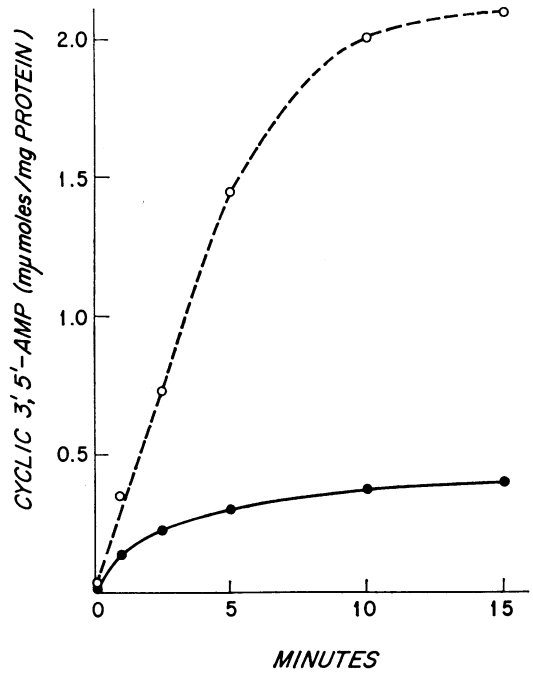


FIGURE 2 Effect of NaF on adenyl cyclase activity in membranes from guinea pig ileal mucosa, expressed as a function of time. Each test mixture contained 3.0 mg of membrane protein. ●——●, no additions; ○---○, + NaF ($1.0 \times 10^{-2}$ M). Two complete and separate experiments were performed and the results represent the mean values.

The membrane fragments used for adenyl cyclase assay were routinely washed four times in the magnesium-containing glycylglycine buffer (see Methods). The results of preliminary experiments demonstrated that repeated washing up to three or four times increased the specific activity of the enzyme incubated in both the presence and absence of NaF. This effect of repeated washings may in part be due to the elimination of non-enzyme-containing fragments, but may also be due to the elimination of contaminating soluble phosphodiesterase activity.

It was also noted that the preservation of enzyme activity required gentle methods of preparation. If the

TABLE I

*Effects of Various Conditions on Adenyl Cyclase Activity*

| Experiment No. | Conditions | Cyclic 3',5'-AMP | | |
|---|---|---|---|---|
| | | Control | NaF | $PGE_1$ |
| | | *mμmoles/mg protein* | | |
| 1 | Method of resuspension | | | |
| | Stirring | 0.26 | 1.61 | 0.56 |
| | Homogenization | 0.19 | 1.15 | 0.28 |
| 2 | Effect of aging | | | |
| | Fresh | 0.33 | 1.97 | 0.61 |
| | After 2 hr | 0.31 | 1.57 | 0.44 |
| | After 48 hr | 0.05 | 0.44 | 0.07 |
| 3 | Effect of pH | | | |
| | 7.0 | 0.50 | 3.15 | |
| | 7.4 | 0.50 | 3.10 | |
| | 7.8 | 0.59 | 3.12 | |
| | 8.2 | 0.47 | 1.90 | |

In experiments 1 and 3, the membranes were from guinea pig ileal mucosa; in experiment 2, from rat duodenal mucosa. In experiment 2 the assays were conducted immediately after preparation of the membranes, following 2 hr of aging on ice, or after 48 hr at —15°C. Shown are the means of values obtained in triplicate determinations in the same experiment.

final pellet was resuspended by gentle stirring, using a thin glass rod, then the base line, NaF, and prostaglandin-stimulated activities (see below) were substantially greater than those obtained with enzyme suspended by using a tightly fitting glass pestle in a Dounce glass homogenizer (Table I).

While both base line activity and stimulation by NaF could be demonstrated with membranes stored for as long as 48 hr in the freezer, there was a considerable decrease in adenyl cyclase activity under these conditions (Table I).

The presence of potassium, in concentrations up to 25 mM did not influence the reaction rate.

The results obtained when the incubation was performed at a variety of pH's between 7.0 and 8.2 are also shown in Table I. The curve is rather flat between pH 7.0 and 7.8, with a decline in the NaF-stimulated activity at pH 8.2. These results are in accord with those obtained in other systems (19, 20).

*Adenyl cyclase activity at various levels of intestine.* In a number of experiments adenyl cyclase activity was measured in membranes prepared from the proximal 20 cm of duodenum, the distal 20 cm of ileum and the middle 20 cm segment of the small intestine (jejunum) with material obtained from the rat, rabbit, and guinea pig. Table II summarizes the results of experiments conducted with guinea pig tissue, but similar results were obtained with the other species. As noted, the base line, NaF and $PGE_1$-stimulated enzyme activities are clearly demonstrable at all levels of the small intestine. There is, however, a slight decline in activity from duodenum to ileum.

*Effects of various prostaglandins on adenyl cyclase activity.* Experiments were undertaken in order to explore the effects, if any, of a variety of prostaglandins on the activity of adenyl cyclase in the small intestine. The results in Table III were obtained with rather high concentrations of prostaglandins added to membranes from guinea pig ileum. The same relative potencies could be demonstrated with enzyme prepared from the rat or rabbit intestine. As noted in the table, $PGE_1$ and $PGE_2$ were clearly the most potent stimulators of adenyl cyclase in the group of compounds tested. With the high concentrations employed, $PGA_1$, $PGF_{2\beta}$, $PGB_1$, and $PGF_{1\beta}$ had only a modest effect, whereas $PGF_{2\alpha}$ and $PGF_{1\alpha}$ had little or no effect.

The activity of adenyl cyclase was measured as a function of the concentration of $PGE_1$ added to the medium. As noted in Fig. 3, concentrations of $PGE_1$ as

TABLE II

*Adenyl Cyclase Activity at Various Levels of the Intestine*

| Level of intestine | Cyclic 3',5'-AMP | | |
|---|---|---|---|
| | Control | NaF | $PGE_1$ |
| | *mμmoles/mg protein* | | |
| Duodenum | 0.31 ±0.03 | 2.00 ±0.28 | 0.68 ±0.11 |
| Jejunum | 0.24 ±0.03* | 1.92 ±0.28* | 0.56 ±0.10* |
| Ileum | 0.24 ±0.03* | 1.75 ±0.27*‡ | 0.47 ±0.09*‡ |

Six experiments; results shown are means ±1 SEM.

* Significantly less than corresponding value in duodenum ($P < 0.05$).

‡ Significantly less than corresponding value in jejunum ($P < 0.05$).

TABLE III

*Effect of Various Prostaglandins on Adenyl Cyclase Activity*

| Prostaglandin | | Increment over control* cyclic 3',5'-AMP |
|---|---|---|
| | | *mμmoles/mg protein* |
| $PGE_1$ | (14)‡ | +0.42 ±0.07§ |
| $PGE_2$ | (11) | +0.30 ±0.03§ |
| $PGA_1$ | (7) | +0.18 ±0.02§‖ |
| $PGF_{2\beta}$ | (7) | +0.16 ±0.02§‖ |
| $PGB_1$ | (8) | +0.10 ±0.02§‖ |
| $PGF_{1\beta}$ | (9) | +0.08 ±0.04§‖ |
| $PGF_{2\alpha}$ | (10) | +0.04 ±0.02‖ |
| $PGF_{1\alpha}$ | (10) | +0.02 ±0.02‖ |

* Control value (15 experiments) = 0.44 ±0.04 mμmoles/mg protein.

‡ Number of experiments in parentheses.

§ Significantly greater than zero ($P < 0.05$).

‖ Significantly less than the mean of pooled data for $PGE_1$ and $PGE_2$, ($P < 0.05$).

low as 0.1 μg/assay (0.2 μg/ml or $5.7 \times 10^{-7}$ M) caused significant stimulation of the enzyme ($P < 0.02$, $n = 6$). A maximal effect was obtained at a concentration of 5.0 μg/assay (10 μg/ml or $2.85 \times 10^{-5}$ M).

Adenyl cyclase activity measured in the presence of $PGE_1$ was a linear function of tissue protein concentration in the range employed in the present study (0–3 mg protein/0.5 ml incubation mixture). When measured as a function of time (Fig. 4), the formation of cyclic 3′,5′-AMP in the presence of $PGE_1$ increased throughout the experiment up to 10 min although the reaction rate gradually decreased. $PGE_1$ caused approximately 2-fold stimulation of the enzyme at every time when tested from 1 min on.

*Effects of various hormones and of $CaCl_2$ on adenyl cyclase activity.* A number of hormones known to stimulate adenyl cyclase in other tissues did not influence the activity of the enzyme in membrane preparations from the small intestinal mucosa of the guinea pig or rat. Thus, bovine insulin (60 μg/ml) and parathyroid hormone (100 μg/ml), as well as crystalline glucagon (10 μg/ml) and thyroid stimulating hormone (200 mU/ml) were all ineffective (three experiments). Porcine thyrocalcitonin (100 μg/ml) and L-thyroxine ($1.0 \times 10^{-5}$ M) were likewise ineffective (three experiments).

Vasopressin, when added in vitro to the serosal surface of stripped rabbit ileal mucosa, causes a large but transient (2–5 min) increase in short-circuit current, which may be due to the stimulation of chloride secretion (1). Epinephrine has been shown to cause a decrease in short-circuit current and an increase in chloride

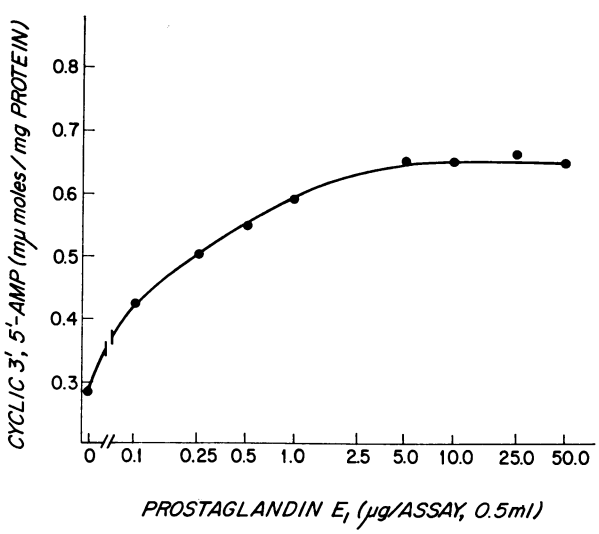


FIGURE 3 Effects of varying concentrations of $PGE_1$ on adenyl cyclase activity in membranes from guinea pig ileal mucosa. Test mixtures contained between 1.1 and 2.4 mg of membrane protein. Four complete and separate experiments were performed and the results represent the mean values.

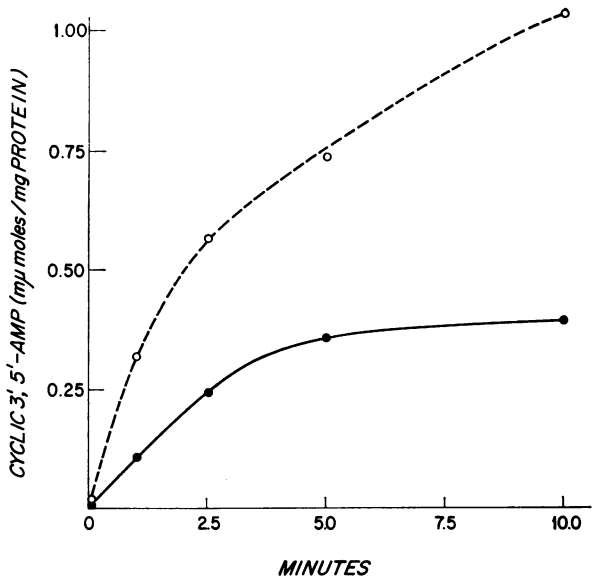


FIGURE 4 Effects of $PGE_1$ on adenyl cyclase activity in membranes from guinea pig ileal mucosa, expressed as a function of time. Incubation mixtures contained between 2.2 and 3.0 mg of membrane protein. ●——●, no additions; ○- - -○, + $PGE_1$ ($2.9 \times 10^{-4}$ M or 50 μg/assay). Results shown represent the means of three separate experiments.

absorption (mucosa towards serosa) under similar circumstances (21). If changes in adenyl cyclase activity were to occur with these two hormones, therefore, one would anticipate stimulation by vasopressin and inhibition by epinephrine. Synthetic vasopressin in concentrations as high as 1.7 units/ml did not alter the activity of adenyl cyclase measured in membranes prepared from the rat, rabbit, or guinea pig ileum. Epinephrine alone, in concentrations varying between $1.0 \times 10^{-6}$ and $1.0 \times 10^{-4}$ M was likewise ineffective. Furthermore, epinephrine in concentrations as high as $1.0 \times 10^{-4}$ M did not diminish the stimulatory effect of $PGE_1$ when the latter was added in concentrations ranging between 2.0 and 100 μg/ml. The effects of these hormones on the intact tissue were not paralleled, therefore, by effects on the adenyl cyclase system in subcellular fragments.

Both inhibitory and stimulatory effects of calcium on adenyl cyclase activity have been demonstrated in other systems (17, 22–25). As noted in Table IV, the addition of $CaCl_2$ at a concentration of $1.0 \times 10^{-3}$ M to the membrane fraction of guinea pig small intestinal mucosa exerted a profound inhibitory effect on both the base line and $PGE_1$-stimulated levels of adenyl cyclase activity without influencing the effectiveness of NaF.

*Effects of cholera enterotoxin in vitro on adenyl cyclase activity.* Concentrations of active toxin as high as 2 μg/ml were totally ineffective in altering adenyl cyclase activity when added directly to the reaction medium,

TABLE IV

*Effects of Calcium on Adenyl Cyclase Activity*

| Calcium concentration | Cyclic 3',5'-AMP | | |
|---|---|---|---|
| | Control | NaF | $PGE_1$ |
| | *mμmoles/mg protein* | | |
| None | 0.26 | 3.24 | 0.72 |
| $1.0 \times 10^{-4}$ M | 0.27 | 3.11 | 0.66 |
| $1.0 \times 10^{-3}$ M | 0.08 | 3.11 | 0.06 |

using membranes prepared from either rabbit or guinea pig ileum.

It is well known that there is a delay in onset of the effects of cholera enterotoxin in vivo (26) as well as a delay in effect in those studies using stripped ileum in vitro (2). Experiments were therefore undertaken in which either active enterotoxin or heat-inactivated enterotoxin were preincubated with mucosal scrapings in vitro before assay of the subsequently prepared membranes for adenyl cyclase activity (see Methods). After a 2.5 hr preincubation, adenyl cyclase activity was greater in membranes from active enterotoxin-treated tissues than in membranes from control tissues preincubated with heat-inactivated enterotoxin (Table V). NaF and $PGE_1$ stimulations were demonstrable, and the levels of activity were uniformly greater with membranes prepared from tissue preincubated with the active toxin.

The levels of adenyl cyclase activity in mucosa preincubated in this manner were invariably lower than those noted previously with membranes prepared immediately after sacrificing the animal. In view of the loss of activity after storage in the cold for even brief periods (Table I), this observation is not at all surprising. Despite a great deal of variability in the levels of activity when separate experiments were compared from day to day, the increment in activity due to preincubation with active toxin was clearly demonstrable within each experiment, and it was generally of the same relative magnitude.

With both rabbit and guinea pig ileal mucosa, an effect of cholera enterotoxin was evident as early as 60 min after the initiation of preincubation, and this effect increased with incubation up to 2.5 hr. The results of a representative experiment using guinea pig ileum are depicted in Fig. 5.

*Effects of cholera enterotoxin in vivo on adenyl cyclase activity.* As noted in Table VI, in vivo preincubation with active enterotoxin leads to an increase in base line adenyl cyclase activity and to corresponding increases in activity measured in the presence of either NaF or $PGE_1$. The addition of $1.0 \times 10^{-3}$ M $CaCl_2$ to the assay mixture led to a 50–60% inhibition of the base line activity of membranes prepared from tissue exposed to either the active or inactive toxin.

*Effect of prostaglandin $E_1$ and cholera enterotoxin on phosphodiesterase activity.* The amount of cyclic AMP-$^{14}C$ present at the end of a 10 min incubation can be influenced not only by its rate of synthesis but also by its rate of destruction. It was of great importance therefore, to test for residual phosphodiesterase activity in the

TABLE V

*Effect of In Vitro Preincubation with Cholera Enterotoxin on Adenyl Cyclase Activity*

| Experimental conditions | Cyclic 3',5'-AMP | Increment over base line | |
|---|---|---|---|
| | Base line | NaF | $PGE_1$ |
| | *mμmoles/mg protein* | *mμmoles/mg protein* | |
| Guinea pig ileum | | | |
| Inactive toxin | 0.09 ±0.03 (9) | +0.38 ±0.06 (6) | +0.10 ±0.04 (4) |
| Active toxin | 0.19 ±0.05 | +0.49 ±0.09 | +0.12 ±0.04 |
| | ($P < 0.01$) | ($P > 0.1$) | ($P > 0.2$) |
| Rabbit ileum | | | |
| Inactive toxin | 0.08 (3) | +0.14 (2) | +0.03 (2) |
| Active toxin | 0.17 | +0.16 | +0.02 |

Mucosal scrapings were preincubated for 2.5 hr in the presence of either active or heat-inactivated cholera enterotoxin (0.5 μg/ml for guinea pig, 1μg/ml for rabbit ileum) and membranes were prepared as described. The number of experiments performed is given in parentheses. The values are given as the means ±SEM 1.

reaction mixture under the conditions used in this study. Phosphodiesterase assays were conducted as described in Methods, with guinea pig ileal membranes from tissue which had been preincubated with either active or inactive toxin, or with membranes incubated in the presence and absence of $PGE_1$ (Table VII). Despite the presence of caffeine, phosphodiesterase activity was demonstrable. It is clear however, that differences in this enzyme activity are in no way responsible for the increased levels of cyclic 3',5'-AMP noted when membranes are prepared after preincubation with active toxin or when they are incubated in the presence of $PGE_1$.

*Effects of cholera enterotoxin on the cyclic AMP concentration in rabbit ileal mucosa.* As noted in Table VIII, the cyclic AMP concentration of tissues incubated for 2 hr in vitro with cholera enterotoxin was increased almost 4-fold. In two of three experiments, theophylline, added 10 min before termination of a 2 hr incubation, also increased cyclic AMP concentration, although the increase was less than that obtained with cholera enterotoxin. When cholera enterotoxin was incubated for 2 hr in the absence of intestinal mucosa (10 μg in 10 ml of Ringer-bicarbonate), no cyclic AMP could be detected in the medium. Heat-inactivated enterotoxin (1 μg/ml) failed to increase the cyclic AMP concentration (animal 4, Table VIII).

*Effects of prostaglandins and cholera enterotoxin on SCC.* Cyclic 3',5'-AMP, theophylline, and a relatively crude preparation of cholera enterotoxin stimulate active chloride secretion and inhibit active Na absorption (2, 3, 21). These agents also increase SCC, indicating that the change in net Cl flux (which increases SCC) is generally greater than the change in net Na flux

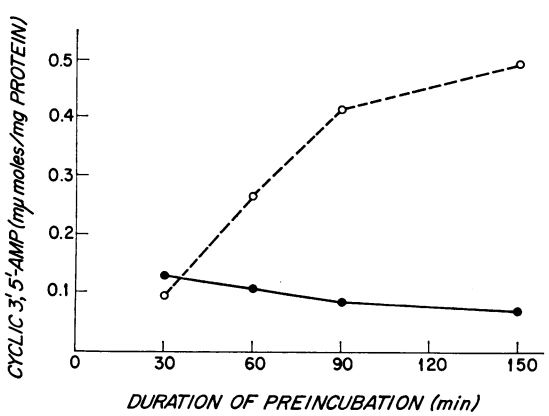


FIGURE 5 Effect of duration of preincubation with cholera enterotoxin in vitro on adenyl cyclase activity. Results are for guinea pig ileal mucosa. Incubation (assay) mixtures contained between 1.2 and 2.3 mg of membrane protein, with no more than a 10% difference between the mean membrane protein concentrations at any given point in time. ●——●, inactive cholera enterotoxin, 1.0 μg/ml; ○- - -○, active cholera enterotoxin, 1.0 μg/ml.

TABLE VI

*Effect on Adenyl Cyclase Activity of in Vivo Preincubation with Cholera Enterotoxin in Rabbit Ileal Loops*

| Experiment No. | Conditions | Cyclic 3',5'-AMP Base line | Change from base line NaF | PGE1 | CaCl2 |
|---|---|---|---|---|---|
| | | *mμmoles/mg protein* | *mμmoles/mg protein* | | |
| 1 | Inactive toxin | 0.24 | +0.21 | +0.02 | −0.09 |
| | Active toxin | 0.42 | +0.20 | +0.06 | −0.23 |
| 2 | Inactive toxin | 0.24 | +0.37 | +0.08 | −0.08 |
| | Active toxin | 0.50 | +0.33 | +0.08 | −0.28 |
| 3 | Inactive toxin | 0.15 | +0.12 | +0.05 | −0.12 |
| | Active toxin | 0.25 | +0.23 | +0.12 | −0.18 |
| Mean | Inactive toxin | 0.21 | +0.23 | +0.05 | −0.10 |
| | Active toxin | 0.39 | +0.25 | +0.09 | −0.23 |

(which decreases SCC). To have measured Na and Cl fluxes with each of the prostaglandins would have proved unusually laborious. Therefore, the SCC was used to determine the magnitude of a secretory stimulus. After the addition of theophylline or cyclic AMP to the serosal side of the stripped ileum, the SCC increases rapidly. In rabbit ileum, the maximally obtainable SCC is no greater with cyclic AMP itself than with theophylline (1). The two agents can be used interchangeably, therefore, to determine the maximum cyclic AMP–dependent secretory capacity of a tissue. An increase in SCC is not proof by itself that active chloride secretion has been stimulated, as an increase in the active absorption of sodium will also increase SCC. In addition to determining the effects of the prostaglandins and cholera toxin on SCC, therefore, we also determined the extent to which prior addition of these agents inhibited

TABLE VII

*Effects of $PGE_1$ and Cholera Enterotoxin on Phosphodiesterase Activity*

| Experimental condition | Disappearance of $^3$H-Cyclic 3',5'-AMP |
|---|---|
| | *mμmoles/mg protein* |
| $PGE_1$ solvent control | 0.15 |
| $PGE_1$ | 0.14 |
| Inactive toxin | 0.25 |
| Active toxin | 0.23 |

Means of two experiments; in each membranes were prepared from guinea pig ileal mucosa. One set of reaction mixtures was boiled immediately after addition of $PGE_1$, $PGE_1$ solvent, inactive toxin, and active toxin. In none of these "zero time" controls was there detectable disappearance of $^3$H-cyclic 3',5'-AMP from the supernate.

TABLE VIII

*Effects of Cholera Enterotoxin and Theophylline on Concentration of Cyclic 3',5'-AMP in Rabbit Ileal Mucosa*

| Animal No. | Cyclic 3',5'-AMP | | |
|---|---|---|---|
| | Control | Cholera enterotoxin | Theophylline |
| | *mμmoles/g wet weight* | | |
| 1 | 0.71 | 11.60 | 2.77 |
| | 1.07 | | |
| 2 | 2.02 | 7.08 | 2.74 |
| | 3.32 | | |
| 3 | 1.25 | 6.02 | 5.51 |
| 4 | 3.55 | 6.39 | — |
| | 3.01* | | |
| 5‡ | 0.47 | 5.86 | — |
| | 0.69 | 3.28 | |
| Mean | 1.79 ±0.41 | 6.70 ±1.11 | 3.67 |

Mucosal strips were incubated in Ringer-bicarbonate containing 5 mM D-glucose at 37°C for 2 hr as described in Methods. Cholera enterotoxin, 0.5–1.0 μg/ml, was added at the beginning of incubation, and theophylline, 2.5 mM, was added 10 min before the termination of incubation.

* Heat-inactivated cholera enterotoxin, 1.0 μg/ml was added at the beginning of incubation.

‡ In four experiments, no distinction was made between cyclic AMP present in the tissue and in the incubation medium. In the fifth experiment, tissue and medium were assayed separately. In the cholera toxin incubations, the amount of cyclic AMP in the medium was less than 2% of the amount present in the tissue. In the control incubations, medium cyclic AMP was 16% of tissue cyclic AMP. Almost all of the cyclic AMP present at the end of incubation was contained in the tissue itself.

the SCC response to the subsequent addition of a maximal concentration of theophylline. Conversely, the extent to which the prior addition of theophylline inhibited the SCC response to the subsequent addition of certain prostaglandins was also determined. If an agent (*a*) increased SCC when added alone; (*b*) reduced the SCC response to the subsequent addition of theophylline; and (*c*) was itself rendered less effective by the prior addition of theophylline, it could reasonably be concluded that the agent had stimulated the chloride secretory process. Finally, in order to ascertain that a reduction in the tissue response to theophylline was due to the prior stimulation of the chloride secretory process and not to a nonspecific inhibition of all transport processes, the SCC response to D-glucose, which stimulates active Na absorption when added to the luminal side, was determined and compared to the glucose response in an appropriate control tissue.

Table IX indicates the changes in SCC caused by the addition of different prostaglandins to the serosal side of stripped guinea pig ileum. At a concentration of $1.5 \times 10^{-7}$ M, only $PGE_1$ and $PGE_2$ caused significant increases in SCC. At a 10-fold higher concentration, a small but significant increase in SCC was also obtained from $PGF_{2\beta}$. At the highest concentration tested, the responses to $PGE_1$ and $PGE_2$ were essentially maximal. With the exception of $PGF_{2\beta}$, the other prostaglandins caused only trivial increases in SCC.

As indicated in Table X, the prior addition of $1.7 \times 10^{-5}$ M $PGE_1$ or $PGE_2$ reduced the subsequent SCC response to 10 mM theophylline by 60%. Conversely, the prior addition of 10 mM theophylline essentially abolished the subsequent response to the prostaglandins. Cholera enterotoxin, at a concentration of 1 μg/ml markedly inhibited the SCC response to theophylline which was added 3 hr later. These results suggest that the prostaglandins, cholera enterotoxin, and theophylline stimulate the same ion transport process. Neither the prostaglandins nor cholera enterotoxin inhibited the subsequent SCC response to glucose.

## DISCUSSION

Recent studies concerned with cholera infection in humans and in animal models have led to the conclusions that the diarrhea is of small intestinal origin (26), that it involves the actual secretion of fluid rather than just a failure to absorb fluid (26–28), and that it is not accompanied by significant morphological alterations in the mucosa (29). Previous in vitro studies by Field and his coworkers have suggested that this enterotoxin-induced fluid production results from the stimulation of a chlo-

TABLE IX

*Effects of Prostaglandins on Short-Circuit Current (SCC) of Stripped Guinea Pig Ileum*

| Prostaglandin | | Concentration, moles per liter | | |
|---|---|---|---|---|
| | | $1.5 \times 10^{-7}$ | $1.6 \times 10^{-6}$ | $1.7 \times 10^{-5}$ |
| | | *ΔSCC, μamps/cm²* | | |
| $PGE_1$ | (9) | 23 ±3 | 44 ±3 | 57 ±5 |
| $PGE_2$ | (5) | 28 ±5 | 55 ±9 | 75 ±18 |
| $PGF_{2\beta}$ | (6) | 0 | 14 ±1 | 44 ±6* |
| $PGF_{2\alpha}$ | (3) | <5 | 6 (0–10) | 11 (4–19) |
| $PGA_1$ | (5) | 0 | <5 | 9 ±1 |
| $PGF_{1\beta}$ | (3) | 0 | <5 | 8 (3–12) |
| $PGF_{1\alpha}$ | (3) | 0 | <5 | 6 (0–19) |
| $PGB_1$ | (3) | 0 | 0 | <5 |

The number of experiments is given in parentheses, and the values are expressed as means ±SEM or means and ranges where n = 3.

* This value is significantly less than the corresponding values for $PGE_1$ and $PGE_2$ ($P < 0.02$).

TABLE X

*Short-Circuit Current (SCC) Responses to Theophylline and Glucose after Prior Additions of Prostaglandins or Cholera Enterotoxin and SCC Responses to Prostaglandins after Prior Addition of Theophylline*

| Animal | First addition | 2d addition | ΔSCC after 2d addition | ΔSCC after addition of D-glucose |
|---|---|---|---|---|
| | | | *μamps/cm²* | *μamps/cm²* |
| Guinea pig | $PGE_1$ or $PGE_2$ | Theophylline | 17 ±2 | 77 ±8 |
| | None | Theophylline | 43 ±4 | 71 ±12 |
| | | | ($P < 0.001$; $n = 6$) | ($P > 0.5$; $n = 4$) |
| Guinea pig | Theophylline | $PGE_1$ or $PGE_2$ | 5 ±4 | — |
| | None | $PGE_1$ or $PGE_2$ | 74 ±21 | — |
| | | | ($P < 0.02$; $n = 4$) | |
| Rabbit | Cholera enterotoxin | Theophylline | 10 ±2 | 55 ±9 |
| | None | Theophylline | 98 ±13 | 49 ±4 |
| | | | ($P < 0.001$; $n = 5$) | ($P > 0.5$; $n = 5$) |

Values are expressed as means ±1 SEM. *n* refers to the number of animals. Controls (no first additions) are paired tissues from the same animals. Theophylline, $1.0 \times 10^{-2}$ M, and the prostaglandins, $1.7 \times 10^{-5}$ M, were added to the serosal side; D-glucose, $1.0 \times 10^{-2}$ M and cholera enterotoxin, 1.0 μg/ml, were added to the luminal side. When the first addition was theophylline or a prostaglandin, the second addition was made within 15 min. When the first addition was cholera enterotoxin, theophylline was added 3 hr later. In all cases, D-glucose was added within 15 min of the second addition.

ride secretory process in the small intestinal epithelium (2). The net secretion of solute is contributed to both by the stimulation of active chloride secretion and by a reduction in active sodium absorption.

Evidence from several previous studies has suggested that the cholera enterotoxin–induced secretory effect may be mediated by cyclic 3′,5′-AMP: (*a*) In vitro studies employing the isolated rabbit ileal mucosa have demonstrated that cyclic 3′,5′-AMP and theophylline produce ion flux changes similar to those produced in the same preparation by cholera enterotoxin (1–3). (*b*) Infusion of theophylline into the superior mesenteric artery of dogs has produced small intestinal fluid secretion comparable in magnitude and composition to that produced by cholera enterotoxin (4). (*c*) Effects similar to those of theophylline and cholera enterotoxin were also obtained by infusing prostaglandins $E_1$, $E_2$, and $F_{2\alpha}$ (4). These prostaglandins have been shown to affect cyclic AMP levels in several tissues (7). (*d*) Cholera enterotoxin has been shown to stimulate processes in other tissues which are thought to be mediated by cyclic 3′,5′-AMP: lipolysis in the isolated fat cell (8), and glycogenolysis in liver and platelets (9) have been stimulated by the addition of enterotoxin in vitro.

The presence of adenyl cyclase activity in the small intestinal mucosa of the rat has recently been reported by Ishikawa and his coworkers (30). In the present study, significant base line and NaF-stimulated levels of adenyl cyclase activity were found in mucosa from all levels of the small intestine of the rat, guinea pig, and rabbit. Fluid secretion in response to cholera enterotoxin has also been found to occur throughout the entire small intestine (26); the highest rate of fluid secretion occurred in the duodenum, which also was found to have the highest level of adenyl cyclase activity.

Despite the circumstantial evidence that cyclic 3′,5′-AMP plays an important regulatory role in intestinal secretion and that it mediates the effects of cholera enterotoxin and the prostaglandins, direct evidence of interactions between these agents and intestinal mucosal adenyl cyclase or cyclic nucleotide phosphodiesterase had not previously been obtained. In the present study both cholera enterotoxin and certain prostaglandins were found to increase the adenyl cyclase activity of membranes prepared from guinea pig and rabbit small intestinal mucosa. Direct measurements of cyclic 3′,5′-AMP concentrations in the ileal mucosa following a 2-hr preincubation with enterotoxin in vitro indicated a 3.5-fold increase over concentrations in comparable controls. Similar increases in cyclic AMP concentration in canine intestinal mucosa have been demonstrated by Schafer, Lust, Sircar, and Goldberg (31) after preincubation with cholera enterotoxin in vivo. These observations suggest that the enterotoxin-induced stimulation of adenyl cyclase activity occurs not only in an in vitro membrane preparation but also in the intact mucosal cell. The central role of adenyl cyclase in this cholera enterotoxin-induced secretory process is emphasized further by the temporal relationship between exposure to enterotoxin in vitro and changes in adenyl cyclase activity (see Fig. 5). The same temporal rela-

tionship has been observed with respect to changes in short-circuit current in vitro (2) and fluid transport in vivo (26).

The stimulation of adenyl cyclase activity by the prostaglandins was also shown to be closely correlated with changes in short-circuit current caused by the same agents in isolated guinea pig ileal mucosa. $PGE_1$ and $PGE_2$ proved to be the most potent of the prostaglandins both with respect to changes in adenyl cyclase activity and changes in short-circuit current. The correlation seems less perfect with the remaining prostaglandins but this may have been a consequence of differences in the concentrations tested. It is noteworthy that the concentration of $PGE_1$ which produced a maximal increase in adenyl cyclase activity (Fig. 3) is nearly the same as the concentration which caused a maximal short-circuit current effect. It is also of interest that the effect on adenyl cyclase activity of preincubating the mucosa with active cholera enterotoxin was additive to the effect of a maximally stimulating concentration of $PGE_1$ (Table V). This observation suggests that the interactions between these two agents and adenyl cyclase may involve different receptors.

The role of mucosal adenyl cyclase in small intestinal fluid production is of interest with respect to the fulminant diarrhea that has been associated with certain tumors such as medullary carcinoma of the thyroid (32), nonbeta cell tumors of the pancreas (33–35), and malignant carcinoid (36). High concentrations of certain prostaglandins including $PGE_2$ have been noted in tumor tissue and in the venous blood draining the tumors in several patients with medullary carcinoma of the thyroid and certain of the pancreatic neoplasms (32, 33). The diarrhea associated with medullary carcinoma of the thyroid has previously been attributed to the stimulation of intestinal motility by the prostaglandins (32). On the basis of the present and other recent studies of intestinal ion transport (4, 5), it would seem likely that the interaction of the prostaglandins or some other hormones with intestinal mucosal adenyl cyclase may contribute to the diarrhea in certain of these syndromes. Further studies in this area are clearly required.

The evidence implicating cyclic 3′,5′-AMP as a mediator for the effects of cholera enterotoxin and certain prostaglandins in the small intestine raises fundamental questions about the nature of their interaction with the adenyl cyclase system. The method employed in the present study for the preparation of a membrane fraction does not permit any conclusions to be drawn about cellular localization of the adenyl cyclase activity. Some brush border structures were noted to be present in the preparation, but whether the adenyl cyclase activity was associated with these structures or with other cell membranes remains to be determined.

The presence of 1 mM calcium has been shown to inhibit the base line as well as the cholera enterotoxin and prostaglandin-induced increases in intestinal mucosal adenyl cyclase activity (Tables IV and VI). Both inhibitory and stimulatory effects of calcium ion on adenyl cyclase activity have previously been demonstrated in a number of other systems (17, 22–25). High concentrations of calcium (1–3 mM) have been shown to abolish the effects of isopropyl norepinephrine on adenyl cyclase activity in lipocytes (23), and the stimulatory effects of parathyroid hormone on skeletal and renal cortical adenyl cyclase (17, 24). Furthermore, when present in high concentrations, calcium ion also depresses base line adenyl cyclase activity (measured in the presence of $Mg^{++}$) in a number of systems (17, 23, 24). These inhibitory effects of calcium ion on both base line and stimulated levels of adenyl cyclase activity are similar to those noted in the present study. It has been proposed that intracellular calcium ion may constitute an important feedback mechanism for the regulation of adenyl cyclase activity within the cell (17). The role of calcium in the mechanisms by which cholera enterotoxin and the prostaglandins affect intestinal mucosal adenyl cyclase remains to be explored.

The 30 to 60 min delay in the onset of the enterotoxin's effect on adenyl cyclase activity and the still longer delay before a peak effect is achieved raises the possibility that a protein synthetic step is involved. Cycloheximide, an inhibitor of protein synthesis, has been shown to block fluid secretion in rabbit jejunum in vivo when administered before cholera enterotoxin (27, 28). This observation, while suggestive, is not sufficient to prove, however, that the inhibitory effect of cycloheximide involves a protein synthetic step. In addition, even if enhanced protein synthesis is required for the effects of the enterotoxin on the intestine to become manifest, there is no evidence based on the results of our studies to suggest that increased synthesis of adenyl cyclase is involved. Although base line adenyl cyclase activity was nearly doubled by preincubation of the intact mucosa with enterotoxin (Tables V and VI), there was not a corresponding doubling when "maximum" stimulation was provided by the addition of $10^{-2}$ M NaF. This suggests that cholera enterotoxin stimulated the existing enzyme rather than causing synthesis of new enzyme. Since the mechanism by which NaF stimulates adenyl cyclase activity is not well understood, however, this conclusion can only be regarded as tentative.

The delay in onset of the cholera enterotoxin–induced stimulation of adenyl cyclase activity could also be due to slow absorption and diffusion of the toxin to its site of action, for the enterotoxin has a molecular weight of about 60,000 (37). While the prostaglandins have a direct effect on the broken cell preparation, adenyl cy-

clase activity was not enhanced by direct addition of toxin to the assay mixture. Unlike mammalian hormones which affect adenyl cyclase systems, it is possible that the enterotoxin, in order to act, must first be converted into an active form by membrane-bound enzyme or must initiate a series of intervening chemical events after attachment to the cell surface.

Other bacterial toxins have been shown to produce small intestinal fluid secretion (38, 39). The possibility that all of these toxins have a common mode of action involving adenyl cyclase stimulation is currently under investigation. Cyclic 3',5'-AMP has also been shown to play an important role in bacterial metabolism (40). While the effects of these bacterial toxins on bacterial adenyl cyclase activity has not been determined, the toxins may in fact represent bacterial compounds which affect growth and metabolism in microorganisms and which affect the tissues of higher organisms only incidentally.

*Note added in proof:* Cholera toxin and $PGE_1$–induced enhancement of intestinal mucosal adenyl cyclase activity has also been observed by Sharp, G. W. G. and S. Hynie. 1971. Stimulation of intestinal adenyl cyclase by cholera toxin. *Nature (London).* **229:** 266. Their results are in general agreement with our own.

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