Effects of Cycloheximide on the Response of
Intestinal Mucosa to Cholera Enterotoxin

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ABSTRACT Prior studies have indicated that effects of cholera enterotoxin (CT) on the small intestine are delayed in onset and involve an interaction with adenyl cyclase in the mucosa. It has also been shown that the administration of cycloheximide to rabbits in doses which inhibit crypt cell mitoses (20 mg/kg), diminishes CT-induced fluid production in jejunal loops. These latter studies have been interpreted as indications that CT-related intestinal secretion is a crypt cell function and that it is mediated by a CT-induced protein.

The present study was undertaken to delineate more precisely the nature of the interaction in the intestine between cycloheximide and cholera toxin. Pretreatment of rabbits with cycloheximide reduced by 60% the secretory response to CT in isolated ileal loops with intact blood supply. Sodium and chloride flux measurements on mucosa isolated from these and control loops indicated that this antisecretory effect of cycloheximide persists in vitro. Measurements of radioactive leucine incorporation into mucosal protein indicated that the dose of cycloheximide employed inhibited protein synthesis by 90%. This inhibitory effect was shown to be independent of any effect of cycloheximide on amino acid uptake across the brush border. Measurements of adenyl cyclase activity and cyclic AMP levels in ileal mucosa of cycloheximide pretreated and control animals indicated that cycloheximide did not diminish the CT-induced increases in these parameters.

These observations demonstrate that cycloheximide reduces CT-induced intestinal fluid production without interfering with the CT-induced augmentation of adenyl cyclase activity or the consequent rise in cyclic AMP concentration. Since the antisecretory effect of cycloheximide persists in vitro, it probably involves a direct interaction of the antibiotic with mucosal cell ion transport mechanisms rather than an indirect effect mediated by other humoral or neurogenic factors. The present observations also suggest that the secretory response of the intestine to CT involves neither the synthesis of new adenyl cyclase nor that of a protein modifying its activity.

INTRODUCTION

There is a great deal of evidence indicating that an active secretory process probably accounts for the gastrointestinal fluid and electrolyte losses resulting from the action of cholera enterotoxin (CT)1 (1, 2; see also references 3-6 for reviews). Employing isolated rabbit ileal mucosa stripped of its muscularis and mounted in modified Ussing chambers, Field and his coworkers have demonstrated that application of CT to the luminal surface eliminates the normal transport of sodium from mucosa to serosa and reverses the normal direction of chloride transport such that net active chloride secretion occurs (4, 7). The active processes for the absorption of sugars and the associated enhancement of sodium absorption are not influenced by secretory stimuli such as CT (4, 7).

Studies performed by Field and his colleagues and others have shown that cyclic 3',5'-adenosine monophosphate (cyclic AMP), theophylline, and certain prostaglandins have effects on small intestinal ion transport which are similar to those noted after addition of CT (4, 8-11). These observations, coupled with the demonstration that the enterotoxin has cyclic AMP-like effects on isolated fat cells (12), liver, and platelets (13), suggested that the CT and prostaglandin-related intestinal

Dr. Kimberg is the recipient of a Research Career Development Award, AM-19377.
Received for publication 5 October 1972 and in revised form 31 January 1973.

1 Abbreviations used in this paper: CT, cholera enterotoxin; cyclic AMP, cyclic 3',5'-adenosine monophosphate; dibutyryl cyclic AMP, N'-2'-O-dibutyryl-cyclic 3',5'-adenosine monophosphate; SCC, short-circuit current.
fluid losses were mediated by means of an interaction with intestinal mucosal adeny1 cyclase. More recent studies in this laboratory (11) and in others (14, 15) have indeed demonstrated that CT and certain prosta
glandins stimulate adeny1 cyclase activity and elevate cyclic AMP levels in small intestinal mucosal cells.

Most effects of endogenous and exogenous hormonal stimuli on cyclic AMP metabolism occur within a mat-
ter of minutes. Characteristically, however, in the in
testine and in the other systems studied, the effects of CT on cyclic AMP metabolism or in producing a
physiologic response are often delayed (4, 7, 11, 12 16–
19). The nature of the events which occur during this lag period are presently unknown. Of interest in this
regard are the observations of Serebro, Iber, Yardley,
and Hendrix (20) and of others (21–23) concerned with
the effects of cycloheximide, an inhibitor of protein syn-
thesis, on CT-induced small intestinal fluid secretion in
in vivo. Serebro and his coworkers (20) demonstrated
that cycloheximide administered intravenously to rab-
bbits 1 h before the intraluminal instillation of CT in
jejunal loops, prevented the enterotoxin-induced pro-
duction of fluid. Glucose absorption was unaffected, and
the major histologic change in the epithelium was a de-
crease in mitoses in the crypts. Also of note are the
observations of Harper, Yardley, and Hendrix (22) who
found that in more prolonged studies, cycloheximide
administration following the instillation of CT could reverse previously induced secretion. The results of
these previous studies employing cycloheximide have been interpreted as indicating that intestinal secretion
due to exposure to CT is mediated by an enterotoxin-
induced protein synthesized in crypt cells (20, 21).

The present studies were undertaken to determine if
cycloheximide administration interferes with the stimula-
tion by CT of intestinal mucosal adenyl cyclase ac-
tivity and cyclic AMP levels, and to relate these effects
(if any) to cycloheximide-induced inhibition of protein
synthesis, and cycloheximide-induced alterations of
intestinal ion transport.

METHODS

Animal preparation. New Zealand white, male rabbits
weighing between 2 and 3 kg were anesthetized with intra
venous pentobarbital sodium and locally administered lido
caine. Two distal ileal loops with intact blood supply were
prepared with ligatures at both ends. Each loop was approxi-
mately 25 cm in length and the two loops were separated by
a skip area of 10–15 cm. Each of the two loops were can-
ulated at both ends with Tygon tubing secured by surgical
silk ligatures, and the tubing was left open at each end.
The loops were flushed with isotonic saline followed by air,
and then returned to the abdomen which was closed. Hy-
dration was provided with intravenous isotonic sodium
chloride (approximately 10–20 ml per h), and body tem-

perature was maintained with a heat lamp. After the
preparation of the ileal loops, animals received either an
intravenous injection of cycloheximide, 20 mg per kg in
isotonic saline, or of isotonic saline alone. 1 h later in a
randomized manner, either the proximal or distal loop in
each animal was filled with approximately 10 ml of an
HCO3-Ringer solution, while the remaining loop was filled
with an identical solution which also contained purified
cholera enterotoxin at a concentration of 1 μg per ml. The
HCO3-Ringer solution (pH 7.4) contained the following
ions in millimoles per liter: Na, 141; K, 10; Ca, 1.25; Mg,
1.1; CI, 127; HCO3, 25; H2PO4, 0.3; and HPO4, 1.65. 1 h
after the instillation of solutions into the loops, the abdom-
en was opened, the loops flushed with air, further drained by
digital pressure, and the abdomen was then reclosed. During
the ensuing 3 h fluid was collected from the cunnace, and
at the end of this time the abdomen was reopened. The loops
were drained and then flushed with air in order to collect
the residual fluid. With the blood supply still intact, dupli-
cate full thickness biopsies from each loop were taken for
cyclic AMP measurements (see below). The intestinal
loops were then removed, their lengths determined, and
mucosa was rapidly obtained for additional studies described
below.

Flux studies. Once excised, a portion of ileum from each
loop was stripped free of muscularis and paired tissues from
each loop were mounted in modified Ussing-type chambers
as described previously (24). The tissues were bathed in the
HCO3-Ringer solution gassed with 5% CO2 in O2 (see
below). In those studies in which mucosa from cyclohexi-
mide pretreated animals was employed, cycloheximide at
a concentration of 5.0 × 10−4 M was added to both the serosal
and mucosal reservoirs. The methods for measuring short-
circuit current (SCC) and fluxes of 22Na and 36Cl (both
obtained from New England Nuclear, Boston, Mass.) have
been previously described (24). Fluxes were determined from
initial samples taken 30–40 min after mounting in vitro and 20 min after adding radioisotopes and final samples
taken 75–90 min after mounting tissues in vitro. 75–90 min
after mounting the tissue and 5–10 min after completing Na
and Cl flux measurements, 0.5 mM N′,N′-dibutyryl-cyclic
3′,5′-adenosine monophosphate (dibutyryl cyclic AMP) was
added to the serosal compartment and the change in SCC
determined over a 5 min period. Dibutyryl cyclic AMP was
purchased as the monosodium salt from Boehringer Mann-
hem Corp., Mannheim, West Germany.

Protein synthesis. After removal of the control and CT-
exposed loops from the saline and cycloheximide-injected
rabbits, mucosa was stripped from underlying muscularis
with the edge of a glass microscope slide, minced, and then
incubated in 25-ml Erlenmeyer flasks containing 2.5 ml of
the HCO3-Ringer solution described above, to which had
been added 2.0 × 10−4 M glucose and 8.0 × 10−4 M L-leucine,
containing 0.4 μCi of L-[14C]leucine (uniformly labeled).
The L-[14C]leucine was purchased from New England
Nuclear at an original specific activity of 250 μCi per μmol.
Triplicate flasks were incubated with the mucosa (about
300 mg wet wt per flask) from each loop. Each flask was
agitated at 80 rpm and incubated at 37°C for 30 min while
being continuously gassed in an atmosphere of 95% O2–5%


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CO₂. At the termination of the incubation protein was precipitated with 5% trichloracetic acid (TCA), isolated by the method of Siekevitz (25), and dissolved in 2 ml of 1 N NaOH for determination of protein (26) and for suspension in 10 ml of Bray's solution (27) and 2% CaB-O-Sil (Cabot Corp., Boston, Mass.). Samples were counted in a model SL40 Intertechnique Liquid Scintillation Spectrometer at an efficiency of 70-80% as determined by internal standardization.

In certain experiments the incorporation of L-[³⁵Cl]leucine into protein was also assessed in mounted preparations of stripped ileal mucosa. These studies were undertaken in order to insure that the cycloheximide effect on protein synthesis persisted in tissues mounted in the chambers in vitro and exposed for prolonged periods to large volumes of cycloheximide-containing medium in the reservoirs. Furthermore, it was considered desirable to ascertain whether the cycloheximide effect on L-[³⁵Cl]leucine incorporation noted with ileal mucosa exposed to incubation medium in Erlenmeyer flasks could simply be due to an influence of the antibiotic on the uptake of L-leucine at the mucosal surface. For this purpose, ileal mucosa stripped of its muscularis was obtained from control and cycloheximide-injected rabbits 1 h after the in vivo administration of the antibiotic. The tissues were mounted and incubated in the chambers as previously described, except that the reservoirs each contained 7.5 ml of HCO₃-Ringer solution. Cycloheximide, at a concentration of 5.0 X 10⁻⁴ M was present in both the mucosal and serosal reservoirs of those chambers containing tissues from cycloheximide-treated animals. At the initiation of the 2 h incubation, purified CT at a final concentration of 1 µg per ml was added on the mucosal side to one of the two tissues prepared from each animal. 11 h later, L-leucine at a final concentration of 8.0 X 10⁻⁴ M and containing 2 µCi of L-[³⁵Cl]leucine was added to the serosal reservoir. After an additional 30 min of incubation, both reservoirs were clamped, the chambers were removed, and a representative sample of the exposed tissue was rapidly plunged into 5% TCA. The methods for the subsequent preparation of the samples for protein determinations and for liquid scintillation counting have already been described.

Adenyl cyclase assays. Immediately after the removal of the two ileal loops from each of the control and cycloheximide-treated rabbits, ileal mucosa stripped of its muscularis was homogenized and membranes were prepared for assay of adenylyl cyclase activity by a previously described (11) modification of the procedure of Krishna, Weiss, and Brodie (28). In certain experiments, assays for adenyl cyclase activity were conducted with membranes from both the control and CT-exposed loops of control and cycloheximide-pretreated rabbits with 2.85 X 10⁻⁴ M cycloheximide present in the incubation medium. All determinations of adenyl cyclase activity were routinely done in triplicate.

Cyclic AMP measurements. As indicated previously, full-thickness biopsies of each loop were obtained while the circulation was still intact, just before the removal of the loops from the animals. Biopsy samples were immediately plunged into ice-cold 5% TCA containing [³²p]cyclic AMP as a recovery marker. After homogenization and centrifugation, HCl was added to supernates to a concentration of 0.1 N and the TCA was extracted with diethyl ether. Supernates were then evaporated to dryness at 50°C using an evaporimix evaporator (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N. J.). The residues were redissolved in 50 mM acetate buffer, pH 4.0 and cyclic AMP levels

| Table I |
| --- | --- | --- |
| **Effects of CT and Cycloheximide Administration on Ileal Fluid Secretion In Vivo** | | |
| **Animal treatment** | **Fluid accumulation** | |
|  | **Control loop** | **Cholera loop** |
| Control (n = 11) | 0.04±0.01 | 0.63±0.09 |
| Cycloheximide (n = 10) | 0.03±0.02 | 0.25±0.08 |

Values are means ± 1 SEM. n refers to the number of animals. Probabilities represented in this table were determined by Student's t test for paired variates. The method for unpaired variates, the difference between control loops from control and cycloheximide-treated animals was not significant, whereas the difference between the CT-exposed loops was significant with a value of P < 0.005.

were then measured by the protein-kinase binding assay described by Gilman (29). Results were expressed as picomoles cyclic AMP per milligram protein. Protein was determined on the TCA precipitates as described above. Duplicate biopsies were obtained from each loop, and duplicate determinations of cyclic AMP levels were performed on each sample.

**RESULTS**

Effects of CT and cycloheximide on ileal fluid secretion in vivo. The results presented in Table I demonstrate that the in vivo application of CT is capable of inducing a brisk small intestinal secretory response in control animals. Consistent with the observations of others made on rabbit jejunum (20-23), the prior administration of cycloheximide failed to significantly affect the rate of fluid accumulation in control loops of ileum, but it did impair the secretory response in loops exposed to CT.

**Effects of CT (added in vivo) and of cycloheximide (added in vivo and in vitro) on Na and Cl fluxes and SCC measured in vitro.** In order to assess the direct effects of cycloheximide on the mucosal secretory response to CT (as contrasted to possible indirect effects of the drug on mucosal blood flow or hormonal or neurogenic intermediates), it seemed desirable to explore the effects of cycloheximide using isolated ileal mucosa in vitro. For this purpose, ileal mucosa stripped of its muscularis was obtained from isolated intestinal loops prepared in control and cycloheximide-pretreated rabbits, the loops having been exposed in vivo to either HCO₃-Ringer solution or to the same solution containing purified CT. In order to insure continued inhibition of protein synthesis during the in vitro incubation of tissues from cycloheximide-pretreated animals, cycloheximide (5.0 X 10⁻⁴ M) was added to both mucosal and serosal

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reservoirs. As shown in Table II, in tissues obtained from the control loops of the control animals, there were net absorptive fluxes of both Na and Cl, whereas in tissues from CT-exposed loops from the same animals, the small net absorptive Na flux disappeared, and the net absorptive flux of Cl was replaced by a net secretory flux. The residual ion flux (SCC - net Na flux + net Cl flux), probably due to HCO₃⁻ secretion, did not change significantly. The average SCC in the CT-exposed tissues during the period of steady-state flux measurements differed little from the control values. The increase in SCC following the in vitro addition of dibutyryl cyclic AMP to the serosal surface has been shown to reflect an increase in the net Cl secretory response (8). Whereas the addition of this nucleotide increased SCC in tissues from both the control and CT-exposed loops of control animals, the response was of much smaller magnitude in the enterotoxin-exposed tissues, suggesting that an enterotoxin-induced Cl secretory response was already occurring, and that this response involved a pathway common to that following cyclic AMP addition (7).

As shown in Table II, when mucosa from the cycloheximide-pretreated animals was studied in vitro in the continued presence of the antibiotic, CT induced changes in the net Na and Cl fluxes and in the SCC similar in direction to those which occurred in the control animals. Furthermore, in a manner characteristic of a tissue responding to CT, the SCC-response to dibutyryl cyclic AMP was blunted in mucosa obtained from the enterotoxin-pretreated loop. Of great significance however, is the fact that the net Cl secretory flux in the enterotoxin-exposed mucosa from the cycloheximide-pretreated animals was substantially less than that noted in enterotoxin-treated mucosa from control animals. It seems clear then, that exposure to cycloheximide interferes with the secretory response of the intestinal epithelium to CT and that this effect is probably not mediated by secondary humoral, neurogenic, or vascular influences in the intact animal.

Effects of cycloheximide on L-[¹⁴C]leucine incorporation into mucosal protein. Cycloheximide in the doses employed in the present study has been used extensively in experiments concerned with the mechanisms underlying CT-induced intestinal fluid and electrolyte losses (20-23). Furthermore, the effects of the antibiotic in this system have been attributed to its ability to inhibit the synthesis of a protein or proteins induced by the enterotoxin and required in the secretory process (20, 21). It seemed desirable therefore, to obtain more direct evidence than the histologic data previously provided (20-22) to support the contention that the treatment regimen employed does indeed inhibit the synthesis of protein in rabbit mucosa.

The results of initial experiments performed in this regard are shown in Table III. In these studies ileal mucosa stripped of its muscularis was minced and incubated in Erlenmeyer flasks containing L-[¹⁴C]leucine as described in the section on Methods. Rates of L-[¹⁴C]leucine incorporation into mucosal protein from animals, pretreated with cycloheximide were less than 10% of

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### Table II

<table>
<thead>
<tr>
<th>Animal treatment</th>
<th>Na fluxes</th>
<th>Cl fluxes</th>
<th>Conductance</th>
<th>+ cyclic DbAMP Δ SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m → s</td>
<td>s → m</td>
<td>net</td>
<td>m → s</td>
</tr>
<tr>
<td>Control animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 4)</td>
<td>10.1±1.4</td>
<td>9.0±0.7</td>
<td>1.0±0.8</td>
<td>8.3±1.0</td>
</tr>
<tr>
<td>Cholera loop</td>
<td>6.4±0.6</td>
<td>7.5±0.8</td>
<td>-1.1±0.3</td>
<td>4.4±0.4</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 6)</td>
<td>12.5±0.7</td>
<td>12.1±0.6</td>
<td>0.4±0.7</td>
<td>11.6±1.2</td>
</tr>
<tr>
<td>Cholera loop</td>
<td>9.8±0.7</td>
<td>10.7±1.1</td>
<td>-0.9±0.6</td>
<td>8.0±0.9</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.02</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

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Effects of Values are means (SEM). n refers to the number of animals. Probabilities represented in the table were determined by Student’s t test for paired variates. By the method for unpaired variates, the effects of cycloheximide in tissues from both the control and CT-exposed loops were significant at the level of P < 0.01.

In order to eliminate the possibility that cycloheximide affected L-[14C]leucine incorporation into protein by virtue of a primary effect on leucine uptake across the brush border, and in order to assure that mucosal protein synthesis was inhibited under the conditions employed for the in vitro flux studies (see above), incorporation of L-[14C]leucine into mucosal protein of tissues mounted in flux chambers was determined with leucine added on the serosal side only. The results of these studies presented in Table IV indicate that quite independently of possible effects of cycloheximide on mucosal leucine uptake, the antibiotic is a potent inhibitor of protein synthesis under conditions similar to those employed in the flux studies. Of note is the fact that less than 0.1% of the radioactivity added to the serosal reservoir appeared in the mucosal reservoir.

Effects of CT and cycloheximide on adenyl cyclase activity and cyclic AMP levels. In view of the 3-4 h delay in obtaining a peak physiologic effect of the enterotoxin on the intestine (4, 7, 11, 16, 19) and the known effects of cycloheximide on this physiologic response (20-23), the effects of this inhibitor of protein synthesis on the CT-induced increase in intestinal mucosal adenyl cyclase activity and cyclic AMP levels were examined. Previous studies conducted in this laboratory suggested that CT stimulates existing adenyl cyclase in the intestinal mucosa rather than causing the synthesis of new enzyme (11); this conclusion, however, was regarded as only tentative.

As shown in Table V, despite the profound degree of inhibition of protein synthesis caused by cycloheximide, adenyl cyclase activity in mucosal membranes prepared from the control and cycloheximide-treated animals was very much the same. Moreover, even though cycloheximide was quite effective in reducing CT-stimulated fluid secretion in vivo and in diminishing the net Cl secretory flux in vitro, this agent failed to prevent the enterotoxin-related increase in cyclase activity. Of note too is the fact that cycloheximide added in vitro to the adenyl cyclase reaction mixtures in concentrations as high as 2.85 × 10^-4 M failed to exert any influence on adenyl cyclase activity with membranes prepared from either the control or enterotoxin-exposed mucosa from either control or cycloheximide-treated animals (results not shown).

### Table III

<table>
<thead>
<tr>
<th>Animal treatment</th>
<th>L-[14C]leucine incorporated (nmol leucine × 10^-4/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>188.2±44.9</td>
</tr>
<tr>
<td>Cycloheximide (n = 4)</td>
<td>15.6±3.8</td>
</tr>
</tbody>
</table>

Control loops: (P > 0.1)

Cycloheximide loops: (P < 0.01)

Values are means ±1 SEM. n refers to the number of animals. Probabilities represented in the table were determined by Student’s t test for paired variates. By the method for unpaired variates, the effects of cycloheximide in tissues from both the control and CT-exposed loops were significant at the level of P < 0.005.

### Table IV

<table>
<thead>
<tr>
<th>Animal treatment</th>
<th>L-[14C]leucine incorporated (nmol leucine × 10^-4/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>36.3±4.6</td>
</tr>
<tr>
<td>Cycloheximide (n = 4)</td>
<td>6.6±1.7</td>
</tr>
</tbody>
</table>

Control loops: (P > 0.5)

Cycloheximide loops: (P < 0.5)

Values are means ±1 SEM. n refers to the number of animals. Probabilities represented in the table were determined by Student’s t test for paired variates. By the method for unpaired variates, the effects of cycloheximide in tissues from both the control and CT-exposed loops were significant at the level of P < 0.005.

### Table V

<table>
<thead>
<tr>
<th>Animal treatment</th>
<th>Adenyl cyclase activity (nmol cyclic AMP formed/mg protein per 10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>0.23±0.04</td>
</tr>
<tr>
<td>Cycloheximide (n = 4)</td>
<td>0.24±0.05</td>
</tr>
</tbody>
</table>

Control loops: (P < 0.05)

Cycloheximide loops: (P < 0.05)

Values are means ±1 SEM. n refers to the number of animals. Probabilities represented in the table were determined by Student’s t test for paired variates. By the method for unpaired variates, the effects of cycloheximide in tissues from both the control and CT-exposed loops were significant at the level of P < 0.05.
Cyclic AMP levels measured in biopsies of control and enterotoxin-exposed loops in control and cycloheximide-treated rabbits are shown in Table VI. Cyclic AMP levels were determined in full thickness biopsies in order to avoid the secondary effects of the delay in removing mucosa. Cyclic AMP levels measured on mucosal scrapings gave similar but more erratic results (results not shown). As one might anticipate from the results of the adenyl cyclase assays (Table V), cycloheximide administration in vivo did not prevent the usual CT-induced increase in intestinal cyclic AMP levels.

**DISCUSSION**

The present study confirms and extends previous observations (20–23) concerned with the effects of cycloheximide on small intestinal fluid and electrolyte secretion after exposure to CT. Thus, the administration of cycloheximide to rabbits in doses shown in this study to be capable of inhibiting intestinal mucosal protein synthesis by greater than 90%, does indeed reduce the small intestinal secretory response to subsequent challenge with CT. Furthermore, the experiments concerned with the influence of cycloheximide administration on the in vitro fluxes of Na and Cl across ileal mucosa from control and CT-exposed small bowel loops suggest that the in vivo effects of this antibiotic are due, in part at least, to a direct interaction with the active ion transport processes of the intestinal epithelium.

The delayed stimulation of intestinal mucosal adenyl cyclase appears to be an essential step in mediating the effects of CT on intestinal ion transport (4, 11, 14, 15, 19). The precise manner in which the enterotoxin stimulates this activity has not been fully resolved. The following possibilities deserve consideration: (a) the enterotoxin may, either directly or indirectly, cause a delayed stimulation of existing membrane cyclase; (b) the enterotoxin may stimulate the synthesis of new cyclase; (c) once combined with a component of the cell membrane, the enterotoxin may itself catalyze the formation of cyclic AMP in a manner analogous to the way in which diphtheria exotoxin catalyzes the cleavage of nicotinamide adenine dinucleotide to nicotinamide and adenosine diphosphoribose (30).

The results of the present study clearly demonstrate that the administration of cycloheximide does not prevent the CT-induced increase in intestinal mucosal adenyl cyclase activity and cyclic AMP levels, suggesting that the synthesis of new adenyl cyclase or of a protein which modifies its activity is not involved. Furthermore, the effects of cycloheximide noted in this study do not seem to be mediated simply by preventing the interaction of CT with existing adenyl cyclase. Analogous results have been obtained in another study concerned with the effects of cycloheximide administration in the rat on hepatic adenyl cyclase activity and cyclic AMP levels.

The changes in SCC produced by dibutyryl cyclic AMP provide further evidence that the effect of cycloheximide on intestinal ion transport cannot be due to an interference with the CT-adenyl cyclase interaction. Addition of dibutyryl cyclic AMP to CT-treated tissues from cycloheximide animals did not result in a significant increase in SCC. If the inhibition by cycloheximide of CT-induced secretion had been secondary to an effect on the specific adenyl cyclase involved with ion transport, then addition of dibutyryl cyclic AMP should have by-passed this step and resulted in an increase in SCC (as seen in the tissues not treated with CT). This was not the case, however, suggesting that the secretory pump itself had been affected.

The cycloheximide inhibition of the secretory process must be considered as partial rather than total. This is reflected by both the volume fluxes measured in vivo (Table I) and the Na and Cl fluxes measured in vitro (Table II). It is also reflected by the SCC changes observed in vitro (Table II). Dibutyryl cyclic AMP did increase the SCC of control loop tissues from cycloheximide animals, but the sum of the base line SCC of these tissues and the increment in SCC produced by dibutyryl cyclic AMP (1.18 + 1.91 = 3.09 μeq/h cm²) is distinctly less than the corresponding sum for control loop tissues of control animals (2.54 + 2.74 = 5.28 μeq/h cm²).

Whether or not the effect of cycloheximide on active fluid secretion is directly related to the antibiotics' action as an inhibitor of protein biosynthesis or to selective damage to crypt cells, concepts which have been suggested by others (20–22), remain to be determined.

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**Table VI**

**Effects of Cycloheximide Administration and CT In Vivo on Intestinal Cyclic AMP Levels**

<table>
<thead>
<tr>
<th>Animal treatment</th>
<th>Cyclic AMP level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control loop</td>
</tr>
<tr>
<td>Control (n = 11)</td>
<td>7.75±0.47</td>
</tr>
<tr>
<td>(P &lt; 0.02)</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide (n = 10)</td>
<td>8.49±0.93</td>
</tr>
<tr>
<td>(P &lt; 0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ±1 SEM. n refers to the number of animals. Probabilities represented in the table were determined by Student’s t test for paired variates. The effects of cycloheximide on cyclic AMP levels were not significant by the method for unpaired variates (P > 0.5).

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Perhaps of significance in this regard are the observations of MacDonald and Ellis (31) and Evans (32). MacDonald and Ellis (31) recently demonstrated that cycloheximide is 10 times more effective on a molar basis than is 2,4-dinitrophenol in stimulating oxygen uptake by red beet discs incubated in vitro. These authors suggested that cycloheximide-related inhibition of chloride uptake by beet discs may not be a direct result of inhibition of protein synthesis, but rather may be the result of interference with metabolic processes such as energy transfer. Evans (32) has provided evidence to support the concept that cycloheximide-related inhibition of 2,4-dinitrophenol uptake by *Euglena gracilis* is not a consequence of the inhibition of protein synthesis. While cycloheximide is known to inhibit protein biosynthesis, it does not follow a priori that its effects in many given systems are the result of this particular action of the antibiotic. One must consider the possibility that cycloheximide may exert effects on membrane transport which are independent of its action as an inhibitor of protein synthesis.

The major alteration in small intestinal morphology after administration of the dose of cycloheximide employed in the present study and in others (20–23) is a decrease in mitotic figures in the undifferentiated crypt cells (20–22). Based upon the observation of these morphologic changes in association with cycloheximide-related inhibition of fluid production by isolated small intestinal loops exposed to CT, Serebro and his coworkers (20) and others (22) suggested that crypt cells are responsible for the secretory response to the enterotoxin. The observation that glucose absorption from isolated loops in vivo was unaffected by cycloheximide administration was interpreted as further evidence in support of the concept that the antibiotic exerted a localized effect on the crypt cells (20). Studies by Frizzell, Nellans, Acheson, and Schultz (33) have provided reason for a great deal of caution in drawing conclusions concerning the site of enterotoxin-induced secretion from morphological evidence alone. These workers clearly demonstrated that the administration of the same dose of cycloheximide to rabbits markedly inhibited the mucosal in-flux processes for Na, Cl, alanine, 3-O-methylglucose, and iron across brush borders. These influx processes are, for the most part, localized in the mature villus absorptive cells. The results, while consistent with the notion that cycloheximide may inhibit the synthesis of protein(s) which are either directly or indirectly involved in carrier-mediated transport, also indicate that the effect of the antibiotic is not restricted to the crypt cell population.

Based on the results of the present study it can be concluded that the cycloheximide inhibition occurs at some step in the secretory process other than the activation of adenyl cyclase by CT. Whether or not the antibiotic inhibits secretion by virtue of its action as an inhibitor of protein synthesis, and whether or not the enterotoxin-stimulated secretory process is largely a crypt cell function remain uncertain; further studies will be required in order to resolve these important issues.

**ACKNOWLEDGMENTS**

This work was supported by Grants AM-13696, AM-05114, and CA-10736 from the National Institutes of Health, Department of Health, Education and Welfare, and by the United States-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (Grant AI-09029).

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