On the Role of the Ileal Epithelium in the Pathogenesis of Experimental Cholera

H. Thomas Norris, M.D., and Guido Majno, M.D.

STUDIES on the pathogenesis of human cholera have been greatly facilitated by several advances: three experimental models have become available to study the disease, and it has become possible to produce a syndrome indistinguishable from that of the infectious disease by using highly purified nonviable products of the vibrio.

Dutta and Habbu [1] demonstrated that rabbits less than 10 days old would uniformly respond with diarrhea to the instillation of viable organisms into the lumen of the small bowel. De and Chatterje [2] showed that ileal loops of the adult rabbit would produce large amounts of fluid in response to intraluminal instillation of the organism. These models also produce fluid in response to nonviable moieties of the vibrio.[3-5] Recently a canine model [6] has also been introduced that responds in a similar manner.

Although several nonviable preparations of cholera vibrio will cause experimental cholera,[3,7,8] only one, choleragen,[3,9] has been purified to such an extent that 0.5–1.0 μg./gm. of body weight will produce diarrhea in the infant rabbit; 5 μg. will cause fluid production in ileal loops of the adult rabbit. In addition, choleragen causes a syndrome in human volunteers indistinguishable from the infectious disease.[10]

Of the many concepts that have been proposed to explain the accumulation of fluid,[11,12] five have the most significance. The oldest concept, epithelial denudation,[13] received its coup de grace when Gangarosa *et al.*[14] demonstrated by biopsy that the intestinal epithelium was intact in patients with cholera. Despite this, speculation continued

---

From the Department of Pathology, Harvard Medical School, Boston, Mass.

Supported by Grants AI 07523-01 and HE 08794-03 from the U. S. Public Health Service.

Presented in part at the sixty-fourth annual meeting of the American Association of Pathologists and Bacteriologists, Washington, D.C., March 1967; American College of Physicians, San Francisco, April 1967; the International Inflammation Club, Augusta, Mich., June 1967; and the U. S.-Japan Cooperative Medical Science Program Symposium on Cholera, Palo Alto, Calif., July 1967.

Accepted for publication May 6, 1968.

Address for reprint requests: Dr. Norris, Department of Pathology, University of Washington, c/o Veterans Administration Hospital, 4435 Beacon Ave. South, Seattle, Wash. 98108.

that, using more refined techniques, epithelial abnormalities might be found to be present.[15–17]

A second concept called for an inhibition of the sodium pump: the vibrio allegedly inhibited active transport of sodium across the epithelial lining of the bowel.[18,19] Recently it has been demonstrated that there is no inhibition of active sodium transport in the experimental model; [20,21] similar preliminary findings have been reported in patients with cholera.[22]

A third concept holds that the microcirculation of the lamina propria is primarily affected in cholera. Two possible mechanisms have been proposed: (1) There is an altered endothelium in the vessels of the villus [15–17,23] with resulting increased permeability to macromolecules. This concept received indirect support from the observation that various choleragenic agents caused a delayed and prolonged type of increase in vascular permeability in the vessels of the skin.[7,24] It has also been suggested that similar alterations are present in the vessels of the choleraic small bowel of the infant rabbit.[25] (2) The response is a simple increase in capillary blood flow, resulting in increased production of fluid.[26]

The fourth concept, hyperproduction of gastrointestinal hormones, postulates that the vibrio causes excessive production of gastrin and secretin,[27,28] and that these two hormones increase the secretion of the pancreas, liver, and upper gastrointestinal tract with resulting diarrhea.

The fifth concept, direct stimulation of the epithelial cell, was initially proposed by Cohnheim in 1889.[29] It had little support until recently, when it was re-emphasized that secretion by the epithelial cell, per se, may play a significant role in the production of fluid.[8,30]

The purpose of this communication is to explore these concepts in relation to experimental cholera induced with choleragen in the rabbit.

Materials and Methods

Preparation of Rabbits

Adult Rabbits. These were females, 2–3 kg. in weight. Under Diabutal (sodium pentobarbital) anesthesia, a laparotomy was performed and constricting ligatures were applied to the ileum so as to segment it into a series of closed loops, 6 cm. in length. Care was taken not to compromise any of the vascular arcades. Between adjacent test loops a 4-cm. segment of ileum was interposed. Control loops received 1 ml. of physiologic saline or were left empty. Doses of choleragen ranging from 5 to 150 μg. were diluted in physiologic saline, so that a total volume of 1 ml. was instilled into the lumen of each of the experimental loops (referred to hereafter as choleraic loops). These were then returned to the abdominal cavity, and the abdominal wall was closed with sutures. Most rabbits were

sacrificed by intravenous injections of Diabutal 2, 4, 6, 9, and 12 hr. after the instillation of choleragen. Because the adult rabbit loop model is more versatile, the majority of the studies were performed with it; over an 11-month period, a total of 352 ileal loops were studied in 70 animals.

Infant Rabbits. These were prepared by lavaging the stomach to remove all remainders of milk. Choleragen, in a dose of 1 μg./gm. of body weight, was then instilled into the stomach. A total of 42 rabbits less than 10 days old were studied by this method.

Choleragen was received in lyophilized form and diluted with physiologic saline so that the standard stock solution contained 1000 μg./ml. This was kept frozen and thawed immediately before use when aliquots were removed. Initially, doses between 5 and 40 μg./ml. would cause fluid production in the ileal loops. After repeated freezing and thawing of the stock solution, higher doses were needed.

Morphologic Studies

The majority of control and choleraic loops were surveyed histologically after fixation in chilled, buffered formalin, embedding in paraffin, and staining with hematoxylin and eosin.

To study changes in vascular permeability, 47 choleraic loops and 58 control loops were examined in nine animals. The choleraic loops were inoculated with four different doses of choleragen (5, 10, 20, and 40 μg./ml.). The control loops were inoculated with physiologic saline or left empty. Both choleraic and control loops were studied 2, 4, 6, and 12 hr. after instillation of various test solutions. Multiple areas from at least two loops were studied at each time and dose interval. At 1 hr. prior to sacrifice, each animal received an intravenous injection of Evans blue (2.5% solution diluted in 0.45% NaCl, 30 mg./kg.) and saccharated iron oxide (40% solution diluted in sterile distilled H_2O, 2.5 ml./kg.). All the loops were examined grossly for the extravasation of Evans blue dye into the bowel wall, or for discoloration of the loop effluent. Histologic examination of all the loops was carried out after routine fixation, processing, and staining for iron by the Turnbull blue method,[31] as well as with hematoxylin and eosin. In addition, the mucosa and submucosa of 5 choleraic and 5 control ileal loops from 3 animals were studied by electron microscopy. The animals were sacrificed 2 and 6 hr. after the instillation of choleragen, either 20 or 40 μg./ml., into their choleraic loops.

Tissues for electron microscopy were fixed in modified Karnovsky fixative [32] for 4 hr. at room temperature, washed four times in chilled 0.1 M sodium cacodylate buffer (pH 7.4), and left at 4° C. overnight. Thereafter, they were postfixed in osmium-Veronal [33] for 2 hr. at 4° C., then slowly brought to room temperature, dehydrated in graded alcohols through four changes of absolute alcohol, and placed in 1:1 (v/v) mixture of propylene oxide and Epon 812 (4:6) for 2 hr., before being left overnight at room temperature in undiluted Epon 812. New Epon was then substituted and the tissues were polymerized at 56° C. Sections were cut on an LKB 2 Ultrotome with glass or diamond knives, placed on parlodion-coated grids, stained with uranyl acetate and lead citrate, carbon coated, and studied in a Philips 200 electron microscope.

Dry Weights

The dry weight of portions of 54 choleraic and 41 control loops from nine rabbits was determined. At least two loops were examined at each dose and time interval. Wet weights were determined after blotting the tissue carefully. Portions

of loops were dried in an oven at 120° C. until constant weights were obtained (7–8 days); the percentage of dry residue was then calculated.

Analytic and Metabolic Techniques

Sodium and potassium concentrations of the effluent and serum were determined by flame photometry, chloride by the mercuric chloranilate method,[34] bicarbonate by the microgasometric procedure, phosphorus by the method of Fiske and Subbarow,[35] and protein content of the effluent by the Lowry modification of the method of Folin and Ciocalteu.[36]

Oxygen utilization was studied by classic manometric techniques with the Warburg apparatus. Four choleraic loops and 5 control loops from 5 rabbits were studied. Effluent production was induced by choleragen, 50, 100, or 120 μg./ml. Control and choleraic loops were immediately removed from rabbits that had been sacrificed with intravenous Diabutal 6 hr. after instillation of choleragen. The tissue was placed on moistened filter paper and laid over a cooling plate kept at 4° C. Wet weights were determined after blotting the tissue carefully; 30–150 mg. of tissue was placed in each vessel.

Each Warburg vessel (main chamber) contained 3 ml. of Krebs-Ringer solution buffered with phosphate; glucose was added to raise the osmolarity to 300 mosm; a 5.0% KOH solution was placed in the center well (0.2 ml.). Fifty-one analyses were carried out; in 15 the side arm of the reaction flasks contained 21–28 μg. of additional choleragen in 0.2 ml. of solution. This was tipped into the main chamber after initial readings had been taken. Oxygen uptake was expressed as μl. O_2 per 100 mg. fresh tissue per 60- or 90-min. interval.

Results

Gross Observations on Intestinal Loops

In the adult rabbits, both the choleraic and the control loops became dry (i.e., the injected fluid was absorbed) within 1 hr. after laparotomy. The control loops remained dry throughout the experiment. The choleraic loops, instead, began to accumulate new fluid toward the fourth hour. By the sixth hour they were noticeably distended, and by the twelfth hour even more so; their walls, however, did not show evidence of gross ischemia until the eighteenth hour. At this time, most of the animals were comatose.

Light and Electron Microscopy

By the sixth hour, the villous and submucosal vessels were severely congested. No inflammatory cells were present in the lamina propria. The goblet cells had discharged their mucus.

By electron microscopy, no abnormal separation was seen between individual epithelial cells or between the epithelial cells and the lamina propria (Fig. 1). The apical portion of the epithelial cells contained the usual complement of rough endoplasmic reticulum and longitudinally oriented mitochondria (Fig. 2). Numerous unaltered desmo-

somes and tight epithelial junctions were present. There were no alterations in the microvilli.

Vascular Permeability

No extravasation of Evans blue into the wall of the ileum or into the effluent could be seen (Fig. 3). In histologic sections, using the Turnbull blue stain, only 1 of 47 choleraic loops showed any extravasation of saccharated iron oxide (S.I.O.) into the lamina propria. No extravasation was seen in the lamina propria of any of the control loops.

There was an occasional extravasation of S.I.O. into the serosa and muscular layers, probably as a result of operative trauma. By electron microscopy, with rare exception, no deposition of tracer particles was seen in the junction between individual endothelial cells, against the vascular basement membrane, or in the extravascular spaces. There were no alterations in the organelles of the endothelial cells or in the junctions (Fig. 4). The basement membrane and surrounding pericytes appeared unaltered.

Protein determinations on the effluent revealed very low amounts of protein (Tables 1 and 2). The dry weights (Tables 3 and 4) of the small bowel were studied statistically (analysis of variance); they

Table 1. Effluent of Choleraic Loops: Average Fluid Content and Total Protein

Dose (µg.)	Post choleragen (6 hr.)			Post choleragen (12 hr.)		
	Volume (cc.)	Protein (mg.)	L/A*	Volume (cc.)	Protein (mg.)	L/A*
5	4.5	23	1/1	3.0	13	1/1
10	4.0	15	1/1	11.0	32	1/1
20	5.0	15	2/2	12.0	40	2/2
40	14.5	12	2/2	26.3	60	2/2
80	10.4	24	5/3	22.3	35	2/2

* L/A: number of loops studied/number of animals used.

Table 2. Average Concentration of Protein

Condition	Protein (mg./100 ml.)	L/A*
Choleraic effluent		
6 hr.	274	11/4
12 hr.	281	8/2
Passive congestion [27]	400	—
Inflammatory edema [27]	6000	—

* L/A: number of loops studied/number of animals used.

Table 3. Tissue Fluid in Control and Choleraic Ileum

Condition	% Tissue fluid post choleragen				Mean (%)
	2 hr.	4 hr.	6 hr.	12 hr.	
		Choleraic Ileum			
Dose (µg.)					
5	82.1	80.5	75.7	75.5	
10	80.6	80.4	77.8	78.4	
20	81.3	80.2	79.9	80.2	
40	82.3	79.3	80.1	80.7	
Mean	81.6	80.1	79.1	78.4	79.6
		Control Ileum			
Empty	80.0	78.9	77.2	76.3	
Saline	81.8	80.6	80.4	73.1	
Mean	80.9	79.8	78.7	74.7	78.4

Table 4. Analysis of Variance for Dry Weights of Small Bowel

Source of variance	DF	Sum of squares	Mean square	F	p
Doses	5	17.16	3.43	0.46	NS
Hours					
Regression	1	39.61	39.61	5.30	< 0.05
Deviations from regression	2	6.61	3.31	0.44	NS
Doses × hours	15	149.63	9.97	1.33	NS
Residual	24	179.55	7.48	—	—
Total	47	392.56			

showed that there was no accumulation of fluid in the wall of the bowel during the first 12 hr. of the experiment.

There was no significant change in the rate of oxygen utilization by the small bowel which had been exposed to choleragen in vivo for 6 hr. However, there was slight but significant increase in oxygen utilization when control tissue was exposed to choleragen in vitro (Table 5).

Table 5. Oxygen Utilization by Ileal Loops

Total time (min.)	Oxygen utilization (mean ± S.D.)*			p
	Choleragen added in vivo	Choleragen added in vitro	Intact controls	
90	106.6 ± 19.1	—	97.5 ± 11.4	> 0.05
60	73.8 ± 12.0	—	68.2 ± 9.2	> 0.05
60	—	80.6 ± 10.0	68.2 ± 9.2	< 0.01

* Measured in µl. O_2 per 100 mg. wet tissue. There was no significant oxygen utilization when choleragen was added to reaction flask with no tissue, and no increase when tissue exposed to choleragen in vivo was again exposed in vitro.

Electrolyte Concentrations in Effluent. There appears to be very little variation in the electrolyte content of effluents produced in response to various doses of choleragen (Table 6). The amount of fluid, however, does appear to be somewhat dose dependent (Table 1).

Table 7 compares the sodium, potassium, chloride, bicarbonate, and phosphorus concentrations in the blood, in the choleraic effluents, and in the fluid that accumulates in the lumen of the gastrointestinal tract above the highest loop, 6 and 12 hr. after the instillation of choleragen. The predominant cation and anion present in the choleraic effluent are sodium and bicarbonate. When compared with rabbit serum, the effluent also shows a concomitant decrease in chloride. The osmolarity of the choleraic effluent at 6 hr. is 305 mosm.

Discussion

In our experimental models, choleragen induced important fluid production, but no change that could be recognized by electron microscopy in the epithelium or endothelium of the ileal lamina propria.

There was no visible increase in the permeability in the ileal vessels, as studied by intravenous markers—Evans blue, which binds with

Table 6. Electrolyte Concentrations in Loop Effluents at 6 hr.

Dose of choleragen (μg.)	Electrolyte concentrations (mEq./L.)				
	Na^+	K^+	HCO_3^-	Cl^-	L/A*
5	140	6.0	45	86	1/1
10	140	6.0	57	79	1/1
20	145	6.4	60	72	2/2
40	151	4.9	66	63	6/4
80	149	5.7	69	60	9/6
> 80	153	4.9	70	60	23/12
Mean	151	5.2	67	62	42/19

* L/A: number of loops studied/number of animals used.

Table 7. Electrolyte Concentrations of Ileal Loop Effluents in Experimental Cholera

Specimen	Electrolyte concentrations (mEq./L.)				mg./100 ml.	
	Na^+	K^+	HCO_3^-	Cl^-	phosphorus	L/A*
6 hr.						
Serum	135	5.9	24	100	6.0	—/19
Cholera loop effluents	151	5.2	67	62	6.1	42/19
Fluid above loops	145	7.6	74	59	20.5	3/3
12 hr.						
Serum	136	7.0	20	100	6.2	2/2
Cholera loop effluents	168	6.1	106	72	9.1	8/2
Fluid above loops	165	11.3	95	53	25.5	2/2

* L/A: number of loops studied/number of animals used.

albumin; and saccharated iron oxide, the smallest colloidal vascular tracer presently available for study by electron microscopy (diameter 25–75 Å). This observation correlates well with the finding that very little protein is present in the choleraic effluent—on the average, 274 mg./100 ml., which is well below that seen in passive congestion or in inflammatory edema.[37] The lowest protein concentrations corresponded to the most abundant effluents.

In addition, a thorough study of tissue fluid content in the choleraic bowel wall proper showed that there is no trace of edema during the time that the gut is producing large amounts of fluid. In fact, there was a significant trend in the opposite direction; the bowel wall actually became "drier" during this process.

The findings are in disagreement with observations made in other models of experimental cholera, in which severe damage of the epithelium,[15–17] endothelium,[15–17,23] or both [15–17,23] has been reported. Thus, it became essential to explore the possible reasons for these differences. In the other experimental models, drastic modification of the animal is necessary; e.g., starvation for as long as 5–7 days [15,16] or the use of heavy doses of opium.[15,16] Secondary changes due to severe distention of the loop could also account for the discrepancies. In addition, the use of the whole vibrio,[15–17,23] rather than a purified protein, may also complicate the picture.

Furthermore, some of the evidence that has been published in favor of vascular leakage is susceptible to other interpretations. In one such study [17] the single electron micrograph purported to show "gaps between endothelial cells" does not warrant such conclusion. In a study based on vascular labeling,[25] light microscopy alone was used; hence it was not possible to decide whether the labeling material was truly within the vascular wall (indicating leakage) or simply in the lumen. As to the increase in vascular permeability that occurs when choleragen or similar products are injected into the skin,[24] this experimental model is too far removed from the choleraic loop to be directly pertinent.

In summary, then, there is no inherent contradiction between our results and those of other investigators. Our studies simply show that the loss of fluid in cholera *can occur in the absence of vascular leakage.*

As to the theory ascribing fluid production to a stimulation of the mucosa by gastrointestinal hormones,[26,27] it is again not compatible with our model, because the production of effluent occurs only in those loops that have received choleragen.

If there is no epithelial abnormality, no increase in vascular permeability, and fluid production is not mediated by gastrointestinal hormones, how does choleragen induce the production of fluid?

An important clue in understanding the pathogenesis of this disease, both natural and experimental, is provided by the electrolyte content in choleraic effluent. If the latter represented a transudate, one would expect it to resemble serum ultrafiltrate with slight modifications due to the Donnan equilibrium. This is not the case. At 6 hr., choleraic effluent has a significantly higher concentration of sodium and bicarbonate than does the serum, with a concomitant decrease of chloride. At 12 hr. the difference is even more striking. The serum is not significantly altered in its electrolyte composition; in the effluent, it is obvious that the major cation and anion at 12 hr. are sodium and bicarbonate. On the other hand, the choleraic effluent is similar in electrolyte composition to the fluid that accumulates *above* the most cephalad loop. The fluid consists of the secretions not only of the gastrointestinal tract, but also of the liver and pancreas, thus accounting for the higher concentration of phosphorus. Choleragen, therefore, induces the net accumulation of an isotonic solution of sodium and bicarbonate in the rabbit ileum.

Is this the natural secretion of the ileum, or is it an abnormal fluid manufactured only in response to choleragen? The fluids secreted by the ileums of man and dog have been analyzed: [38] they contain sodium, and a significantly higher concentration of bicarbonate than does the jejunum. Recently, the physiologic secretion of the rabbit ileum has also been studied: [39] It is a solution of sodium and bicarbonate with a small amount of potassium; in fact, it is very similar to the choleraic effluent.

We can, therefore, collate two separate pieces of information: (1) The effluent induced by choleragen in the rabbit ileum is a solution containing large quantities of sodium and bicarbonate with lesser amounts of potassium; and (2) this is similar to the physiologic secretion of the ileum. This similarity, combined with the other facts discussed above, leads us to conclude that *choleragen has a marked functional effect on the epithelial cell of the ileum.* Whether it be malabsorption, hypersecretion, or both has yet to be determined. Evidence indicates that the primary effect is probably one of hypersecretion, as the process can be readily reversed by the action of intraluminal glucose and Diamox.[39] This conclusion is similar, in a number of respects, to that of Leitch, Iwert, and Burrows,[8] who induced fluid production in rabbit loops with whole-cell lysates of the vibrio. In their study, diminution in effluent production was seen in rabbits who received Diamox in their drinking water.

This postulated functional effect has no morphologic equivalent that we could detect on our electron micrographs. It has been stated that

ileal epithelium becomes more basophilic in cholera; [5] this would be compatible with a hyperactivity, but our histologic material showed no difference.

The concept of epithelial stimulation fits well with the slight but significant increase in oxygen uptake after exposure of ileal mucosa to choleragen in vitro. An increased oxygen uptake has been noticed in vitro when rabbit ileal mucosa was exposed to the cathartic cascara.[40]

We are certainly not implying that inflammation of the intestinal mucosa plays no role in cholera. Some inflammation may well occur, especially in later stages; certainly a hyperemia of the intestinal wall is grossly visible in many of the experimental models,[3,5,6,15-17] which suggests that at least a circulatory component should be taken into account. Our main point, however, is that the *primary* events occur in the epithelial cell.

Could this pathogenesis be relevant to the human disease? Evidence from isolated experiments indicate that a similar process is probably present in patients with cholera. The rice water stool [41] is quite similar in its electrolyte concentration (sodium, 140 mEq./L.: chloride, 90.1 mEq./L.; potassium, 10.1 mEq./L.; bicarbonate, 50 mEq./L.; and phosphorus, 6.7 mg./100 ml.) to the choleraic effluent at 6 hr. Intravenous injections of Evans blue [42] or ^{131}I-labeled polyvinylpyrrolidone [14] do not appear in the stools of cholera patients, suggesting that vascular permeability for macromolecules is not increased. An average stool protein concentration of 85 mg./100 ml.[41] is also well below the levels seen in passive congestion or inflammatory edema.

Furthermore, electron microscopic studies of the small bowel in a few patients with cholera showed epithelial changes which can be categorized at best as degenerative and probably secondary.[23]

So far, intravenous supportive therapy has been the method of choice in treating cholera. If stimulation of ileal epithelium plays a major role in fluid production, pharmacologic agents aimed specifically at decreasing the amount of sodium and bicarbonate in the effluent might be successful in modifying the amount of fluid produced, not only in the experimental model, but also in the patient with cholera. Further studies along these lines are in progress.

Summary

The pathogenesis of fluid production in experimental cholera was studied in infant and adult rabbits by light and electron microscopy, by vascular labeling techniques, and by electrolyte analysis of the effluent.

Choleragen, a material produced by the cholera vibrio and capable of eliciting a syndrome identical to the infectious disease, was used to induce fluid production. No epithelial or endothelial abnormalities or changes in vascular permeability for macromolecules could be demonstrated by light or electron microscopy. Choleragen did not cause a typical mesenchymal inflammatory response, but appeared to act upon the epithelial cell of the ileum, causing it to secrete its usual product, but in larger amounts.

References

1. Dutta, N. K., and Habbu, M. K. Experimental cholera in infant rabbits: A method for chemotherapeutic investigation. *Brit J Pharmacol 10*:153–159, 1955.
2. De, S. N., and Chatterje, D. N. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. *J Path Bact 66*:559–562, 1953.
3. Finkelstein, R. A., Norris, H. T., and Dutta, N. K. Pathogenesis of experimental cholera in infant rabbits. I. Observations on the intraintestinal infection and experimental cholera produced with cell-free products. *J Infect Dis 114*:203–216, 1964.
4. Finkelstein, R. A. "Observations on the Nature and Mode of Action of the Choleragenic Product(s) of Cholera Vibrios." In *Proceedings of the Cholera Research Symposium.* U. S. Public Health Service Publication No. 1328, 1965, pp. 264–270.
5. Norris, H. T., Finkelstein, R. A., Dutta, N. K., and Sprinz, H. Intestinal manifestations of cholera in infant rabbits: A morphologic study. *Lab Invest 14*:1428–1436, 1965.
6. Sack, R. B., Carpenter, C. C. J., Steenburg, R. W., and Pierce, N. F. Experimental cholera. A canine model. *Lancet 2*:206–207, 1966.
7. Craig, J. P. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. *Nature (London) 207*:614–616, 1965.
8. Leitch, G. J., Iwert, M. E., and Burrows, W. Experimental cholera in the rabbit ligated ileal loop: Toxin-induced water and ion movement. *J Infect Dis 116*:303–312, 1966.
9. Finkelstein, R. A., Atthasampunna, P., Chulasamaya, M., and Charunmethee, P. Pathogenesis of experimental cholera: Biologic activities of purified Procholeragen A. *J Immun 96*:440–449, 1966.
10. Benyajati, C. Experimental cholera in humans. *Brit Med J 1*:140–142, 1966.
11. Pollitzer, R. *Cholera.* World Health Organ. Monogr. No. 43, Geneva, 1959, pp. 504–510.
12. Najib-Farah. Patent bileducts in cholera. *Lancet 1*:206–207, 1966.
13. Virchow, R. *Gesammelte Abhandlungen aus dem Gebiete der Öffentlichen Medizin* (Vol. 1). Berlin, 1879, p. 151.
14. Gangarosa, E. J., Beisel, W. R., Benyajati, C., Sprinz, H., and Piyaratn, P. The nature of the gastrointestinal lesion in Asiatic cholera and its relation to pathogenesis: A biopsy study. *Amer J Trop Med 9*:125–135, 1960.

15. DAMMIN, G. J., BENENSON, A. S., FELDMAN, D., FORMAL, S. B., GOLDSTEIN, H. B., MERRILL, T. G., and SPRINZ, H. "Clinical and Histopathologic Correlations in Acute Diarrheal Disease." In *Proceedings of the Cholera Research Symposium*. U. S. Public Health Service Publication No. 1328, 1965, pp. 205–211.
16. GOLDSTEIN, H. B., MERRILL, T. G., and SPRINZ, H. Experimental cholera. Morphologic evidence of cytotoxicity. *Arch Path* (*Chicago*) *82*:54–59, 1966.
17. PATNAIK, B. K., and GHOSH, H. K. Histopathological studies on experimental cholera. *Brit J Exp Path 47*:210–214, 1966.
18. FUHRMAN, G. J., and FUHRMAN, F. A. Inhibition of active sodium transport by cholera toxin. *Nature* (*London*) *188*:71–72, 1960.
19. PHILLIPS, R. A. The patho-physiology of cholera. *Bull WHO 28*:297–305, 1963.
20. NORRIS, H. T., SCHULTZ, S. G., CURRAN, P. F., and FINKELSTEIN, R. A. Active sodium transport across rabbit ileum in experimental cholera induced by choleragen. *J Infect Dis 117*:193–196, 1967.
21. LEITCH, G. J., BURROWS, W., and STOLLE, L. C. Experimental cholera in the rabbit intestinal loop: Fluid accumulation and sodium pump inhibition. *J Infect Dis 117*:197–202, 1967.
22. SACHAR, D. B., SAHA, J. R., and HARE, K. W. Transmural electric potentials and their response to sugars in the intestine of normal subjects and acute cholera patients. *Fed Proc 26*:384, 1967.
23. SHEEHY, T. W., SPRINZ, H., AUGERSON, W. S., and FORMAL, S. B. Laboratory *Vibrio cholerae* infection in the United States. *JAMA 197*:321–326, 1966.
24. FINKELSTEIN, R. A., NYE, S. W., ATTHASAMPUNNA, P., and CHARUNMETHEE, P. Pathogenesis of experimental cholera: Effect of choleragen on vascular permeability. *Lab Invest 15*:1601–1609, 1966.
25. KEUSCH, G. T., ATTHASAMPUNNA, P., and FINKELSTEIN, R. A. A vascular permeability defect in experimental cholera. *Proc Soc Exp Biol Med 124*: 822–825, 1967.
26. GREENOUGH, W, B., III The relevance of cholera to American medicine. *Milit Med 132*:596–601, 1967.
27. GORDON, R. S., JR. "Cholera as an Experiment of Nature." In *Proceedings of the Cholera Research Symposium*. U. S. Public Health Service Publication No. 1328, 1965, pp. 293–296.
28. GREENOUGH, W. B., III Pancreatic and hepatic hypersecretion in cholera. *Lancet 2*:991–994, 1965.
29. COHNHEIM, J. F. *Lectures on General Pathology, A Handbook for Practitioners and Students*, MCKEE, A. B., Transl. The New Sydenham Society, London, 1890, pp. 953–960.
30. NORRIS, H. T., and MAJNO, G. The advance of cholera. *Lancet, 1*:1229–1230, 1967.
31. MALLORY, F. B. *Pathological Technique*. Saunders, Philadelphia, 1938, pp. 137–138.
32. KARNOVSKY, M. J. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. (abst.). *J Cell Biol 27*:137A–138A, 1965.
33. CAULFIELD, J. B. Effects of varying the vehicle for OsO_4 in tissue fixation. *J Biophys Biochem Cytol 3*:827–830, 1957.

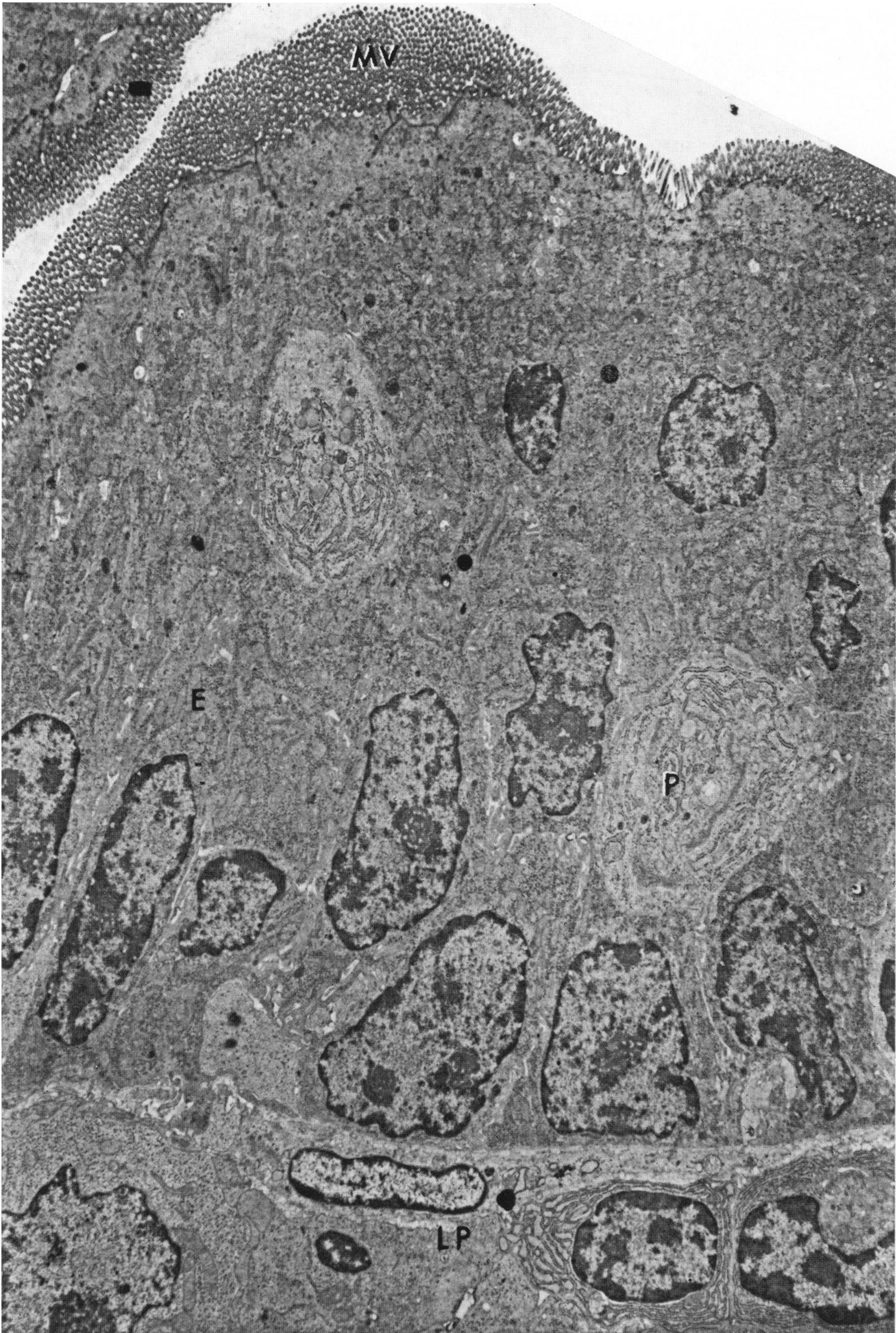
34. Baginski, E. S., Williams, L. A., Jarkowski, T. L., and Zak, B. Polarographic and spectrophotometric micro serum determinations of chloride. *Amer J Clin Path 30*:559–563, 1958.
35. Fiske, C. H., and Subbarow, Y. The colorimetric determination of phosphorus. *J Biol Chem 66*:375–400, 1925.
36. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. *J Biol Chem 193*:265–275, 1951.
37. Page, L. B., and Culver, P. J., Eds. *A Syllabus of Laboratory Examinations in Clinical Diagnosis* (rev. ed.). Harvard Univ. Press, Cambridge, 1960, p. 266.
38. Code, C. F., Phillips, S. F., and Swallow, J. H. The final common sorption fluids of small and large bowel. *J Physiol (London) 187*:42P–43P, 1966.
39. Norris, H. T., Curran, P. F., Schultz, S. G., and Majno, G. "Observations on the Actions of Choleragen in the Ileum of the Adult Rabbit." In *Symposium on Cholera*. U. S.-Japan Cooperative Medical Science Program, Office of International Research. National Institutes of Health, 1967, pp. 177–178.
40. Phillips, R. A., Love, A. H. G., Mitchell, T. G., and Neptune, E. M., Jr. "Cathartics and the Sodium Pump." In *Proceedings of the Cholera Research Symposium*. U. S. Public Health Service Publication No. 1328, 1965, pp. 85–86.
41. Fresh, J. W. "Chemical Studies of Stools: A Comparison of Normal Stools, Cholera Stools, Other Diarrheal Stools, and Artificially Induced Diarrheal Stools." In *Proceedings of the Cholera Research Symposium*. U. S. Public Health Service Publication No. 1328, 1965, pp. 296–299.
42. Saha, H., and Das, A. Observations on the nature of cholera stools. *J Indian Med Ass 21*:464–467, 1952.

We are indebted to Dr. Ramzi Cotran, Department of Pathology, Harvard Medical School, for advice and encouragement during the study; to Dr. Richard A. Finkelstein, Department of Bacteriology, SEATO Medical Research Laboratory, Bangkok, Thailand, for a generous supply of choleragen; to Dr. Theodore Colton, Department of Preventative Medicine, Harvard Medical School, for aid in statistical analyses; to Miss Virginia Gilmore and Miss Juliette Spurgeon for their assistance; and to Mr. Eduardo Garriga for preparing the figures.

[*Illustrations follow*]

Legends for Figures

Fig. 1. Low-power electron micrograph of intestinal villus from a choleraic ileal loop, demonstrating the junction between epithelium (*E*) and underlying lamina propria (*LP*). No discontinuity is present between individual epithelial cells (*E*) or between the epithelium and lamina propria (*LP*). Microvilli (*MV*), Paneth cell (*P*). × 4500.



1

2

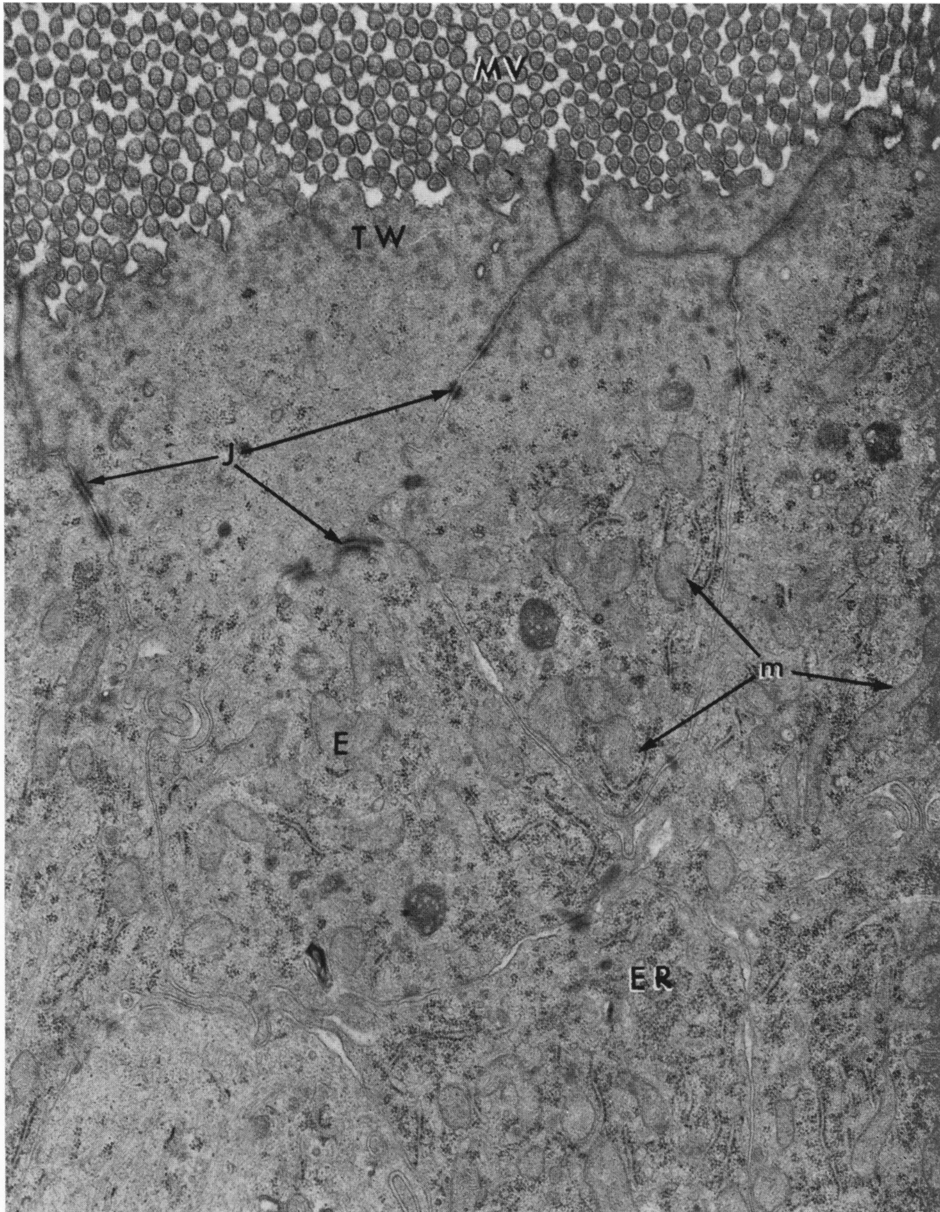
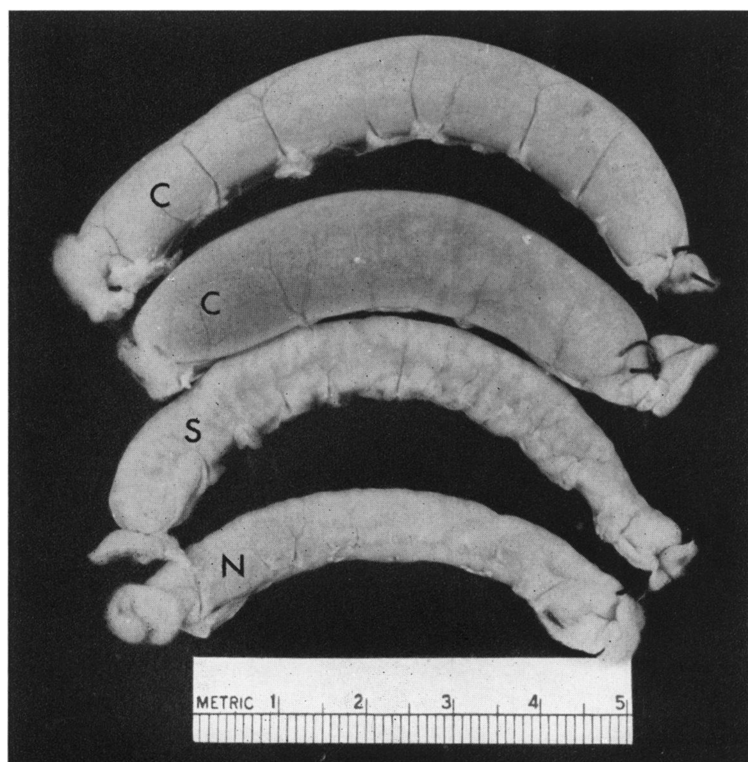


Fig. 2. Apical portion of epithelial cells (*E*) from Fig. 1. The usual complement of unaltered mitochondria (*m*), rough endoplasmic reticulum (*ER*), and epithelial junctional complexes (*J*) is present. Terminal web (*TW*) is devoid of organelles. Microvilli (*MV*) are unaltered. × 18,000.



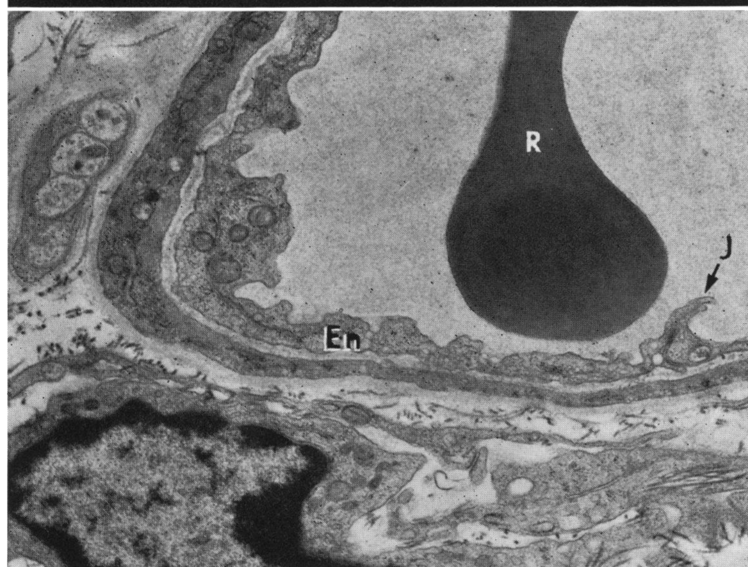


Fig. 3. Four ileal loops (gross specimens) removed 6 hr. after instillation of choleragen. One hour prior to sacrifice, vascular markers were given I.V. Two choleraic loops (*C*) are moderately distended by fluid resembling rice water stool. Control loops *S* and *N* are empty: *S* received 1 ml. physiologic saline; *N* was left empty at laparotomy. There was no extravasation of the dye markers in ileal wall or effluent. **Fig. 4.** Capillary of lamina propria from segment of choleraic ileum. No extravasation of saccharated iron oxide, although granules are present in lumen adjacent to erythrocyte (*R*). Intercellular junction (*J*) between two endothelial cells (*En*) appears unaltered. × 14,000.