# REDUCTION OF ENTEROTOXIN-REACTIVITY BY BRUSH BORDER MEMBRANES AND ENTEROTOXIN-INDUCED ALTERATION OF BRUSH BORDER CHEMISTRY

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# CHAPTER I

## INTRODUCTION

Recent studies suggest that those strains of Escherichia coli (E. coli) which produce an enterotoxin (a bacterial exotoxin) cause a major portion of severe acute diarrheal disease in developing nations (1-3) and in the United States (4). Adults and children alike are afflicted by these organisms (5 - 7). Neither are the organisms respectors of species. They are implicated also, as the cause of acute diarrhea in newborn calves, lambs, and piglets (8, 9).

Patients infected with these pathogenic strains of E. coli experience the same physical and metabolic abnormalities as patients infected with Vibrio cholerae (V. cholerae). However, Pierce and Wallace (10) reported that the duration of the E. coli induced disease is shorter than the duration of V. cholerae disease. The favorable aspect of a shorter time of incapacitation with E. coli-induced diarrhea is offset by its increased incidence; noncholera diarrhea is responsible for greater morbidity than cholera on the Indian subcontinent, as well as in other Asian, African, and South American nations (11, 12).

The pathophysiology induced by enterotoxins is reviewed in chapter II. Although much is known about the actions of E. coli-enterotoxins on the small intestine, the binding of the enterotoxin to microvillous membranes has not been demonstrated, steming undoubtedly from the fact that the enterotoxin has not been purified. Cholera-enterotoxin has

been purified and, by labeling it with $^{125}I$, Peterson (13) and Walker (14) have shown that it specifically binds to intestinal mucosal components and to intestinal microvillous membranes.

The instillation of E. coli-enterotoxin or cholera-enterotoxin into small intestinal loops of rabbits causes a net secretion of isotonic fluid into the lumina of the infected loops. Staley et al. (15), working with E. coli-enterotoxin, and Serebro et al. (16) working with cholera-enterotoxin, reported that net secretion of fluid also occurs in adjacent noninfected loops. This observation led to the speculation that a "blood borne agent" may be released from the infected loops (15, 16).

Recently, Said and Mutt (17, 18) extracted a vasoactive intestinal peptide (VIP) from normal small intestinal mucosa. Its intravenous infusion in small quantities into dogs causes vasodilatation, glycogenolysis (hyperglycemia), and net secretion of isotonic fluid into the lumen of the small intestine (19, 20). However, there is no evidence, to date, that VIP is the "blood borne agent" mentioned above.

The objectives of the present study are answering the following questions: are the reactivities of E. coli-enterotoxin and cholera-enterotoxin decreased by incubation with small intestinal mucosal components; can these enterotoxins alter the biochemical composition of brush border membranes; and finally, what effect does instillation of these enterotoxins into ligated small intestinal loops have on blood glucose concentration?

Answering these questions may help delineate the mechanisms involved in enterotoxin-related diseases. Such delineation is a requisite to controlling these diseases which are probably the greatest

single cause of premature death and loss of productivity over a geographic area that includes half of the world's population (21).

# CHAPTER II

# REVIEW OF LITERATURE

## Enterotoxins and Plasmids

*E. coli* may produce two types of enterotoxin. Smith and Gyles (22) have reported that some strains produce only a heat stable enterotoxin, while other strains elaborate both heat stable and heat labile enterotoxins. Smith and Halls (23) have shown that the biosynthesis of enterotoxins in many of these strains of *E. coli* is mediated *via* a transmissible plasmid. Plasmids are extrachromosal, dispensable genetic elements within many bacteria. Gyles et al. (24) have isolated and characterized a class of plasmids which codes for the production of enterotoxin; the plasmids were homogenous, consisting of a single DNA species with an approximate molecular weight of $6.0 \times 10^7$ daltons.

## Characterization of *E. Coli*-Enterotoxins

The enterotoxins of *E. coli* have been partially characterized as to molecular weight, heat and pH stability, susceptability to inactivation by protolytic enzymes, neutralization by antitoxin, and neutralization by gangliosides. By the use of gel filtration chromatography, the molecular weights of heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) have been estimated to be 100-10,000 and $5 \times 10^6$ daltons, respectively (25-27). Biochemical analysis of the fractions from these gel columns have indicated a LT-fraction composed of 45.8% carbohydrate and

9.3% protein and a ST-fraction composed of 2% carbohydrate and 15% protein (percentages based on mg of carbohydrate or protein per mg of lyophilized peak fraction (25, 26).

Incubation studies conducted by Smith and Gyles (22) and Evans et al. (28) have shown that E. coli-ST resists the effects of heating at 100°C for 30 minutes. After heating, the ST is still capable of causing diarrhea (ST-reactivity). The E. coli-LT on the other hand is neutralized (no LT-reactivity) after the same heat treatment (9, 22, 28).

E. coli-ST is also stable to pH changes. Exposing ST to solutions of pH 1, 3, 5, 7, 8, and 9, for four hours at 37°C has no effect on ST-reactivity (26, 28). However, when E. coli-LT is exposed to acid solutions, it is partially or totally neutralized (25, 28).

Jacks and Wu (26) have shown that the protolytic enzymes "trypsin" and "pronase" have no effect on ST-reactivity, whereas, subjecting E. coli-LT to solutions of pronase neutralizes it. Solutions of trypsin have no effect on LT-reactivity (25, 27).

E. coli-LT is antigenic while E. coli-ST is not. Gyles (29, 30) and Sack (31, 32) report that the injection of LT into rabbits results in antibodies capable of partially neutralizing LT. ST fails to elicit antibody production (22, 29).

Finally, E. coli-LT and E. coli-ST differ in the effect of gangliosides on their reactivities. Gangliosides are large molecular components of the glycocalyx of most normal cells (33). Neutralization of enterotoxin by gangliosides was first indicated from studies conducted by Heyningen et al. (34). They found that ganglioside-like substances isolated from gut mucosal membranes neutralizes cholera-enterotoxin. Holmgran et al. (35), employing different perparations of pure

gangliosides, found a $GM_1$ ganglioside to have the greatest neutralizing effect on cholera-enterotoxin. This has led to the belief that the pathogenesis of cholera is a result of the enterotoxin binding to $GM_1$ receptors on mucosal cell membranes (35-38). However, such an inference has not been possible with respect to E. coli. Pierce (36) and Holmgren (37) have found that LT is only partially neutralized by gangliosides while ST is unaffected by gangliosides.

## Enterotoxins and Diarrhea

Although E. coli-LT and E. coli-ST differ as to molecular weight, stability, etc., they produce similar pathological changes within the gut. LT or ST, when introduced into ligated loops of the small intestine (39), causes an outpouring of isotonic fluids into the lumina of the loops (40, 41). The enterotoxins apparently induce net secretion of fluids into the gut lumen by direct or indirect activation of adenyl cyclase which is known to be associated with the lateral and basal membranes of the gut columnar epithelial cells (42). The activation of adenyl cyclase leads to an increase in the concentration of intracellular adenosine -3', 5'-cyclic monophosphate (cAMP). Increased cAMP levels are implicated directly in triggering the net secretion of electrolytes and water into the gut lumen (43-46). Greenough et al. (47) and Field et al. (48) have shown that not only cAMP, but theophylline and prostaglandins stimulate fluid production within the gut lumen, and this fluid has basically the same electrolytic composition as that fluid secreted when the intestine is challenged with E. coli-enterotoxins. The common stimulant under these conditions is cAMP. Theophylline is known to inhibit the enzymatic action of phosphodiesterase, an enzyme

responsible for the breakdown of cAMP. When so inhibited, concentration of cAMP increases within the mucosal cells. Kimberg et al. (49) and Matuchansky and Bermer (50) also have shown that prostaglandins increase concentrations of cAMP, presumably by the activation of adenyl cyclase.

In order to determine if the net movement of water to the luminal side of the mucosa during E. coli-enterotoxin stimulation is due to the active secretion of certain electrolytes, Al-Awqati et al. (51) employed a technique which made use of a Ussing Chamber (52). They have shown that in normal rabbit intestinal mucosa, sodium and chloride ions are normally transported from luminal-to-serosal surfaces, and in mucosal membranes pretreated in vivo with LT, this is reversed; chloride and possibly sodium ions are transported from serosal-to-luminal surfaces. Field et al. (48) and Carpenter (53) have indicated that this same pattern of ion movement occurs when cholera-enterotoxin, theophylline, or cAMP are added to the Ussing chamber.

Thus, most workers have concluded that the enterotoxins of both E. coli and V. cholerae come in contact with the luminal surface of the small intestine and, as a result of attachment to mucosal receptors, or as a result of enterotoxin absorption, adenyl cyclase is activated, and levels of cAMP increase. Increased concentration of cAMP leads to the reverse movement of chloride ions across the gut mucosal membrane. This causes an electrical gradient to develop across the mucosa which is responsible for the movement of cations into the gut lumen. Water follows this traffic of ions into the lumen for maintenance of an osmotic equilibrium with blood plasma.

The net secretion of electrolytes and water into the gut, stimulated

by E. coli and V. cholerae, involves no morphological damage to the mucosa (54, 55). Consistent with the absence of morphological damage, are the results reported by Pierce et al. (56) and Carpenter et al. (57) that neither the absorption of glucose nor the enhancement of sodium absorption by glucose is altered during clinical or experimental cholera. On the other hand, Sherr et al. (40) observes that LT decreases the active absorption of glucose, as well as glycine.

## Biochemical Composition of Membranes

Although no morphological damage is seen in the mucosa during the diarrhea induced by E. coli-LT or cholera-enterotoxin, cholera-enterotoxin may cause "microchemical lesions" (biochemical alteration) within the microvillous membranes of the intestinal absorptive cell. Before stating the membrane components which might be altered by cholera-enterotoxin, it might be best to review current information concerning biochemical composition and structure of membranes.

Membranes from erythrocytes, hepatocytes, and neoplastic cells grown in culture (HeLa cells and Ehrlich ascites) contain, by weight, approximately 55% protein, 35 to 40% lipid, 5 to 10% carbohydrate, and less than 0.1% RNA (58).

Protein of cell membranes have been classified into two categories: peripheral and integral proteins (59). Peripheral proteins are believed to be held to the membrane proper by weak noncovalent interactions, and they are rather easily removed by mild treatments (59). The main proteins however, are of the integral type. They can be removed only by drastic treatments. Detergents, such as sodium dodecyl sulfate, bile

acids, organic solvents, and protolytic enzymes are some of the reagents and solutions employed to remove these integral proteins (59).

Some of these proteins contain insoluble lipophilic fragments which Javaid and Winzler (60) assume are the membrane-attachment sites. Some have a relatively high carbohydrate moiety (glycoproteins; 61). Javid and Winzler (62) have isolated a glycoprotein from human erythrocytic membranes which contains approximately 102 amino acid residues in the polypeptide chain, two molecules of fucose, two of mannose, 11 of galactose, 12 of n-acetylgalactosamine, six of n-acetylglucosamine, and 17 of n-acetylneuraminic acid (sialic acid).

As indicated above, a large portion of the cell membrane is composed of lipids. These lipids can be divided into phospholipids, neutral lipids (i.e. cholesterol), and glycolipids (lipids which possess covalently linked carbohydrate residues).

The way these proteins and lipids fit together to form a typical membrane is probably best described by Singer and Nicolson's (59) "Fluid Mosaic Model." According to this model, which has much experimental backing, the phospholipids are arranged in a bilayer. The nonpolar fatty acid chains of the phospholipids are sequestered together away from contact with water (hydrophobic interactions). The polar heads of the phospholipid molecules are in direct contact with the aqueous phase of the exterior surfaces of the bilayer. The membranous proteins are of a globular form, and they are partially embedded in the bilayer. The protruding portions of the proteins are of an ionic nature while the nonpolar residues of the proteins are largely in the embedded part. Some of these globular proteins may span the entire thickness of the membrane. The lipid bilayer is fluid, and these globular proteins may

undergo lateral diffusion (59).

Much evidence has been gathered on the asymmetry of membranes. One aspect of this asymmetry is the distribution of membranous oligosaccharides. These chains of carbohydrates are an integral part of membranous glycoproteins and glycolipids (62, 63). Lectins (plant proteins), which bind to specific sugar residues, have been conjugated with ferritin. Electron microscopic studies, conducted by Nicolson and Singer (64), of erythrocytic membranes treated with lectin-ferritin reveal conjugate binding to the outer, but not the inner, surface of the membranes. Etzler and Branstrator (65), using fluorescein-isothiocyanate-labeled lectins, demonstrated an extensive external carbohydrate layer on the apical surface of cells lining the villi of the intestine. This outer carbohydrate-rich component (glycocalyx) of the membrane is dynamic; labeled compounds such as sodium acetate-$^{3}H$, glucose-$^{3}H$, glucosamine-$^{14}C$, and galactose-$^{3}H$ become incorporated into the glycocalyx within short time periods following their detected presence within the cells (33).

There is also evidence indicating asymmetry within the phospholipid bilayer. In general, phosphatidylcholine and phosphatidylethanolamine are the most abundant phospholipids in mammalian cell membranes, but phosphatidylserine and sphingomyelin are also present in significant amounts (63). Using specific chemical agents which react with amino groups, work with erythrocytic membranes has shown that the outer phospholipid layer is relatively devoid of amino phospholipids. One interpretation of these results is that choline phospholipids are concentrated in the outer half of the bilayer leaflet, and the amino phospholipids comprise a significant portion of the inner (cytoplasmic)

half of the leaflet (63).

Information concerning the localization of the neutral lipid "cholesterol" within membranes is limited. Detailed X-ray analysis of myelin membranes reveal a more dense electron profile of the outer lipid layer than of the inner layer. These results were interpreted by Casper and Kirschner (66) as indicating cholesterol located within the external side of the membrane bilayer.

As indicated previously, cholera-enterotoxin appears to alter the biochemical composition of small intestinal microvillous membranes. Leitch (62) reports that exposing the small intestinal mucosa to cholera-enterotoxin *in vivo* results in the significant reduction of brush border sialic acid, phospholipid, cholesterol, and glycolipid:protein ratios. Such a study has not been performed with *E. coli*-enterotoxins. The observation that *E. coli*-LT has an inhibitory effect upon glucose and glycine absorption, might represent a biochemical alteration.

## Summary and Objectives

The literature supports the following summarized concept. Cholera-enterotoxin binds to specific receptors on the intestinal microvillous membrane, adenyl cyclase activity is increased, and the subsequent increase in intracellular cAMP levels leads to the active secretion of chloride ions into the small intestional lumen. The binding *in vivo* of the cholera-enterotoxin to the membranes apparently produces an alteration of the membrane's chemistry. *E. coli*-enterotoxin also increases adenyl cyclase activity in intestinal epithelium and the increase in cAMP concentration is responsible for net chloride ion secretion. However, it is not known if *E. coli*-enterotoxins bind to microvillous

membranes, or if they alter these membranes biochemically.

My thesis is that E. coli-LT binds to intestinal microvillous membranes, and the instillation of E. coli-LT and cholera-enterotoxin into the small intestine causes an alteration of brush border chemistry and a significant increase in plasma glucose concentration via a "blood borne agent."

# CHAPTER III

# MATERIALS AND METHODS

## Bacterial Organisms

The E. coli organisms used for enterotoxin production have been described elsewhere: strain 0148:H28 was isolated from humans and produces both LT and ST (68). Strains 711 (K12, $LAC^-$, $NAL^+$, $ENT^-$) and 711 (K12, $LAC^-$, $NAL^+$, $ENT^+$) were obtained through the courtesy of Dr. Carlton Gyles, Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario, Canada. Strain 711 (K12, $LAC^-$, $NAL^+$, $ENT^-$), referred to in this paper simply as 711, does not produce enterotoxin. Strain 711 (K12, $LAC^-$, $NAL^+$, $ENT^+$), referred to in this thesis as 711 (155), is a 711 strain which received the enterotoxin plasmid from the porcine enteropathogenic strain P 155 (0149:K91, 88ac). Strain 711 (155) produces both LT and ST (22).

## Preparation of Enterotoxin

E. coli-LT was prepared in the following way: a sterile trypticase soy broth nutrient (approximately 6ml in a stoppered test tube) was inoculated by loop transferral of the bacterium from a culture maintained on a nutrient agar slant. The inoculated nutrient broth was incubated for 6 hours at 37°C, after which a 1ml aliquot of the broth culture was used to inoculate stoppered one-liter Erlenmeyer flasks which contained 500ml of sterile trypticase soy broth media (pH of media preadjusted to

8.5 using NaOH). After inoculation, the flasks were placed in a mechanical shaker and incubated at 37°C, with continual shaking, for 18 hours. Following incubation, a cell free supernatant was obtained by centrifuging the cultures at 15,000 x g for 30 minutes at 40°C. The supernatant (broth containing enterotoxins) was then sterilized by filtration through 0.45 um Seitz filters prior to ultrafiltration.

Ultrafiltration consisted of placing the sterile filtrate in an ultrafiltration system (ultrafiltration cell model 402, Amico corp.) fitted with a XM-100 A membrane (membrane retains substances of MW>100,000 daltons). One liter volumes of the sterile filtrate were concentrated to approximately 50ml of retentate, at which time the retentate was washed with 100ml of 0.01M phosphate buffer (pH 7.6). Ultrafiltration was continued until approximately 40ml of retentate remained above the membrane. The retentate was then removed from the ultrafiltration system and lyophilized. The lyophylized material (E. coli-LT) was stored in vials at -70°C until needed.

Lyophilized material was also obtained (for control purposes) from the nonenterotoxin producing 711 strain by the procedure outlined above.

Cholera-enterotoxin (V. cholerae, lot 001) was obtained by the aid of Dr. Carl Miller, National Institute of Health, Bethesda, Maryland.

## Enterotoxin Assay

The reactivities of E. coli-LT and cholera-enterotoxin were determined by the small intestinal ligated loop assay (39). Albino rabbits, which were fasted for 24 hours and weighed approximately 2Kg (8 to 9 weeks of age), were used for all assays.

The assay consisted of instilling 1ml volumes of enterotoxin-

containing solutions into ligated small intestinal loops, approximately 5cm in length, (12 loops per rabbit) which had been previously flushed with warm (37°C) Krebs-Ringer-bicarbonate solution. Ten hours later, the animals were sacrificed, the loops excised, and their total fluid content determined by withdrawing the contents into a syringe. After fluid withdrawal, the lengths of the loops were measured and the loop response reported as a ratio of loop volume (ml) to loop length (cm).

Each rabbit served as its own control. Control and experimental (supernatant) solutions were instilled into the loops in an alternating pattern.

Enterotoxin dose response curves were obtained by using essentially the method reported by Burrows and Musteikis (39). However, the lower region of the jejunum was also used for the assay, and approximately 5cm loops (12 per rabbit) were used instead of 10cm loops. Also, 1ml volumes of enterotoxin solution were instilled into each loop instead of 2ml. The animals were sacrificed 10 hours after the instillation of enterotoxin and the loop response was recorded. The location of loops in the small intestinal segment receiving the same amount of enterotoxin was varied in each rabbit.

## Preparation of Mucosal Homogenate Sediment and Cytosol

The jejunal and ileal segment of the intestine of rabbits fasted 24 hours was removed from the body cavity, opened at its antimesenteric border and spread on a chilled glass plate, mucosa upwards. The mucosa was rubbed gently with saline moistened Kimwipes to remove any adhering mucus. Finally, the mucosa itself was removed by scraping the segment

with the edge of a glass slide. The mucosal scrapings were homogenized in a Waring blender for 30 seconds in 100ml of cold (4°C) Krebs-Ringer-bicarbonate solution.

The mucosal homogenate was centrifuged at 24,000 x g for 10 minutes at 4°C. The sediment was called mucosal homogenate sediment (MHS). The MHS was washed twice with aliquots of cold Krebs-Ringer-bicarbonate solution and stored. The supernatant of the 24,000 x g centrigugation was centrifuged again at 100,000 x g for 60 minutes at 4°C to yield mucosal homogenate cytosol (MHC). MHC was stored at -70°C until needed.

## Preparation of Brush Borders and Brush Border Membranes

Brush borders were obtained from the jejunum and ileum of rabbits using essentially the technique reported by Forster et al. (69). However, instead of washing and suspending the brush borders in 2.5 mM EDTA buffered solution, cold (4°C) Krebs-Ringer-bicarbonate solution was used for these steps. Brush border membranes were obtained by the method reported by Hopfer et al. (70).

## Preparation of Brush Borders and Brush Border Membranes for Electron Microscopy

Pellets of brush borders and brush border membranes were fixed in sodium cacodylate-buffered glutaraldehyde (pH 7.2) overnight at 4°C. The pellets then were washed in four changes of buffer and stored a second night at 4°C in the fifth rinse. The third day the pellets were postfixed in 1% $OsO_4$ for 1 hour at 4°C then dehydrated in a cold graded

series of ethanol concentrations, allowing adjustment to room temperature in 100% ethanol. The dehydrated pellets were then passed through three rinses of propylene oxide before they were infiltrated for one hour in a mixture of equal volumes of propylene oxide and Epon. Finally, the pellets were infiltrated overnight at room temperature in pure Epon and embedded. The pellets were sectioned using a ultramicrotome, and then stained first with saturated uranyl acetate in 50% ethanol and then with lead citrate according to the method reported by Venable and Coggshall (71).

### Mucosal Homogenate Sediment (MHS) Plus Enterotoxins

Solutions of *E. coli* (0148:H28)-LT (2.0mg/ml) and cholera-enterotoxin (1.0mg/ml) were made using Krebs-Ringer-bicarbonate-gelatin (0.2% gelatin) solution (KRBG) as the solvent. Ten ml of each enterotoxin solution were added to separate pellets of MHS each weighing approximately 2 g (wet weight). The pellets were suspended in the enterotoxin solutions and quantitatively transferred to 25ml Erlenmeyer flasks. Ten ml of each enterotoxin solution were added to 25ml flasks to serve as control solutions. The flasks and their contents were incubated at 37°C for 30 minutes with continual agitation (using mechanical shaker).

After incubation, the MHS was resedimented from the enterotoxin solutions as pellets by centrifugation (24,000 x g for 10 minutes at 4°C). The respective supernatants were withdrawn as completely as possible and saved. The pellets were washed twice with 4.5ml volumes of cold KRBG solution. The supernatant washes were added to the appropriate initial supernatant. Finally, the volume of the respective

pooled supernatants (initial supernatant plus two washings) was adjusted to exactly 20ml. The control solutions were diluted one-fold with cold KRBG. The reactivity of enterotoxin within the respective supernatants and control solutions was determined by the ligated loop assay as previously described.

### Mucosal Homogenate Cytosol (MHC) Plus Enterotoxins

Solutions of *E. coli* (O148:H28)-LT (1.0mg/ml) and cholera-enterotoxin (0.5mg/ml) were made using MHC as the solvent. For control purposes, solutions of *E. coli* (O148:H28)-LT (1.0mg/ml) and cholera-enterotoxin (0.5mg/ml) were made using KRBG as the solvent. The solutions were added to 25ml Erlenmyer flasks and incubated (37°C) for 30 minutes with constant agitation. Following incubation, the control and MHC-enterotoxin solutions were assayed for enterotoxin-reactivity using the ligated loop assay.

### Cholera-Enterotoxin-Treated Brush Borders

A solution of cholera-enterotoxin (2.0mg/ml) was made using KRBG as the solvent. Five ml of this solution were added to pellets of brush borders (approximately 50 units of sucrase activity in each pellet). The brush borders were suspended in solution, and the suspensions were transferred to 25ml Erlenmeyer flasks. The flasks were incubated at 37°C, with agitation, for 15 minutes at which time the brush borders were sedimentated (2,000 x g for 15 minutes at 4°C). The brush borders were then washed twice with aliquots of cold KRBG solution, and all of the supernatants were discarded. The pellets were designated as "cholera-enterotoxin-treated brush borders."

## Papain Hydrolysis of Brush Borders

A dilute solution of papain was made according to the method reported by Forstner (72). This solution was added to brush border pellets containing approximately 50 units of sucrase activity (1ml of papain solution per 3mg of brush border protein). The resulting suspensions were incubated for 30 minutes at 37°C with gentle agitation.

Prior to the addition of the papain solution, samples were taken from the brush borders for biochemical analyses of protein, sucrase, and sialic acid.

After incubation, the brush borders were sedimentated by centrifugation (24,000 x g for 30 minutes at 4°C). The pellets were washed twice with cold Krebs-Ringer-bicarbonate solution. The pellets were then suspended in cold idstilled water, and samples were removed for subsequent analyses of brush border protein, sucrase, and sialic acid. The brush borders were again sedimentated (24,000 x g), and the resulting pellets were designated as "hydrolyzed brush borders."

## Brush Borders and Brush Border Membranes Plus Enterotoxins

The enterotoxin solutions referred to below were made using KRBG as the solvent. Four ml of an E. coli (0148:H28)-LT solution (2.0mg/ml) were added separately to pellets of cholera-enterotoxin-treated brush borders,isolated, untreated brushborders, and brush border membranes. Three ml of this enterotoxin solution were added also to pellets of hydrolyzed brush borders. To still other pellets of brush borders, 4ml of a cholera-enterotoxin solution (1.0 mg/ml) were added. The pellets

of brush borders and membranes were suspended in the enterotoxin solutions and quantitatively transferred to 25ml Erlenmeyer flasks. Four ml of each enterotoxin solution alone were also added to 25ml flasks to serve as controls. The flasks and their contents were incubated for 30 minutes at 37°C with continual agitation. Some of the brush border-LT suspensions were incubated for 30 minutes at 4°C, with continual agitation.

Following the above incubation, the brush borders and brush border membranes were sedimentated by centrifugation (2,000 x g for brush border suspensions and 24,000 x g for membrane suspensions). The respective supernatants were removed and saved. All of the resulting pellets were washed twice with 1.5ml volumes of cold KRBG, the supernatant from each washing was added to the original supernatant. The final volume of the respective pooled supernatants was adjusted to 8ml, a one-fold total dilution. The volume of each supernatant from pellets of hydrolyzed brush borders was adjusted to 6ml, also a one-fold dilution. Each control solution was diluted one-fold with KRBG. Enterotoxin-reactivity was determined by the ligated loop assay.

Some of the pellets of brush borders and brush border membranes used in the enterotoxin-incubation study contained from 40 to 70 units of sucrase activity. The loop responses produced by the supernatants from these pellets encompassed a range proportional to enterotoxin concentration (verified by a linear relationship between the loop response values of this range and enterotoxin concentration or dose). This linearity permitted an estimate of loop responses normalized to an amount of brush borders or membranes containing 50 units of sucrase activity. For example, the supernatant from a brush border pellet

containing 70 units of sucrase activity produced a mean loop response of 0.60 ml/cm. The response due to control solution was 1.30 ml/cm. The 0.60 ml/cm value represented a reduction in enterotoxin-reactivity of 0.70 ml/cm. If one assumed that the magnitude of this reduction was proportional to the amount of membrane present (sucrase is a membrane marker), then one could calculate the reduction that would be expected if pellets containing 50 units of sucrase had been used instead of 70-unit-pellets $\left(\frac{0.70 \text{ ml/cm}}{70 \text{ units}} = \frac{0.01 \text{ ml/cm}}{\text{unit}}, \frac{0.01 \text{ ml/cm}}{\text{unit}} \times 50 \text{ units} = 0.5 \text{ ml/cm}\right)$. The normalized experimental response, based upon 50-unit-pellets, would be 0.80 ml/cm (1.30 ml/cm -X = 0.5 ml/cm; X = 0.8 ml/cm).

## Brush Border Chemistry and Blood Glucose Samples

Twenty-four-hour fasted rabbits (each weighing approximately 2.0 Kg) were anesthetized with sodium pentobarbital *via* an ear vein. The small intestine, from the ligament of Treitz to a point approximately 20cm above the end of the appendix, was flushed *in vivo* with warm (37°C) saline. Four, approximately 40cm loops were formed in this segment by ligation. A solution of cholera-enterotoxin (6mg/ml of saline) was instilled into the loops of three rabbits (5ml/loop-four loops per rabbit). Five ml of *E. coli* 711 (155)-LT solution (20mg/ml of saline) were instilled into the loops of six other rabbits. For control purposes, 5ml of a solution of *E. coli* 711-nonenterotoxin material (20mg/ml saline), and 5ml of saline were instilled into loops of separate groups of rabbits (six rabbits per group).

One ml blood samples were collected in heparinized tubes from each

animal, *via* an ear vein, at 0 (just before instillation of control or enterotoxin solutions), 15, 30, 60, 90, 120, and 180 minutes after solution instillation. Microhematocrit tubes were filled from each sample prior to the obtainment of plasma. The plasma samples were labeled and stored at -70°C for future glucose analysis. Hematocrit values on each blood sample were determined by use of a micro-hematocrit reader.

The animals were sutured, and, three hours after instillation of control and enterotoxin solutions, they were sacrificed. The loops were removed and placed on a cold glass plate. The control and enterotoxin responses were recorded. The loops were opened along their antimesenteric borders, and the mucosa was wiped free of mucus using saline moistened Kimwipes. The mucosa was then removed with the edge of a glass slide. Brush borders from the scrapings were obtained by the method reported by Forstner et al. (69), with one modification; the brush borders were suspended in glass distilled water instead of the 2.5nM EDTA buffered solution. Each rabbit (4 loops) comprised one brush border preparation. All brush border preparations were stored at -70°C.

At a later time, the brush borders were thawed and analyzed for protein, sucrase, sialic acid, lipid galactose, lipid phosphorus, and cholesterol.

## Chemical Analysis

Sialic acid content of brush borders was measured using the thiobarbituric acid method of Warren (73). Total lipids were extracted from the brush borders using a chloroform-methanol (2:1, v/v) solvent

(74). This lipid extract was then assayed for cholesterol (75), lipid galactose (76), and lipid phosphorus (77). Protein content of the brush borders was determined by the method reported by Lowry et al. (78). Brush border sucrase was determined by the method reported by Dahlqvist (79). All chemical data were expressed per mg of brush border protein.

Plasma glucose concentration was determined by the use of a glucostat kit (Worthington). The glucose assay values were adjusted so that increased glucose concentration due to hemoconcentration was not reflected in the values recorded. This was done in the following way: total blood volume of each rabbit was obtained on the basis of 63ml per Kg of body weight (80). The initial (zero minute) hematocrit value was used to calculate the total volume of blood cells in a given rabbit [total blood cell vol. (ml) = total blood vol. (ml) X $\frac{\text{initial hematocrit.}}{100}$ It was assumed that the total volume of blood cells remained constant over the experimental time period and that increased hematocrit values were due to the loss of glucose-free and protein-free plasma *via* the enterotoxin stimulated gut. Total blood volume was calculated from each subsequent hematocrit value (total blood vol. (ml) = $\frac{\text{total blood cell vol. (ml)}}{\text{hematocrit value}}$ X 100]. Total plasma volume for each time period could then be calculated as follows: total plasma vol. (ml) = total blood vol. (ml) - total blood cell vol. (ml). Total plasma glucose was obtained by multiplying total plasma volume (calculated from zero minute sample) times the concentration of glucose (mg/ml) in the zero minute sample. The change in glucose concentration due to hemoconcentration at each time period was indicated as follows:

$$\text{glucose (mg\%)} = \frac{\text{total plasma glucose (mg)}}{\text{total plasma vol. of each time period}} \text{ X } 100$$

Using the above formula, any increase in glucose concentration (over the zero minute value) with time, was considered as an increase due to hemoconcentration, and the value of this increment was subtracted from the appropriate measured glucose concentration.

# CHAPTER IV

# RESULTS

## Dose Response Curves

Does response curves were plotted using E. coli (O148:H28)-LT (Fig. 1) and cholera-enterotoxin (Fig. 2). Each point on the curves represents the mean response value ± SEM of at least five loops.

In the incubation experiments, the concentrations of the control solutions chosen with respect to LT and cholera-enterotoxin were 1.0 mg/ml and 0.5 mg/ml respectively. These concentrations and their responses are indicated by the arrows in figures 1 and 2. Since, in the assay, each loop received 1ml of enterotoxin solution, the concentration of enterotoxin (mg/ml) was indicative of the dose (mg/loop). It may be noteworthy to point out that these dose response curves are not typically sigmoid-shpaed.

## Purity of Brush Borders and Brush Border Membranes

The purity of the brush borders and brush border membranes was indicated chemically by measuring the specific activity of the membrane marker "sucrase" (Table I). Specific sucrase activity within mucosal homogenates was consistently around 0.16 units/mg of protein; this represented a purification factor of 1. Specific sucrase activity within the brush borders obtained from this homogenate averaged 2.00

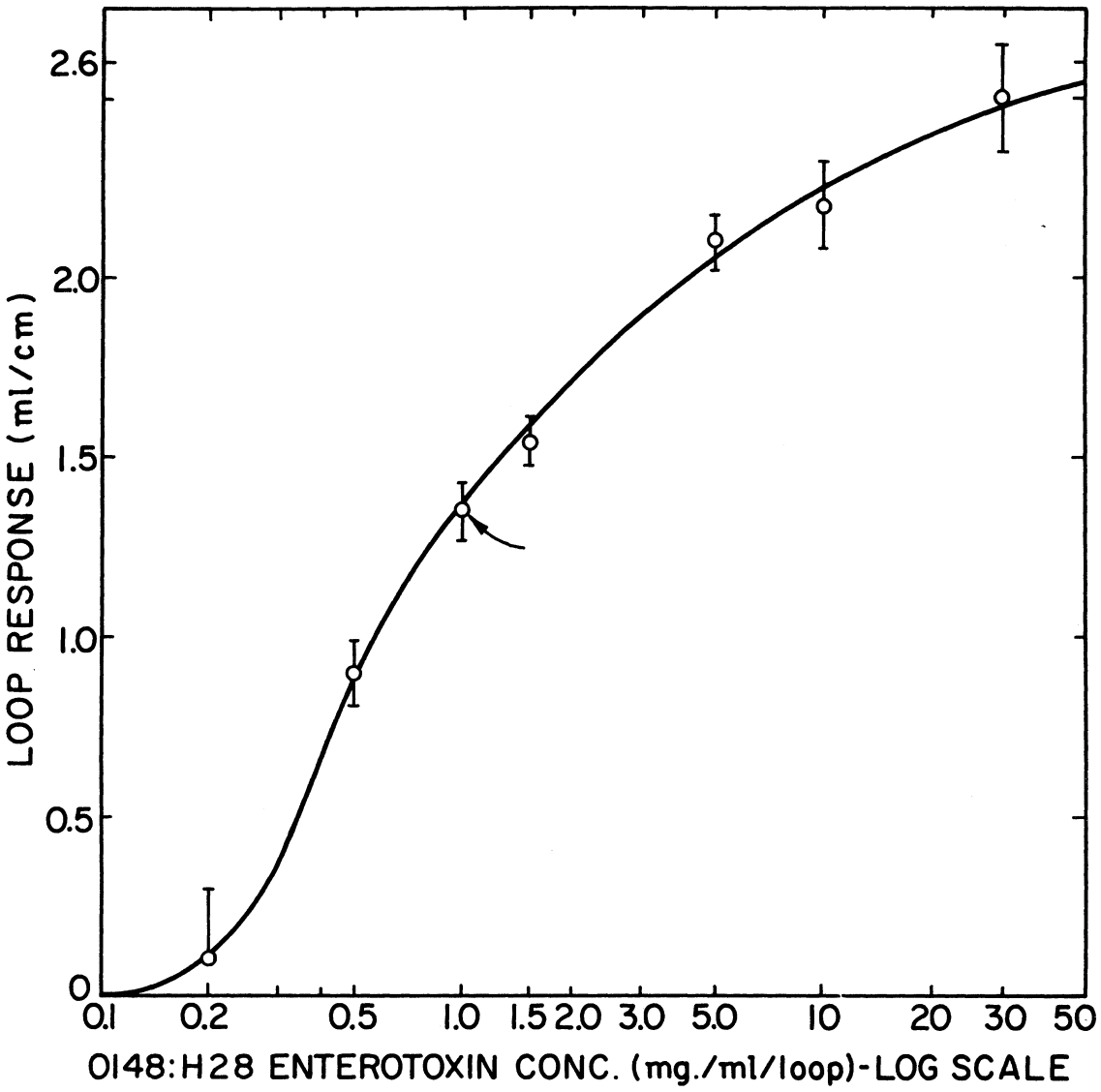


Figure 1. E. Coli (0148:H28)-LT Dose Response Curve. Loop response, ± SEM, is plotted against the logarithm of the enterotoxin concentration. Each point represents the mean value of 5-8 loops. The arrow represents the concentration of enterotoxin used for control solutions.

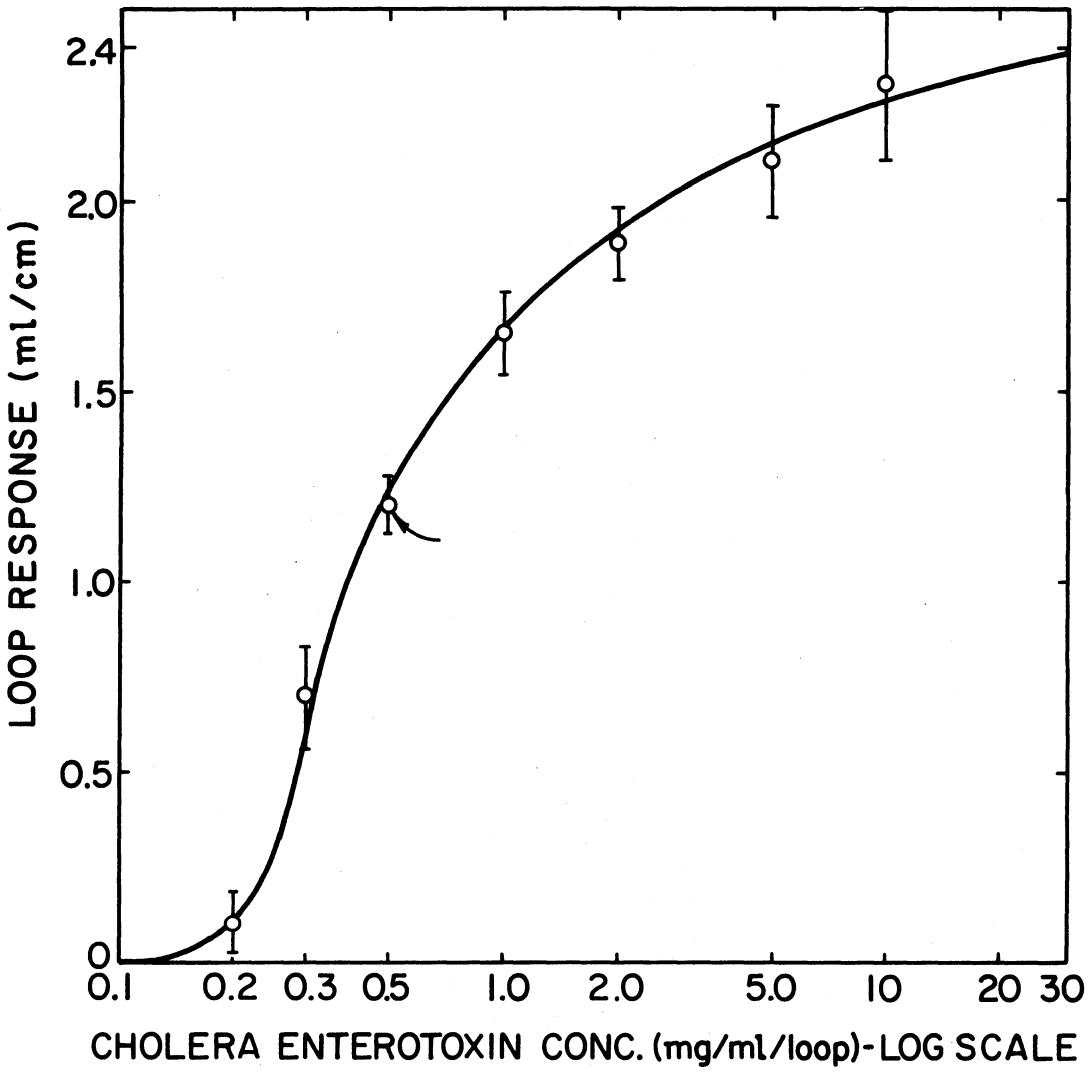


Figure 2. Cholera-Enterotoxin (lot 001) Does Response Curve. Loop response, ± SEM, is plotted against the logarithm of the enterotoxin concentration. Each point represents the mean value of 5 loops. The arrow represents the concentration of enterotoxin used for control solutions.

units/mg of protein. These brush borders represented a 13-fold increase in the specific activity of sucrase. The average specific sucrase activity within brush border membranes was 3.44. This value represented a 22-fold increase in sucrase purification as compared to the homogenate. It is noteworthy that the sucrase specific activity was 1.7 times greater in the brush border membranes than in the intact brush border fraction.

TABLE I

SUCRASE ACTIVITY (RABBIT)

| Fraction | Specific Activity[1] | Purification | Yield |
|---|---|---|---|
| Mucosal Homogenate | 0.16[2] | 1.0 | 100% |
| Brush Borders | 2.00 | 13 | 16% |
| Brush Border Membranes | 3.44 | 22 | 9% |

[1]Micromoles of sucrase hydrolyzed per min. per mg of protein at 37°C and pH 6.0.

[2]Sucrase specific activity represents the mean value of four determinations.

The yield of membranes (Table I) in a given preparation was indicated by measuring total sucrase activity. Mucosal homogenates represented a 100% yield of sucrase. Brush border preparations

represented a 16% yield of this sucrase activity. The brush border membranes represented a 9% yield of this sucrase activity. Ninety-one percent of the sucrase activity present in the initial homogenate of rabbit mucosa was lost during the process of obtaining brush border membranes.

The electron microscopic appearance of the brush borders (Fig. 3 and 4) and brush border membranes (Fig. 5) indicated preparations that were highly pure. In the brush border preparations (Fig. 3 and 4), the terminal web and microvillous core filaments were visible. Terminal web and filamentous material were virtually absent in the membrane preparations (Fig. 5).

## Hydrolyzed Brush Borders

Total brush border protein, sucrase, and sialic acid values of pellets before and after papain digestion are recorded in Table II. Total brush border protein before papain treatment was 30.7mg as compared to 8.1 mg after treatment; this represented a reduction in total protein of 73%. Total brush border sucrase was 77.4 units as compared to 2.0 units after papain treatment. This reflected a 97% decrease in total sucrase. Brush border sialic acid before papain treatment was 236muM, and after treatment it was 37muM. These values represented an 84% reduction in total sialic acid.

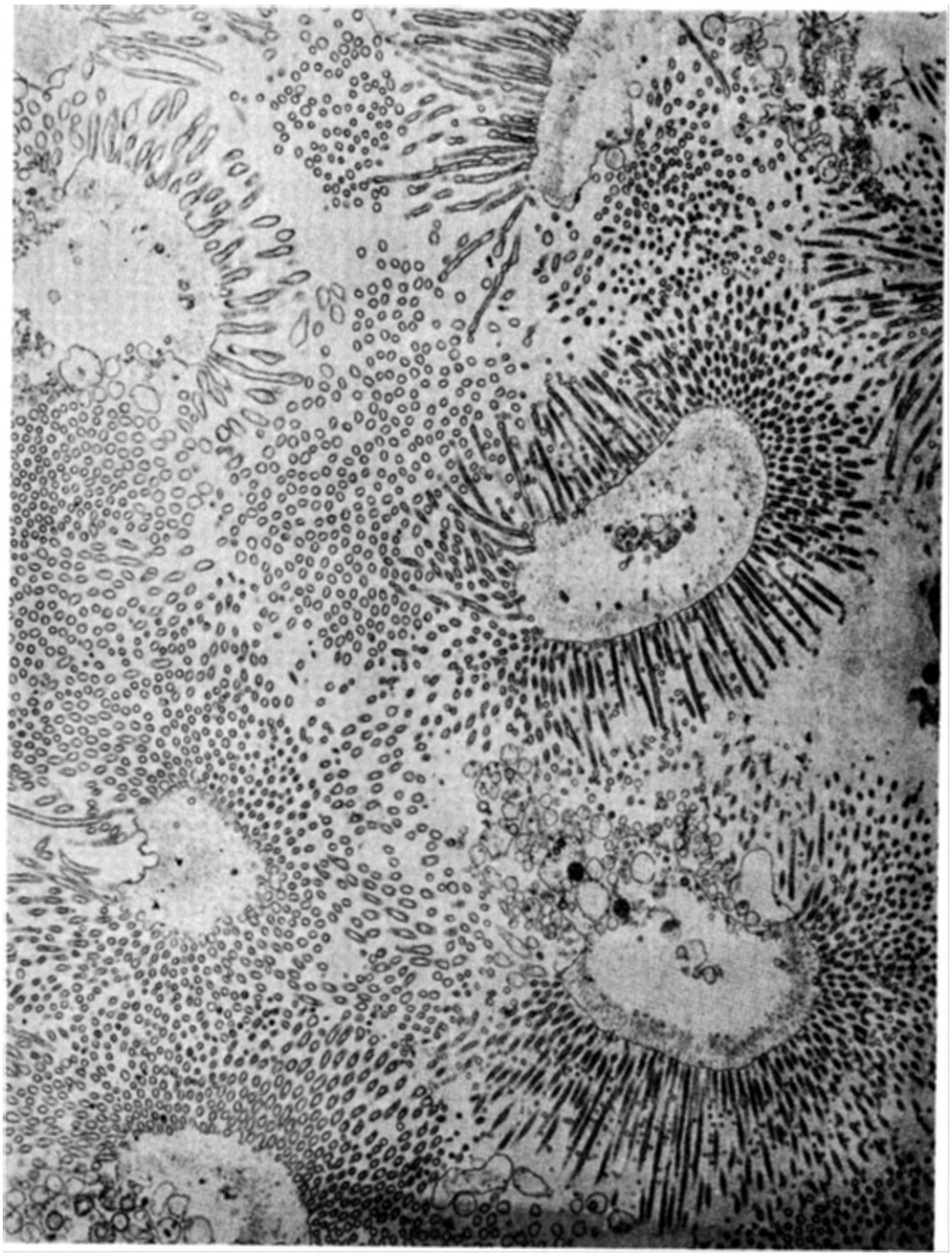

Figure 3. Electron Micrograph of Rabbit Brush Borders (approximate magnification X 10.000).

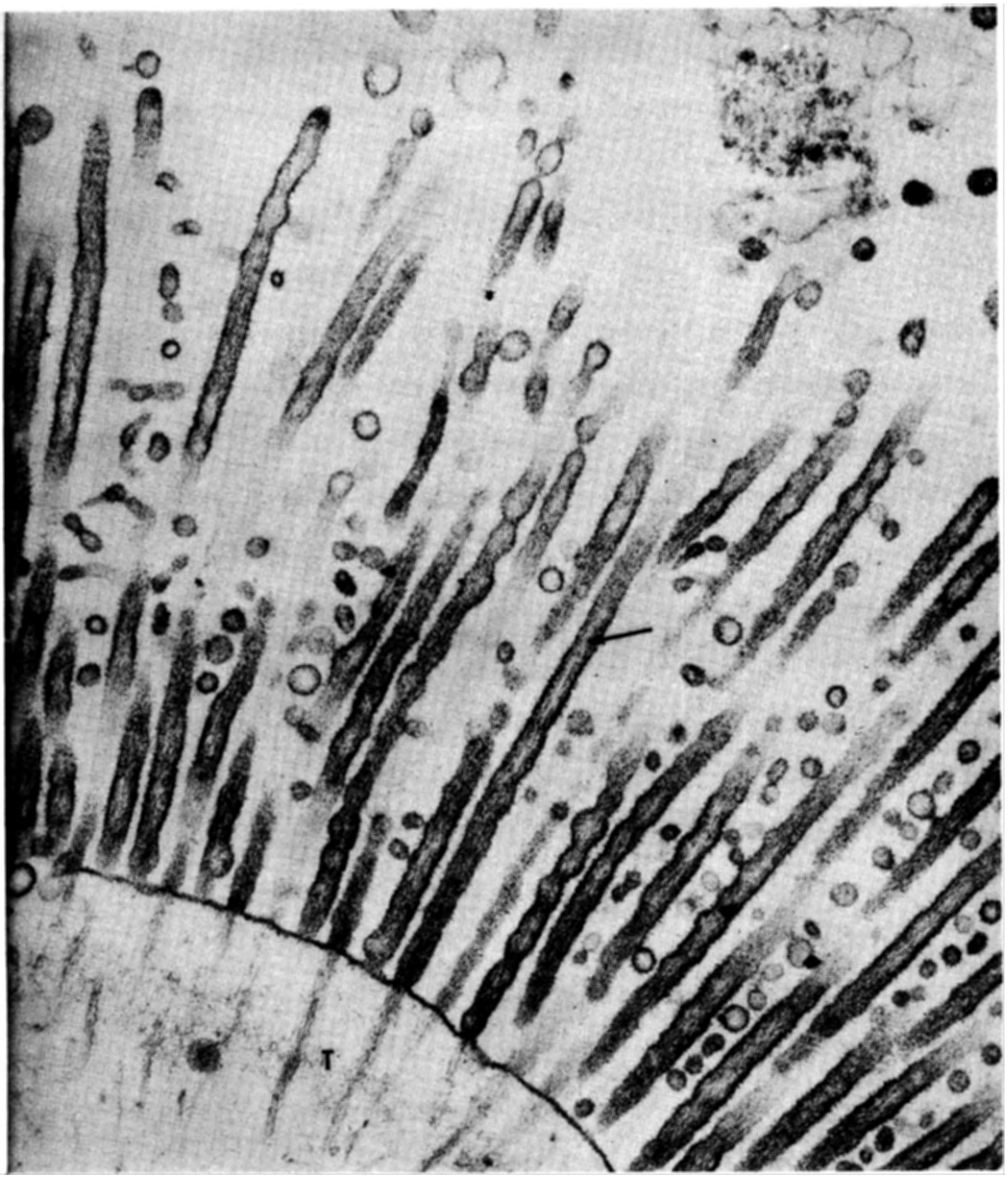


Figure 4. Electron Micrograph of Rabbit Brush Borders (approximate magnification X 52,000).

→ = microvillous filaments
t = terminal web

Figure 5. Electron Micrograph of Rabbit Brush Border Membranes (approximate magnification X 35,000).

TABLE II

PAPAIN DIGESTION OF RABBIT BRUSH BORDERS

| Preparation | Total Protein (mg) | Total Sucrase units [2] | Total Sialic Acid (muM) |
|---|---|---|---|
| Brush Borders Before Papain Treatment | 30.7[1] | 77.4 | 236 |
| Brush Borders After Papain Treatment | 8.1 | 2.0 | 37 |
| Mean Percent Reduction | 73% | 97% | 84% |

[1]Each numerical value represents a mean of three brush border preparations.

[2]Micromoles of sucrase hydrolyzed per minute at 37°C and pH 6.0.

## Effects of Mucosal Components on Enterotoxin-Reactivity

The effects of mucosal components of rabbit small intestine on the reactivities of E. coli (O148:H28)-LT and cholera-enterotoxin are recorded in Table III. As a preliminary step, both enterotoxin solutions were added to mucosal homogenate pellets, the latter being resuspended in these solutions and incubated at 37°C. Both cholera-enterotoxin and E. coli-LT were completely removed from the solution and/or neutralized in it by components of the mucosal homogenate sediment (MHS). The enterotoxin responses (the control responses) that one would have expected if no enterotoxin had been removed or

TABLE III

EFFECTS OF MUCOSAL COMPONENTS ON ENTEROTOXIN-REACTIVITY (RABBIT)

| Preparation | Mean Control Response (ml/cm) | Mean Supernatant Response[1] (ml/cm) | Response Reduction[2] | Percent Decrease in Reactivity[3] |
|---|---|---|---|---|
| Cholera Enterotoxin Plus MHS @ 37°C [4] | 1.10 ± .04 (21)[5] | 0 (21) | 1.10 | 100 |
| 0148:H28-LT Plus MHS @ 37°C [6] | 1.40 ± .06 (18) | 0 (18) | 1.40 | 100 |
| Cholera Enterotoxin Plus MHC @ 37°C | 1.28 ± .07 (14) | 1.22 ± .08 (14) | 0.06 | 5 |
| 0148:H28-LT Plus MHC @ 37°C | 1.50 ± .05 (13 | 1.50 ± .05 (13) | 0 | 0 |
| Cholera Enterotoxin Plus Brush Borders @ 37°C | 1.30 ± .05 (24) | 0.90 ± .06 (24) | 0.40 | 31 |
| 0148:H28-LT Plus Brush Borders @ 37°C | 1.34 ± .06 (15) | 0.12 ± .04 (15) | 1.22 | 91 |
| 0148:H28-LT Plus Brush Borders @ 4°C | 1.38 ± .10 (12) | 0.88 ± .06 (12) | 0.50 | 36 |
| 0148:H28-LT Plus Brush Membranes @ 37°C | 1.38 ± .10 (14) | 0.33 ± .09 (14) | 1.05 | 76 |

TABLE III (Continued)

| Preparation | Mean Control Response (ml/cm) | Mean Supernatant Response[1] (ml/cm) | Response Reduction[2] | Percent Decrease in Reactivity[3] |
|---|---|---|---|---|
| O148:H28-LT Plus Brush Borders Pretreated with Cholera Enterotoxin @ 37°C | 1.45 ± .09 (14) | 0.47 ± .08 (14) | 0.98 | 68 |
| O148:H28-LT Plus Brush Borders Pretreated with Dilute Papain Solution @ 37°C | 1.57 ± .13 (12) | 0.51 ± .09 (12) | 1.06 | 68 |

[1] In preparations involving brush borders and membranes, this response is based upon supernatants from pellets which contained 50 units of sucrase activity.

[2] Response reduction = mean control response-mean supernatant response.

[3] Percent decrease in reactivity = response reduction/control response X 100.

[4] Mucosal homogenate sediment.

[5] ± = SEM and ( ) = number of loops involved in determination of mean response.

[6] Mucosal homogenate cytosol.

neutralized was 1.10 ± .04 ml/cm for the cholera-enterotoxin solution and 1.40 ± .06 ml/cm for the LT solution.

Cholera-enterotoxin and E. coli-LT were mixed in mucosal homogenate cytosol (MHC), and the solutions were incubated at 37°C. This was done to determine if soluble components from the mucosa (enzymes, soluble receptors, etc.) were capable of neutralizing the enterotoxins. There was no significant effect. The reactivities of cholera-enterotoxin and LT when suspended in MHC were not significantly different than their reactivities in Krebs-Ringer-bicarbonate-gelatin-solution (KRBG). The cholera-enterotoxin-KRBG solution (control solution) produced a mean loop response of 1.28 ± .07 ml/cm while the cholera-enterotoxin-MHC solution produced a mean response of 1.22 ± .08 ml/cm. The mean loop response to LT-KRBG solution (control solution) was 1.50 ± .05 ml/cm which was identical to the mean response produced by LT-MHC solution.

Purified brush border pellets were obtained in order to determine whether brush borders were capable of decreasing the reactivity of the enterotoxins. Cholera-enterotoxin or E. coli-LT was added to pellets of brush borders, and the mixed suspensions were incubated at 37°C. After recentrifugation, a significant quantity($P < .001$) of both enterotoxins was removed or neutralized. The reactivity of cholera-enterotoxin in supernatants from the brush border pellets was reflected in a mean loop response of 0.90 ± .06 ml/cm. The control solution of cholera-enterotoxin produced a mean loop response of 1.30 ± .05 ml/cm. The significant difference between these two responses was indicated by a 31% decrease in the reactivity of the enterotoxin.

The E. coli-LT containing supernatants from the brush border pellets

produced a mean loop response of 0.12 ± .04 ml/cm. The LT mean control response was 1.34 ± .06 ml/cm. These values reflected a 91% decrease in the reactivity of the enterotoxin. Apparently, equal quantities of brush borders were capable of decreasing the reactivity of LT much more efficiently than they were at decreasing the reactivity of cholera-enterotoxin (91% as compared to a 31% decrease).

E. coli-LT suspensions were incubated at 4°C. The mean supernatant response was 0.88 ± .06 ml/cm, and the mean control response was 1.38 ± .10 ml/cm. The difference between these responses was significant ($P < .001$). The percent decrease was 36% at 4°C as compared to a 91% decrease at 37°C.

At this point, it was not known if the decrease in reactivity of E. coli-LT was due to the enterotoxin reacting with the brush border membrane and/or brush border filaments (terminal web and microvillous filaments - Fig. 4). Therefore, brush border membranes were obtained which were free of filamentous material (Fig. 5). LT-supernatants from pellets of these membranes previously incubated at 37°C, produced a mean loop response of 0.33 ± .09 ml/cm. The mean control response was 1.38 ± .10 ml/cm. The difference between these responses was highly significant ($P < .001$). Pellets of these membranes were responsible for decreasing the reactivity of the supernatant by 76%. Thus, the filamentous material seems to have little effect on the reactivity of LT.

In order to determine if membrane receptors of cholera-enterotoxin were involved in reducing the reactivity of E. coli-LT, brush borders were previously incubated with cholera-enterotoxin and washed. LT was added to these pellets, and the resulting suspensions were incubated at

37°C. The mean supernatant response was 0.47 ± .08 ml/cm, and the mean control response was 1.45 ± .09 ml/cm. The mean response value of the supernatants represented a significant difference ($P < .001$). The decrease in the reactivity of the supernatant was 68%. Therefore, the receptors for the cholera-enterotoxin appear to be separate from those of LT.

E. coli-LT was added to pellets of brush borders that were treated previously with papain to determine if the decrease in reactivity of LT was dependent upon an intact glycocalyx. The mean supernatant and control responses were 0.51 ± .09 ml/cm and 1.57 ± .13 ml/cm respectively. These values represented a significant decrease (68%, $P < .001$) in the reactivity of LT. This indicated that the receptors or neutralizers were associated more with the membrane proper than the glycocalyx.

## Enterotoxin-Induced Alterations in Brush Border Chemistry

Table IV contains the results of the biochemical assays performed on brush borders from control animals (E. coli 711-nonenterotoxin-treated rabbits) and experimental animals [cholera-enterotoxin or E. coli 711 (155)-LT-treated rabbits]. All biochemical values were expressed as quantity per mg of brush border protein. Sucrase activity within control brush borders was 2.00 ± .20 units/mg protein. Brush borders from rabbits treated with cholera-enterotoxin and E. coli 711 (155)-LT contained 2.31 ± .13 and 2.33 ± .12 units of sucrase activity per mg of protein respectively. Although these values represented a 15% and 16% respective increase in sucrase activity over the control value,

TABLE IV

ENTEROTOXIN-INDUCED ALTERATIONS IN BRUSH BORDER CHEMISTRY[1]

| Biochemical Component of Brush Borders | *E. coli* 711-Non Enterotoxin Brush Borders (Control) | Cholera-Enterotoxin Brush Borders | *E. coli* 711 (155)-Brush Borders |
|---|---|---|---|
| Sucrase units[2]/mg prot. | 2.00 ±[3].20 | 2.31 + .13<br>15%↑[4]<br>P < .2[5] | 2.33 + .12<br>16%↑<br>P < .2 |
| Sialic Acid mμM/mg prot. | 21.1 ± .6 | 16.5 + 1.7<br>22%↓<br>P < .05 | 19.1 + 1.2<br>10%↓<br>P < .2 |
| Lipid Phosphorus μg/mg prot. | 4.87 ± .18 | 4.35 + .13<br>11%↓<br>P < .05 | 4.37 + .25<br>10%↓<br>P < .2 |
| Lipid Galactose μg/mg prot. | 50.3 ± 1.1 | 41.2 + 1.6<br>18%↓<br>P < .005 | 37.9 + 2.0<br>25%↓<br>P < .001 |
| Cholesterol μg/mg prot. | 53.6 ± 1.1 | 47.3 + 0.8<br>11%↓<br>P < .001 | 48.1 + 2.1<br>10%↓<br>P < .05 |
| Mean Loop Response | 0.07 ± .01 | 0.22 + .01<br>214%↑<br>P < .001 | 0.24 + .03<br>243%↑<br>P < .001 |

[1]The enterotoxin and control solutions were instilled into small intestinal loops of rabbits (one solution per animal); three hours later, brush borders were obtained from these loops and analyzed for chemical components. Each chemical value represents the mean result of 6 brush border analyses. Each response value represents the mean response of 24 loops (4 loops per rabbit).

[2]Micromoles of sucrase hydrolyzed per min. at 37°C and pH 6.0.

[3]± = SEM.

[4]% values = percent increase (↑) or decrease (↓) with respect to the control values.

[5]Student t test was used to determine if the % increase or decrease of any parameter was significant.

this increase was not statistically significant.

Brush borders from the control rabbits contained 21.1 ± .6 muM of sialic acid per mg of protein. Sialic acid content of brush borders treated with cholera-enterotoxin was 16.5 ± 1.7 muM per mg of protein which represented a significant decrease ($P < .05$) of 22% with respect to the control value. Sialic acid content of brush borders from rabbits treated with E. coli 711 (155)-LT was 19.1 ± 1.2 mUM per mg of protein. Even though this value represented a 10% decrease, this decrease was not statistically significant.

The lipid phosphorus content of brush borders from control rabbits was 4.87 ± .18 ug per mg of protein. For cholera-enterotoxin and E. coli 711 (155)-LT-treated brush borders, it was 4.35 ± .13 and 4.37 ± .25 ug per mg of protein respectively. The 11% decrease in the lipid phosphorus content of cholera-brush borders was significant ($P<.05$). The 10% decrease in the lipid phosphorus content of E. coli 711 (155)-brush borders was not statistically significant.

Brush borders from rabbits treated with E. coli 711-nonenterotoxin material (control brush borders) contained 50.3 ± 1.1 ug of lipid galactose per mg of protein. Cholera-brush borders contained 41.2 ± 1.6 ug of lipid galactose per mg of protein, and E. coli 711 (155)-brush borders contained 37.9 ± 2.0 ug of lipid galactose per mg of protein. These values represented a cholera-enterotoxin and E. coli 711 (155)-LT induced decrease in brush border lipid galactose of 18% ($P<.005$) and 25% ($P<.001$) respectively.

Brush borders from control rabbits contained 53.6 ± 1.1 ug of cholesterol per mg of protein. Cholesterol content of cholera-brush borders was 47.3 ± 0.8 ug per mg of protein, which represented a

significant ($P < .001$) 11% decrease. Cholesterol content of E. coli 711 (155)-brush borders was 48.1 ± 2.1 ug per mg of protein. This value reflected a significant ($P < .05$) 10% decrease in cholesterol content.

Before the brush borders were isolated from the treated rabbits, the loop response was determined (Table IV) to establish the presence of enterotoxin. The mean loop responses were as follows: 0.07 ± .01 ml/cm for E. coli 711-nonenterotoxin (control) loops, 0.22 ± .01 ml/cm for cholera-enterotoxin loops, and 0.24 ± .03 ml/cm for E. coli 711 (155)-LT loops. The responses of enterotoxin stimulated loops were significantly greater than the responses of the control loops indicating that enterotoxin was virtually absent from the control solutions and present in the experimental solutions.

## Enterotoxin-Treated Rabbits and Plasma Glucose Levels

Before the brush borders were obtained for chemical analysis, blood samples were collected at spaced time intervals from the rabbits following instillation of control and enterotoxin solutions.

Figure 6 displays the plasma glucose concentrations after the instillation of E. coli 711-nonenterotoxin (control) and E. coli 711 (155)-LT solutions. In rabbits treated with E. coli 711-nonenterotoxin material, the plasma glucose concentrations were 160 ± 7 mg% at zero minutes (blood sample collected just before instillation of solution into loops), 187 ± 10 mg% at 15 minutes, 188 ± 14 mg% at 30 minutes, 173 ± 12 mg% at 60 minutes, 173 ± 16 mg% at 90 minutes, 162 ± 2 mg% at 120 minutes, and 172 ± 4 mg% at 180 minutes. In rabbits treated with E. coli 711 (155)-LT, the plasma glucose concentrations were 161 ± 6 mg%

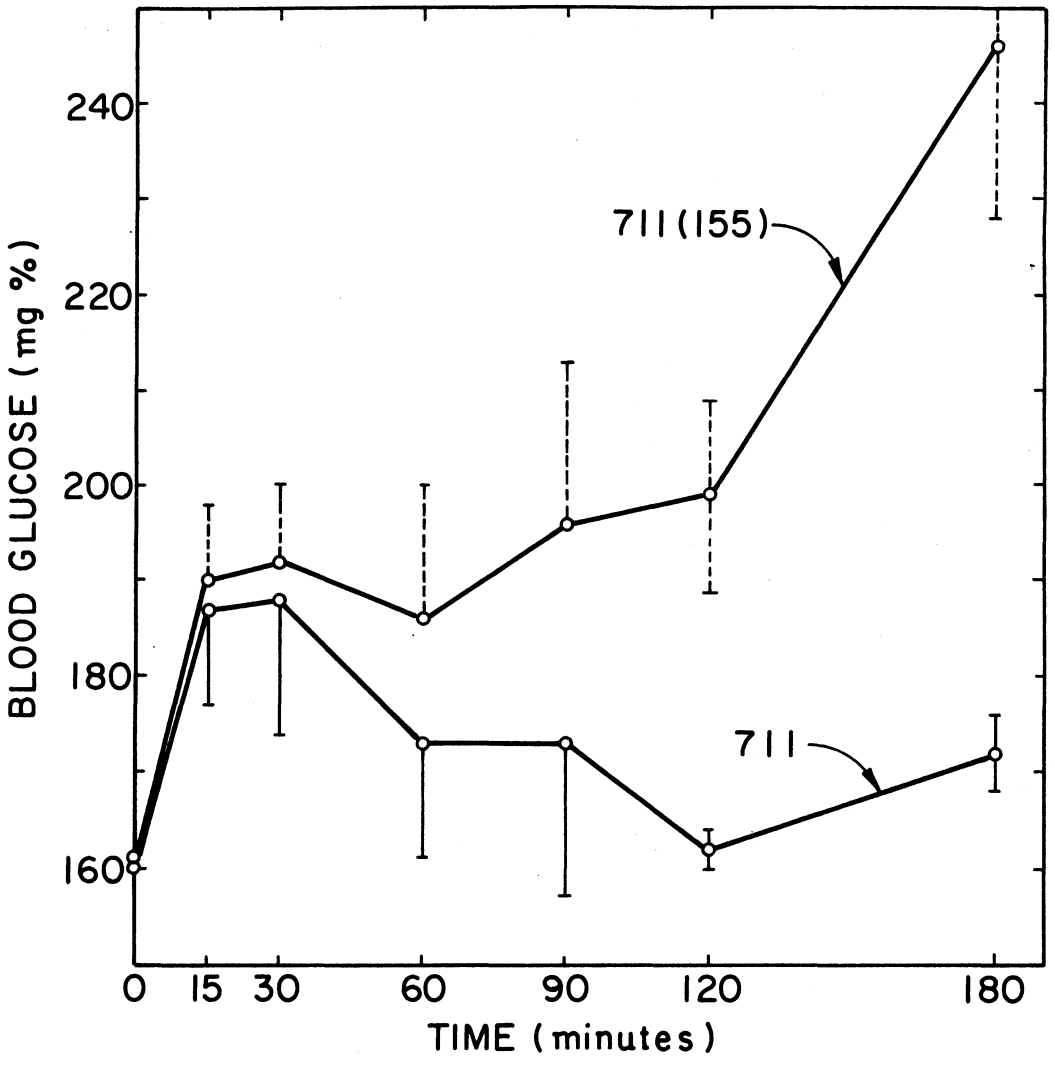


Figure 6. Plasma glucose concentrations are plotted against the time lapse after instillation of E. coli 711 (155)-LT and E. coli 711-nonenterotoxin solutions into rabbit small intestinal loops. Each point represents the mean result, ± SEM, of six determinations (six rabbits per mean).

at zero minutes, 190 ± 8 mg% at 15 minutes, 192 ± 8 mg% at 30 minutes, 186 ± 14 mg% at 60 minutes, 196 ± 17 mg% at 90 minutes, 199 ± 10 mg% at 120 minutes, and 246 ± 18 mg% at 180 minutes. Considering plasma glucose levels of rabbits treated with E. coli 711-nonenterotoxin material as control values, plasma glucose values of rabbits treated with E. coli 711 (155)-LT were significantly higher ($P < .005$) at 120 and 180 minutes after enterotoxin instillation.

Plasma glucose levels, after instillation of saline and cholera-enterotoxin, are portrayed in Figure 7. This figure also depicts the plasma glucose values of rabbits treated with E. coli 711-nonenterotoxin material. The concentrations of glucose in plasma from rabbits treated with cholera-enterotoxin were 161 ± 13 mg% at zero minutes, 225 ± 10 mg% at 15 minutes, 181 ± 20 mg% at 30 minutes, 237 ± 4 mg% at 60 minutes, 238 ± 13 mg% at 90 minutes, 278 ± 34 mg% at 120 minutes, and 356 ± 52 at 180 minutes. Considering plasma glucose concentrations of rabbits treated with E. coli 711-nonenterotoxin material as control values, plasma glucose values of rabbits treated with cholera-enterotoxin were significantly higher at 15 ($P < .05$), 60 ($P < .005$), 90 ($P < .025$), 120 ($P < .001$), and 180 ($P < .001$) minutes after enterotoxin instillation.

The concentrations of glucose in plasma from rabbits treated with saline were 158 ± 9 mg% at zero minutes, 162 ± 7 mg% at 15 minutes, 160 ± 7 mg% at 30 minutes, 151 ± 8 mg% at 60 minutes, 155 ± 12 mg% at 90 minutes, 174 ± 16 mg% at 120 minutes, and 167 ± 12 mg% at 180 minutes (Figure 7). E. coli 711-nonenterotoxin material, when compared with saline, was responsible for increasing the concentration of plasma glucose at 15 minutes ($P < .10$) and 30 minutes ($P < .10$).

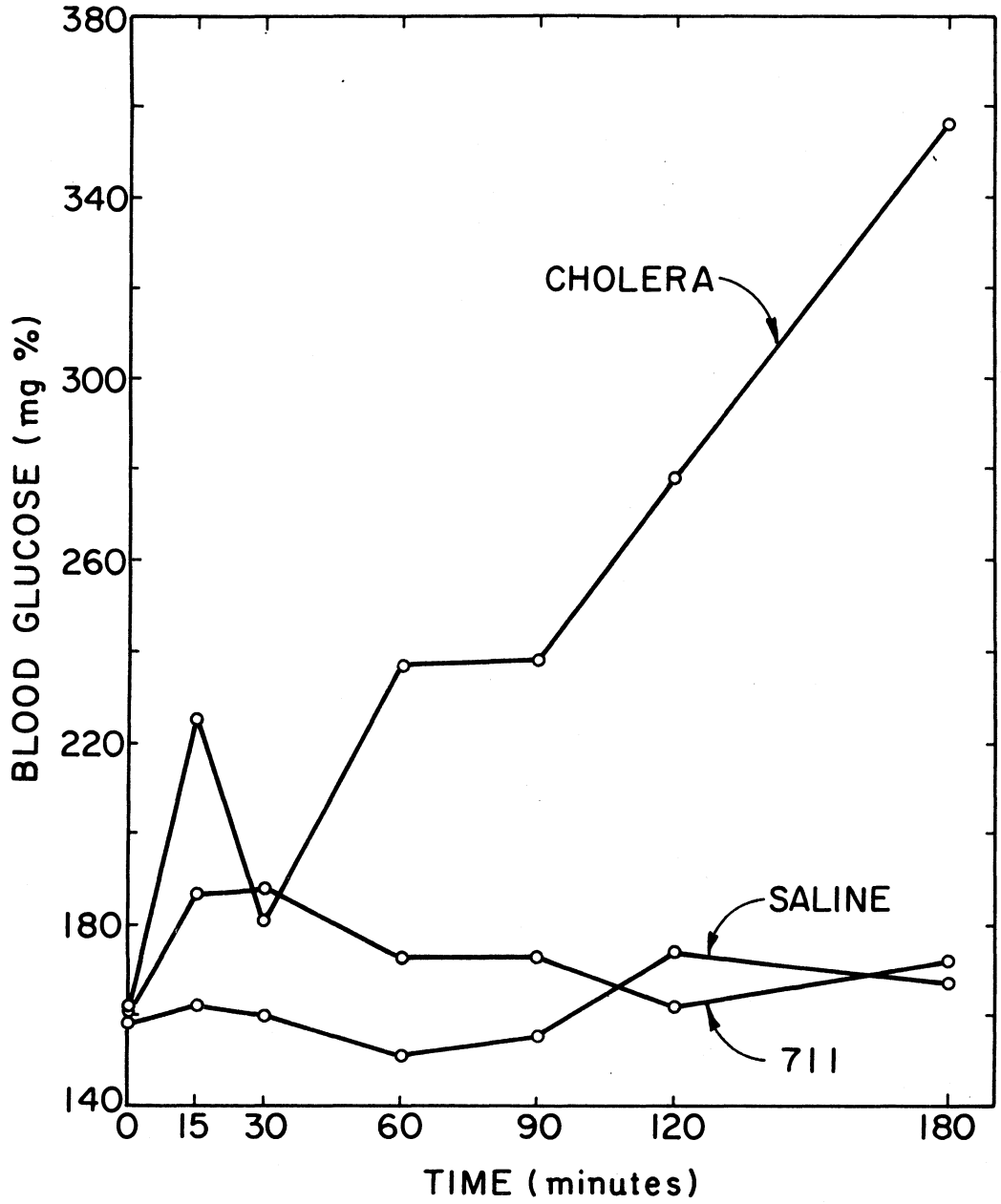


Figure 7. Plasma glucose concentrations are plotted against the time lapse after instillation of cholera-enterotoxin, saline, and E. coli 711-nonenterotoxin solutions into small intestinal loops of separate rabbits. Each point forming the cholera "curve" represents the mean result of 3 determinations (3 rabbits per mean). Each point forming the other "curves" represents the mean result of 6 determinations (6 rabbits per mean).

# CHAPTER V

# DISCUSSION

## Dose Response Curves

Burrows and Musteikis (39) obtained cholera-enterotoxin dose response curves which were typically sigmoid-shaped. The atypical sigmoid-shpaed dose response curves obtained in this work may have been due to the different assay procedure. The reason for modifying the assay procedure described by Burrows and Musteikis was to permit a more efficient use of animals.

## Membrane Purity and Yield

Forstner et al. (69) and Johnson (81) have demonstrated that the enzyme "sucrase" is located predominantly in the microvillous membrane. Therefore, sucrase activity was measured in the different mucosal prepartions in order to determine the purity and yield of the membranous component within these preparations. Rabbit brush borders (Table I) represented a mean sucrase purification of 13 (sucrase specific activity in brush borders divided by sucrase specific activity in mucosal homogenate). Although no comparable data exists for the rabbit, these results compare favorably with the 11-fold purification obtained in guinea pig (82), the 13.6-fold purification reported in the hamster (83), and the 17.4-fold purification in the rat (70).

Rabbit brush borders were used for the starting material for

membrane isolation. With respect to brush borders, the rabbit membrane preparations represented a further mean increment in specific sucrase activity of 41%; this value is comparable to the 44% value obtained by Hopfer et al. (70) using rats.

The total yield of membranes (as indicated by total sucrase activity) was somewhat reduced in the rabbit brush border and membrane preparations as compared to yields obtained by Hopfer et al. (70) using rats. The sucrase yields in rat brush border and membrane preparations were 38% and 26% respectively (70). The yields obtained from rabbits (Table I) were much lower than these values. Since much of the total sucrase activity was lost during the obtainment of a pure membrane pellet, two animals were routinely required to obtain one membrane preparation.

## Effects of Mucosal Components on Enterotoxin-Reactivity

Cholera-enterotoxin binds to specific receptors in microvillous membranes (13, 14, 35-38). With this knowledge in mind, incubation studies were carried out on cholera-enterotoxin suspensions of mucosal homogenate sediment (MHS) and brush borders for three reasons: (1) to substantiate that cholera-enterotoxin is removed from solution by mucosal components, (2) to determine if the intestinal loop assay system is sensitive enough to detect the removal of the enterotoxin from solution, and (3) to compare the degree of the removal of cholera-enterotoxin (as evidenced by loss of enterotoxin-reactivity in the supernatant) with the degree of removal of E. coli-LT.

Washed pellets of MHS removed or neutralized all of the cholera-

enterotoxin and E. coli-LT added to them (Table III). At this point, it was not known if the abolishment of supernatant-reactivity was due to binding of the enterotoxins to the MHS or due to an enzymatic neutralization of the enterotoxins.

To clarify this uncertainty, cholera-enterotoxin and E. coli-LT were suspended in mucosal homogenate cytosol (MHC) and incubated at 37°C in order to determine if soluble components from the mucosa were capable of neutralizing the enterotoxins. Also, there was the possibility that homogenization of the mucosa may have released a soluble enterotoxin receptor. Schneider et al. (84) have shown that ileal receptors which bind vitamin $B_{12}$ complex can be released from brush borders in soluble form. Also, Peterson (13) has reported that a soluble cholera-enterotoxin receptor is released by homogenization of brain tissue and large intestinal mucosa. The binding of the enterotoxins to such receptors could, in effect, neutralize the enterotoxins by preventing the enterotoxins from reacting with the mucosa in the assay system.

MHC did not significantly alter the reactivities of either cholera-enterotoxin or E. coli-LT (Table III). These results indicate that enterotoxin receptors were not released in soluble form in significant amounts and that soluble mucosal components such as enzymes, mucins, etc. have no effect on the enterotoxins' reactivities.

Since MHS was capable of diminishing the reactivity of enterotoxin-supernatant, the next step was to see if brush borders could do the same thing. Any binding or neutralization of enterotoxin by brush borders would be physiologically significant since it is the brush border with which the enterotoxins must first interact in causing the disease.

Brush border pellets containing equal quantities of microvillous membranes, measured by sucrase activity, were responsible for decreasing the reactivities of cholera-enterotoxin and LT-supernatant significantly (Table III). One may assume that the diminution of the reactivity of cholera-enterotoxin supernatant was due to the binding of the enterotoxin to the brush borders, since cholera-enterotoxin receptors are known to exist in brush borders (13, 14, 35-38). However, one cannot unequivocally say at this point that the decrease in the reactivity of LT-supernatant was due to the binding of E. coli-LT to the borders. Jones et al. (85) have reported that incubation of endotoxin at 37°C with isolated rat liver membranes causes a disaggregation of the endotoxin without a significant amount of the endotoxin being permanently bound to the membranes. The disaggregation of endotoxin by these membranes is believed to be an enzymatic process since disaggregation could be prevented by heat inactivation of the membranes, and was found to be pH, time, temperature (disaggregation did not occur at 4°C), and concentration dependent (85).

Considering the possibility that E. coli-LT may have been neutralized by disaggregation via brush border enzymes, brush borders and LT were incubated at 4°C. It was assumed that a 4°C, no enzymatic neutralization of LT would occur. The decrease in the reactivity of LT-supernatant was significant (Table III). If this decrease in reactivity was not due to neutralization of the LT by enzymatic activity or by soluble components from the brush borders (mucosal homogenate cytosol had no effect on LT-reactivity), it must have been due to the binding of the enterotoxin to the brush borders. Assuming that binding did occur, it is not known if this binding was "specific" or "nonspecific."

Heyningen and Mellanby (86) have interpreted specific binding as that which will occur at low temperature in the presence of a protective protein. Since gelatin was present in all incubated preparations, the binding of E. coli-LT to brush borders at 4°C could be specific. Peterson (14) has indicated that "protective protein" decreases non-specific electrostatic adsorption between brush borders and enterotoxin.

The reduction of the reactivity of LT-supernatant due to binding of the enterotoxin to brush borders at 4°C was somewhat less than the decrease in the reactivity of LT-supernatant from brush borders incubated at 37°C (36% decrease as compared to a 91% decrease). It is tempting to conclude that some enzymatic neutralization of the E. coli-LT occurred at 37°C. However, the difference in the magnitude of these reactivity-reductions may be explained on the basis of different kinetic energies of the two suspensions; the kinetic energy of the 4°-LT-brush border-suspension was much lower than that of the 37°C suspension. It is conceivable that essentially all of the LT may have become bound to the brush borders if the incubation time at 4°C had been lengthened.

Thus, it is apparent that E. coli-LT may bind to brush borders, and once bound, it is not removed by washing (all brush borders and other preparations were washed twice). Since brush borders are composed of microvillous membranes, terminal web, and microvillous filaments (Figure 4), is was necessary to ask the question: does LT bind to the microvillous membrane or to the filamentous material? LT was incubated at 37°C with purified brush border membranes in attempt to answer this question. Since the reduction in the reactivity of LT-supernatant from the membranes was comparable to that obtained with brush borders,

it is concluded that LT binds predominantly to the microvillous membrane.

The ability of choleragenoid to block the action of cholera-enterotoxin but not E. coli-LT (36, 37) suggests different receptor sites may exist for the enterotoxins. The existence of two different receptors is supported by the results obtained with preparations of brush borders pretreated with cholera-enterotoxin (Table III). If one assumes cholera receptors were "tied up" with cholera-enterotoxin, then the removal of LT from the supernatant was due to a different receptor site.

Holmgren et al. (35) have indicated that the specific cholera-enterotoxin receptor may reside in the glycocalyx of microvillous membranes as part of a ganglioside ($GM_1$). Since Forstner (72) and Louvard et al. (87) have shown that many of the glycocalyx components can be hydrolyzed with a dilute papain solution, brush borders were treated with papain solution to determine what effect, if any, the removal of the glycocalyx had on the binding of E. coli-LT.

Papain, a protease from papaya latex, hydrolyzes a number of peptide and ester bonds (88). Johnson (81, 89) has reported that papain digestion of brush borders removes surface 60 Å enzyme particles as well as the fuzzy coat (glycocalyx) without altering unit membrane structure. Forstner (72) has shown that papain digestion of labeled brush borders for five minutes releases 90% of the surface sucrase and almost one half of the brush border glycoprotein label, and that the released sucrase may itself be a glycoprotein.

The major components of the glycocalyx of cell membranes, as mentioned previously, are glycoproteins and glycolipids. Like the

phospholipids of membranes, the glycolipids possess polar heads and two long hydrocarbon chains, which are probably present in the outer layer of the lipid bilayer structure. Lehninger (90) has indicated that the polar head groups of gangliosides (a glycolipid) are composed of oligosaccharides containing one or more residues of sialic acid. The glycoproteins also contain sialic acid residues. The sialic acid residues of these glycolipids and glycoproteins are negatively charged at pH 7.0 (90).

In order to determine if papain treatment of rabbit brush borders were responsible for removing much of the glycocalyx, brush border sucrase, protein, and sialic acid content was determined before and after papain digestion. Since a large percent of total brush border sucrase, protein, and sialic acid was lost due to papain digestion (Table II), it was concluded that the glycocalyx was effectively removed.

Incubation of E. coli-LT with these papain-treated brush borders resulted in a significant reduction of the reactivity of the LT-supernatant. The magnitude of this reduction could not be compared with the reduction of the reactivity of LT-supernatant from untreated brush borders since different quantities of LT were added to these preparations (6mg of LT were added to treated brush borders and 8mg were added to untreated brush borders). However, it seems that the binding of a significant quantity of LT by brush borders is not dependent on an intact glycocalyx. Also, the removal of negative charges (loss of negatively charged sialic acid residues) from the brush borders did not prevent the binding of LT.

## Enterotoxin-Induced Alterations in Brush Border Chemistry

LT preparations from E. coli strain 711 (155) were used for determining the effects of LT on brush border chemistry because impurities present in the LT preparations could be adequately controlled. Non-enterotoxin material was obtained from E. coli strain 711 (plasmid free organism) in the same way as LT material was collected. Since E. coli strain 711 was identical to the enterotoxin plasmid induced strain 711 (155), the nonenterotoxin material contained the same impurities (endotoxin, bacterial enzymes, etc.) as the enterotoxin preparation, thus affording a near perfect control. The observed decreases in brush border lipid glactose and cholesterol: protein ratios were, therefore, due to the LT and not to the impurities present in the LT preparation.

Since the preparations of cholera-enterotoxin contained impurities, chemical assay values of brush borders from rabbits treated with these preparations were compared with the control values obtained from brush borders of rabbits treated with E. coli 711-nonenterotoxin material. The alterations in the biochemical composition of the brush borders due to cholera-enterotoxin were of the same nature as reported by Leitch (67). However, the magnitude of these alterations were different. The magnitude of the lipid:protein decreases were in the order of lipid galactose > lipid phosphorus $\geq$ cholesterol. The magnitude of the decreases obtained by Leitch were in the order of phospholipids $\geq$ cholesterol $\geq$ glycolipids. These result differences may be explained on the basis of animal age and experimental design; Leitch used rabbits

which were much older, jejunal brush borders were not used, and his "control brush borders" were from saline-treated rabbits.

It would seem then that cholera-enterotoxin and E. coli-LT can bind to microvillous membranes and in some way alter the biochemical composition of the membranes. Further research is required to disclose how these chemical alterations come about. Ideas of possible mechanisms may be obtained from the research results of others. Burger (91) has reported that tumor viruses can produce changes in the architecture of tissue culture cell membranes. Cumar et al. (92) have reported that in mouse cell cultures, a DNA virus may alter the cell membrane by blocking the synthesis of gangliosides. Membranes are dynamic structures; injected $C^{14}$-labeled glucosamine, a precursor of sialic acid, is quickly incorporated into glycoproteins and gangliosides of some membranes (90). This observation suggests that the turnover rate for some membrane components is very rapid and any block in the synthesis of one of these components would rapidly be reflected in the composition of the membrane.

Experimental evidence obtained by Abrahams and Holtzman (93) and Amsterdam et al. (94) indicates that the removal of plasma membrane with associated material during endocytosis can be compensated by incorporation of secretory vesicle membranes. Whaley et al. (95) have proposed that such "new" membrane segments may carry different specificities into the plasma membrane.

Masur et al. (96) have reported that in toad bladder, oxytocin and dibutyryl cAMP stimulates the fusion of granule membranes with the apical surface membrane of the mucosal epithelium. They proposed that the hormone and dibutyryl cAMP-induced fusion of membranes could result in an altered, more permeable, apical membrane. It must be determined

whether or not enterotoxin-induced cAMP is involved in the biochemical alteration of brush border membranes.

It is important to consider the significance of an enterotoxin-induced alteration in brush border chemistry; is this occurrence a trivial one, or is it some how involved in enterotoxin pathogenesis? E. coli-LT and cholera-enterotoxin were both responsible for decreasing brush border cholesterol:protein ratios significantly. Alteration of cholesterol content of membranes can have pronounced effects on membrane permeability and membrane transport systems. Bruckderfer et al. (97) have reported that the partial removal of cholesterol from the membranes of erythrocytes results in increased osmotic fragility and glycerol permeability. Conversly, Kross and Ostwald (98) reported that erythrocytes with an increased cholesterol to phospholipid ratio shows decreased permeability to nonelectrolytes and also decreased passive and active sodium transport. From work conducted by Oldfield and Chapman (99) on purified model membranes, it appears that the addition of cholesterol condenses the packing of the phospholipid molecules, producing a more solid-like membrane.

There is evidence that the activity of several membrane enzymes and transferases depends upon the presence of a fluid phospholipid environment (100). Kimelberg and Papahadjopoulos (100) have shown that the addition of equimolar amounts of cholesterol to phospholipid can inhibit significantly the activation of $Na^+ + K^+$ ATPase. It is conceivable that in the enterotoxin challenged gut, the inhibition of active sodium absorption and the inducement of active chloride secretion by increased intracellular cAMP levels (48) may be ultimately dependent upon an altered membrane structure.

Bennett et al. (101) have recently proposed that the reactivity of cholera-enterotoxin depends upon the fluidity of cell membranes. They have reported that membrane cholera-enterotoxin receptors ($Gm_1$ gangliosides) may be ubiquitous, and enterotoxin-reactivity may involve translational diffusion of the toxin-ganglioside complex with ultimate formation of stable complexes between the enterotoxin and adenyl cyclase. Increased membrane fluidity, due to an alteration in the biochemical composition of microvillous membranes, could enhance the enterotoxin disease process if the above proposition is true.

## Enterotoxin and Blood Glucose Levels

Cholera-enterotoxin and E. coli 711 (155)-LT, when instilled into rabbit small intestinal loops, significantly increased blood glucose concentration after 60 and 120 minutes, respectively (Figures 6 and 7). This significance was determined with respect to blood glucose concentration of rabbits treated similarly with nonenterotoxin material from E. coli 711. Thus, any nonspecific increase in blood glucose concentration due to endotoxin or other impurities present in the enterotoxin preparations were compensated. Using blood glucose levels of saline treated rabbits for comparison, it was apparent that endotoxin and/or other impurities present in the solutions of E. coli 711 (155)-LT and E. coli 711-nonenterotoxin material were responsible for increasing glucose concentration at 15 and 30 minutes after loop instillation (Figure 7). Truszcynski and Pilaszek (102) and Homma et al. (103) have reported that endotoxin is absorbed from the small intestine, and Thomas (104) has reported that endotoxin increases blood glucose concentration.

The observed increases in blood glucose due to cholera-enterotoxin

and E. coli-LT may have been due to several factors: (1) the enterotoxins or enterotoxin subunits might have been absorbed from the small intestine, (2) the enterotoxins may have caused the release of a "blood borne agent" from the intestinal mucosa which was glycogenolytic and/or lipolytic, and (3) the enterotoxins may have released a "blood borne agent" from the intestinal mucosa which stimulated the pancreas to release glucagon.

The possibility of enterotoxins being absorbed from the small intestine is supported by the work of Williams and Dohadwalla (105) who demonstrated, in rabbits, that parenterally administered cholera-enterotoxin was responsible for net secretion of fluids into the small intestinal lumen. They also showed that an uninfected ten day old rabbit developed choleric signs when cross circulated with blood from a littermate infected intraintestinally with V. cholerae, Inaba 569B (106). On the other hand, Pierce et al. (107) demonstrated that, in dogs, parenterally administered purified cholera-enterotoxin did not cause diarrhea, but blood glucose levels became elevated. Zieve et al. (108), working with rats, indicated that cholera-enterotoxin increased glycogenolysis within platelet lysates and liver homogenates. Kae et al. (109), using fluorescein labeled antibody stains, presumable demonstrated the absorption of cholera-enterotoxin by small intestinal epithelial cells. However, Peterson et al. (38), using immunohistochemical techniques and autoradiography with highly purified tritium labeled cholera-enterotoxin, could not demonstrate absorption of the enterotoxin by intestinal epithelial cells.

Conclusive evidence for enterotoxin induced release of a "blood borne factor" from the mucosa is lacking. However, the observation that

cholera-enterotoxin or E. coli-LT applied to one loop of the gut causes net secretion from another loop not challenged with enterotoxin (15, 16) supports the "blood borne agent" concept. Two likely candidates for such a "blood borne factor" are vasoactive intestinal peptide (VIP) and enteroglucagon. VIP has been isolated from the small intestine, and Barbezat and Grossman (20) have shown that ug quantities of this peptide infused intravenously into dogs, causes diarrhea. Said and Mutt (19) have reported that parenterally administered VIP causes hyperglycemia. Also, Gutman et al. (110) have shown that enteroglucagon can activate hepatic adenyl cyclase causing a subsequent rise in blood glucose concentration.

Further work must be done to determine if hyperglycemia due to enterotoxins is due to the release, from gut mucosa, of VIP and/or enteroglucagon. Also, an enterotoxin-induced release of an intestinal mucosal factor which may stimulate the release of glucagon from "A cells" in the pancreatic islets cannot be discounted at this time.

# CHAPTER VI

## SUMMARY AND CONCLUSIONS

This study has shown that the reactivities of cholera-enterotoxin and E. coli-LT-supernatants were significantly decreased by brush borders incubated at 37°C. The reduction of the reactivity of LT-supernatant from these brush borders was several magnitudes greater than the reduction of the reactivity of cholera-enterotoxin-supernatant (91% vs. 31% reduction). Since cholera-enterotoxin binds to specific receptors in microvillous membranes (35-38), the reduction of the reactivity of cholera-enterotoxin-supernatant was probably due to the binding of the enterotoxin to the brush borders.

The reduction of the reactivity of LT-supernatant was probably due to the E. coli-LT binding to the microvillous membranes of the brush borders. This statement is made only after considering the following results: soluble brush border components (mucosal homogenate cytosol) did not alter the reactivity of LT; the reactivity of LT-supernatant from brush borders incubated at 4°C was significantly reduced; and brush border filamentous material was not responsible for significantly decreasing the reactivity of LT-supernatant. Since the reduction of the reactivity of LT-supernatant was not due to soluble components from the brush borders, or due to enzymatic neutralization of the LT (this is assuming that no enzymatic neutralization of LT occurred with brush borders incubated at 4°C), it was apparently due to

the removal of the LT from solution by the brush borders. This binding of the LT to brush borders was largely a function of the microvillous membranes since the reduction of the reactivity of LT-supernatant was not dependent upon brush border filamentous material.

More work needs to be done to determine whether or not this binding of E. coli-LT to microvillous membranes is a specific phenomenon. Heyningen and Mellanby (86) have interpreted specific binding as that which will occur at low temperature in the presence of a protective protein. Since gelatin was present in all of the incubated preparations, the binding of LT to brush borders at 4°C would fit their definition of specific binding.

Heyningen, et al. (34) and Holmgran et al. (35) have reported that the binding of cholera-enterotoxin to brush border membranes is probably dependent upon a component (a ganglioside) of the glycocalyx. The binding of E. coli-LT is apparently not dependent upon an intact glycocalyx as evidenced by binding of the enterotoxin to brush borders which had been previously digested with a dilute papain solution. Further evidence for the involvement of different membrane receptors in the binding of these two enterotoxins was obtained from incubating brush borders with a "saturating" solution of cholera-enterotoxin. After incubation, these washed brush borders were capable of binding a significant amount of LT. If one assumes that cholera receptors were "tied up" with cholera-enterotoxin, then the binding of LT was due to a different binding site.

In vivo instillation of cholera-enterotoxin and E. coli-LT into rabbit small intestinal loops led to an alteration of the biochemical composition of the brush borders from these loops. Cholera-enterotoxin

was responsible for significantly decreasing brush border sialic acid, lipid phosphorus, lipid galactose, and cholesterol:protein ratios. LT significantly reduced brush border lipid galactose and cholesterol: protein ratios. It is not know, at present, if these biochemical alterations are involved in enterotoxin-reactivity. However, Bennett et al. (101) have proposed that the reactivity of cholera-enterotoxin may be dependent upon translational diffusion of the cholera-enterotoxin-receptor-complex in a fluid membrane. If their proposal is correct, reactivity of cholera-enterotoxin is a function of membrane fluidity. Although Oldfield et al. (99) have reported that decreasing the cholesterol content of membranes increases the fluidity of the membranes, increased fluidity of microvillous membranes due to enterotoxin-induced alteration in membrane composition must be determined.

Cholera-enterotoxin and E. coli-LT, when instilled into rabbit small intestinal loops, significantly increased blood glucose concentration after 60 and 120 minutes respectively. These increases in blood glucose levels may have been due to the absorption of the enterotoxins from the small intestine or to an enterotoxin-induced release of a "blood borne factor" from the intestinal mucosa.

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VITA

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