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The effect of Escherichia coli heat-stable enterotoxin on sodium efflux from mature and immature enterocytes isolated from swine jejunum

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The effect of <u>Escherichia coli</u> heat-stable

enterotoxin on sodium efflux from mature and immature

enterocytes isolated from swine jejunum

by

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INTRODUCTION

Enterotoxins from Escherichia coli have been shown to produce diarrhea in animals and man. Two types of toxin may be elaborated, a heat stable (ST) and a heat labile (LT) form.

LT is believed to act through adenylate cyclase, increasing cAMP levels and producing secretion similar to cholera toxin. The site of intestinal secretion in cholera toxin induced diarrhea is believed to be the crypts. Crypt cells are precursor cells to villus cells and are markedly different in morphology and enzyme characteristics. Cyclic AMP is believed to inhibit a coupled NaCl influx into the cell. In addition, an active secretory efflux is also presumed to be affected by CT and LT to explain the net secretory flux observed with these toxins in the intestine.

ST is considerably smaller than LT and is believed to act through guanylate cyclase and cGMP. It is believed to inhibit a coupled NaCl influx at the brush border of the intestinal cell. It is unknown whether there is any crypt involvement with ST.

These experiments were designed to test the hypothesis that E. coli ST enterotoxin has a differential effect on sodium transport in isolated crypt and villus cells.

LITERATURE REVIEW

The gastrointestinal (GI) tract is composed of a number of tubular
organs and supporting glands. It is responsible for the digestion and ab-
sorption of all nutrients such as proteins, carbohydrates, fats, minerals,
electrolytes, and water.

Although there is some absorption in the pharynx, esophagus, and
stomach, most absorption occurs in the large and small intestine, with the
small intestine having the greater role. The small intestine in man re-
ceives approximately eight liters of fluid a day, of which three liters are
from small intestinal secretion (Guyton, 1971). Bywater (1973) has reported
bi-directional fluxes of fluid in the small intestine of the one-to three-
week-old calf to be approximately six liters per hour. Obviously, any
disease of the gut that disturbs the normal processes of absorption and se-
cretion will have profound effects on the fluid and electrolyte balance of
the animal.

Functional Anatomy

The major points of anatomy of the small intestine can be found in
any anatomy or histology text. Only a few special points will be discussed
here. In general, all the tubular organs of the digestive tract have a
similar morphology, being composed of four main layers of tissue with some
variation in a particular layer depending on the organ.

The four layers are: tunica mucosa, tunica submucosa, tunica muscu-
laris, and tunica serosa. The tunica mucosa is adjacent to the lumen of
the intestine and is composed of a sheet of columnar epithelium overlying

a thin layer of connective tissue, the lamina propria. A thin layer of muscle, the muscularis mucosa, may be present.

The tunica submucosa is immediately adjacent to the mucosa. This is a strong tissue primarily composed of connective tissue, although some glandular tissue may be present.

The tunica muscularis, located between the submucosa and serosa, consists of smooth muscle necessary for the peristaltic and mixing movements facilitating digestion.

The outermost tissue of the tubular organs is the tunica serosa, an epithelial layer with some connective tissue in it. The serosa is a serous membrane that allows organ movement in the process of digestion. It also prevents the formation of adhesions between various organs in the peritoneal cavity, which would interfere with the normal function of these organs.

The small intestine has a number of anatomical features that increase the surface area of the small intestine, thereby increasing the absorptive capacity of this organ. The proximal intestine has circular folds known as plica circularis, or valvulae conniventes, in the relaxed state. A second feature is the projection of numerous finger-like villi from the mucosa. The villi are composed of an epithelium surrounding a core of the lamina propria containing blood and lymph vessels. A final increase in surface area is provided by minute foldings on the surface of each columnar epithelial cell. These microvilli are known collectively as the brush border. The brush border contains numerous enzymes enmeshed in the glycocalyx, a thin carbohydrate layer covering the microvilli.

The mucosa can be differentiated further into villi and crypts. The crypts are located at the base of the villi and are composed of a number of

cell types including Paneth, argentaffin, and precursor cells for the cells of the villi. Paneth cells and argentaffin cells are secretory in nature, but are not found in all species (Stinson and Calhoun, 1976, Trier, 1968). The primary cells of the crypts are undifferentiated cells which serve as precursors for the two main cell types of the villi, absorptive cells and goblet cells.

Trier (1963, 1968) reported morphological differences in crypt and villus cells. Crypt cells had sparse, poorly differentiated microvilli, little interdigitation of lateral plasma membranes, and were shorter than the tall columnar cells of the villus epithelium. As the cells migrated from crypt to villus, they abruptly differentiated into absorptive cells as indicated morphologically by development of microvilli, increased inter-digitation with neighboring cells, and an increase in cell height.

There is a continuous turnover of cells on the intestinal villus with absorptive cells sloughing from the tips of the villi and cells moving up from the crypt to the villus.

LeBlond and Messier (1958) labeled mitotic activity in the crypts with tritiated thymidine and followed the subsequent migration and loss of the labeled cells autoradiographically from the villus tips. The process took approximately three days in the mouse. In the human, normal migration time is three to five days (Trier, 1968). Imonde et al. (1969) reported further evidence of high mitotic activity in the crypts when they demonstrated thymidine kinase, an enzyme involved in deoxyribonucleic acid (DNA) syn-thesis, was restricted primarily to crypt cells.

Numerous enzymes develop in association with the brush border and

cytoplasm as the crypt cells mature in their migration. Nordstrom et al. (1968) used a microtome technique in acquiring a gradient of cells from villus to crypt and found the highest activities for disaccharidases, dipeptidases, and alkaline phosphatase in the villi and lowest activity in crypt cells. Similarly, Dahlquist and Nordstrom (1966) reported disaccharidase activity to be present in significant amounts only in the villi.

Weiser (1973) demonstrated an increasing gradient of activity for sucrase from crypt to villus, and also reported thymidine kinase activity was confined to the crypt cells. He labeled cells with tritiated thymidine and found that those cells initially taking up the labeled thymidine also had the highest thymidine kinase activity and were the basal crypt cells. He labeled glycoprotein precursors and found the highest activity in glycoproteins of the villus cells, specifically the plasma membranes of those cells. The results indicated that incorporation of these compounds was closely associated with membrane bound enzymes of the brush border. These changes were considered to be consistent with maturation of cells from crypt to villus. Further evidence of cellular differences in crypt and villus cells was a greater activity of sodium-potassium adenosine triphosphatasa (Na^+-/K^+-ATPase) in the villi of rat jejunum and ileum than in the crypts (Charney et al., 1974). This suggested an important difference in sodium transport between crypt and villus cells as (Na^+-/K^+)-ATPase plays an essential role in sodium transport.

Water and Electrolyte Movement

A key concept in intestinal fluid movement is that water movement, absorption or secretion, is a passive phenomenon secondary to solute move-

ment. Schultz and Curran (1968) reviewed the literature pertaining to water movement and concluded there was no indication of independent water transport in the absence of net solute transport. They restated the idea that there is a coupling between water and solute transport, primarily sodium and chloride, and that water movement can be explained by physical forces generated within the cell or tissue by active transport of solute. Three criteria must be satisfied for this linkage of water and solute:

1) Net water transport may take place in the absence of, or against, an osmotic pressure difference.

2) Water transport is dependent on the presence of active solute transport.

3) Under many conditions, the fluid transported is approximately isotonic with the solution bathing the mucosal surface of the tissue (Schultz and Curran, 1968).

Solute movement across membranes may occur by passive diffusion, facilitated diffusion, or active transport. Passive diffusion is movement down an electrochemical gradient without expending energy. The end point of passive diffusion is a steady-state equilibrium. Facilitated diffusion is transfer of a solute from a region of low concentration to high concentration without direct expenditure of energy. A transport molecule is presumed to be in operation in this case, as well as in active transport. Active transport is transport of a solute against an electrochemical gradient with the expenditure of energy. Solute absorption can conceivably follow any one or all three of these processes.

Curran and MacIntosh (1962) proposed a three compartment model that coupled active solute transport to water movement. In this model, shown in Figure I, two membranes or barriers separate three compartments in series. Membrane A is selectively permeable while Membrane B is extremely permeable to solute. In this model, solute is actively transported from compartment I to compartment II, which increases the osmotic pressure in compartment II. This in turn causes water movement from I to II, and if compartment II is constrained in size, an increase in hydrostatic pressure will cause fluid to move from II to III. The anatomical correlates to I, II, and III are the intracellular environment, the lateral space, and the capillary lumen. Membranes A and B correspond to the basolateral membranes of the cell and the basement membrane/capillary wall (Diamond and Bossert, 1967).

Sodium transport has been investigated extensively and is the best understood of all solute transport in the intestine. Studies with radioactive isotopes have revealed that movement of solutes such as sodium and chloride is marked by large unidirectional fluxes many times larger than the net fluxes observed (Visscher et al., 1944; Bywater, 1973). Any net movement of solute will be determined by differences in unidirectional flux from mucosa to serosa and from serosa to mucosa. These fluxes will be determined by the properties of the membrane itself, concentration of solute, temperature, pressure, and the electrical potential difference across the membrane (Schultz and Curran, 1970).

Sodium transport in the intestine is linked inextricably to the movement of other solutes. It has been known for many years that glucose added to the mucosal side of rabbit ileum in vitro will increase the rate

Figure 1. Model coupling active transport of solute to water movement
           (After Curran and MacIntosh, 1962)

        I.   Epithelial cell interior

       II.   Lateral space

      III.   Vascular lumen

        A.   Basolateral cell membrane

        B.   Basement membrane and endothelial cell membrane

A   B

I   II   III
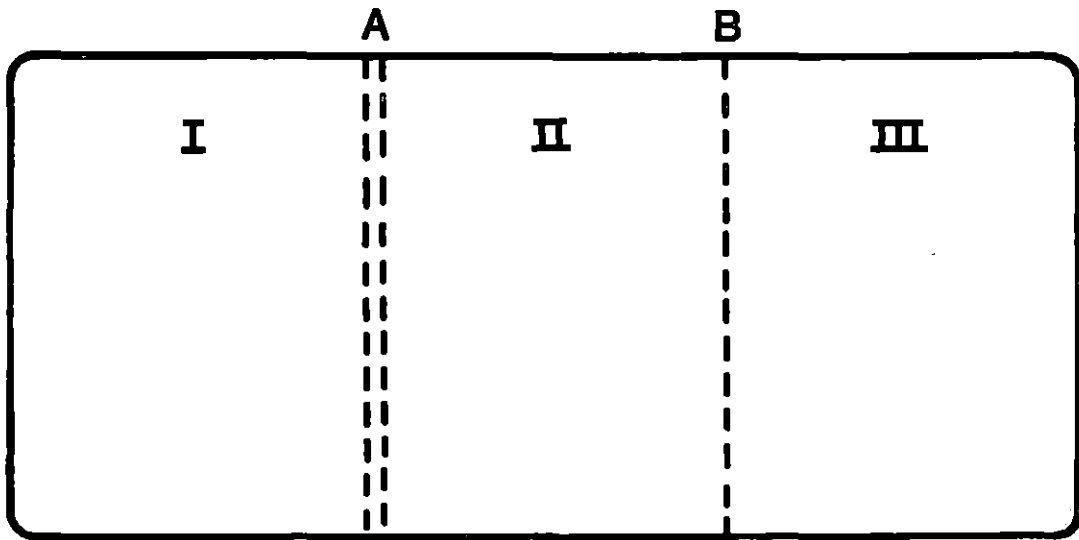
of sodium transport from mucosa to serosa as measured by the increase in short circuit current (SCC or I_{sc}) (Schultz and Zalusky, 1964b). Schultz and Zalusky (1964b) interpreted the increase in potential difference across isolated rabbit ileum when glucose was added to the mucosal solution to indicate increased sodium absorption. The increase in sodium absorption was demonstrated to be proportional to the sodium concentration in the bathing solution, and was a saturable function of the sugar concentration. This increase in sodium absorption into the cell and concomitant solute absorption has been demonstrated for amino acids, glucose, and non-metabolizable sugar in rabbit ileum in vitro (Peterson et al., 1970; Goldner et al., 1969). This phenomenon has also been observed with isolated human jejunum (Binder, 1974). Perfusion studies by Fordtran (1975) have demonstrated that glucose-stimulated sodium absorption holds true in the live animal.

This relationship of sodium and other solutes has been shown to be reciprocal. Other studies have shown the dependence of nonelectrolyte transport on sodium transport. Schultz et al. (1967) studied the influx of alanine from the mucosal solution using rabbit ileum in vitro and found a 75% reduction in influx in the absence of sodium. Goldner et al. (1969) observed an increase in transport of the non-metabolizable sugar 3-0-methyl glucose in the presence of sodium in rabbit ileum in vitro.

The mechanism for coupling of solute and sodium was first proposed by Crane (1962). He proposed a system whereby both glucose and sodium would enter the mucosal cell via a mobile carrier system located in the cell membrane.

Schultz and Zalusky (1964a, b) expanded Crane's idea, using a sodium gradient for driving sugar absorption. In its simplest form, this mechanism has two main features:

1) A carrier mediated process at the luminal border for coupling the entry of sodium and solute into the cell.

2) An energy dependent, ouabain sensitive mechanism at the basolateral membrane capable of actively transporting sodium out of the cell (Schultz and Zalusky 1964a, b).

In order for sodium to move from the lumen of the small intestine through the cell to the serosal side of the cell, it must overcome an electrochemical gradient. Sodium concentration is lower in the cell than the blood or intestinal lumen by approximately 100 millimoles (Schultz and Curran, 1970). In addition, the interior of the cell is negative with respect to the mucosal solution (by -36mV) and the serosal solution (by -39mV) (Schultz and Curran, 1970; Rose and Schultz, 1971). Sodium will move down an electrochemical gradient into the cell, but must be transported actively out of the cell. It was proposed that the sodium pump maintaining the low intracellular sodium concentration was the same as the (Na^+/K^+)-ATPase found in other cells. The electrochemical gradient for sodium would provide the energy for "uphill" movement of sugar into the cell (Schultz and Zalusky, 1964a, b).

In recent years, work with isolated basolateral and brush border mucosal membranes has added credibility to both coupled influx into the cell and the location of the Na^+/K^+)-pump on the basolateral membrane (Fujita

et al. 1972; Quigley and Gotterer, 1969). Using glucose as an example, it has been proposed that three different membrane movements are essential in the overall movement of solute. Each is a specific carrier or enzyme-mediated process and reflects the asymmetry of the brush border and baso-lateral membranes. These are:

1) Na^+-D-glucose co-transport across the brush border membrane via a Na^+-dependent carrier (Hopfer et al., 1973; Sigrist-Nelson et al., 1975; Hopfer et al., 1976).

2) D-glucose transport across the basolateral plasma membrane via a Na^+-independent carrier (Murer and Hopfer, 1974; Murer et al., 1974); and

3) Na^+ transport out of the cell across the basolateral membrane by (Na^+/K^+)-ATPase (Fujita et al., 1972).

Absorption of amino acids is believed to follow the scheme outlined above. Active transepithelial transport and intracellular accumulation of amino acids are abolished in the absence of sodium (Schultz and Curran, 1970). The influx of amino acids into the cell appears to be carrier mediated and an increase in amino acid influx is associated with an increase in sodium influx (Curran et al., 1967; Schultz and Curran, 1970).

Based primarily upon in vitro experiments, several models of solute transport have been proposed. The model currently most acceptable for transport across epithelia includes two routes for solute movement; a transcellular and a paracellular route (Ussing and Windhager, 1964). The paracellular route is referred to as the shunt pathway and is believed to correspond to the tight junctions and intercellular lateral spaces (Frömterand Diamond, 1972). The shunt appears to play a major role in movement of water and small electrolytes and non-electrolytes.

In work with isolated rabbit ileum, up to 85% of total tissue conductance is believed to be via this shunt (Frizzell and Schultz, 1972). This paracellular shunt has been demonstrated to be a part of many epithelia characterized by high hydraulic conductance, low resistance, and low transepithelial potential difference (Frömter and Diamond, 1972).

In addition to glucose-coupled sodium absorption and active absorption of sodium not directly coupled to other solutes, a third transcellular route of sodium transport has been proposed by Nellans et al. (1973). Working with rabbit ileum in vitro, they observed a 20% decrease in sodium and chloride influx across the brush border when either ion was missing from the mucosal bathing solution. When sodium was replaced by choline, chloride influx decreased. Similarly, replacement of chloride by sulfate and mannitol decreased sodium influx. Theophylline, a phosphodiesterase inhibitor, inhibited the coupled influx of sodium and chloride, and had no further effect if either ion was absent other than the 20% decrease noted originally from the absence of sodium or chloride. This "neutral coupled NaCl absorption" is present in mammalian gall bladder (Frizzell et al., 1975) and flounder small intestine as well (Field et al., 1978b; Frizzell et al., 1979a, b). It may account for a high percentage of chloride influx into the cell on a daily basis (Schultz, 1980).

Electrolyte Movement in Other Tissues
and Models of Absorption and Secretion

Recent work on epithelial tissues other than mammalian intestine has broadened our understanding of the mechanisms involved in intestinal absorption and secretion. Frizzell et al. (1976) demonstrated that the

diuretic amiloride abolished sodium absorption and Isc in rabbit colon _in vitro_ when placed in the mucosal bathing solution. Chloride transport was not affected by this agent, nor was the residual ion flux that is believed to be HCO_3^-. Ion replacement studies and ouabain studies further demonstrated that sodium was solely responsible for Isc. Cyclic AMP (cAMP) stimulated electrogenic chloride secretion, abolished HCO_3 secretion, but did not change the rate of sodium absorption. These results were consistent with a $Cl^- - HCO_3^-$ exchange mechanism independent of sodium.

Further work with the rabbit colon has implicated the possible role of calcium in secretory diarrhea. Addition of the calcium ionophore A23187 to the mucosal surface of rabbit colon _in vitro_ reversed active Cl^- absorption to active secretion without changing active Na^+ absorption (Frizzell, 1977). These results were similar to those observed previously in rabbit colon in response to cAMP (Frizzell _et al._, 1976). The effect of A23187 was abolished when calcium concentration was reduced to $10^{-6}M$, but the response to cAMP was not affected by the low calcium concentration.

The ionophore had no effect on cAMP levels in the colon. Cyclic AMP addition to colonic strips preloaded with ^{45}Ca caused an increase in calcium efflux. These results suggested that an increase in intracellular calcium concentration stimulates colonic secretion and that the effect of cAMP may be due partly to intracellular release of calcium.

Bolton and Field (1977) studied the effect of A23187 in isolated rabbit ileal mucosa. Their results were similar to those reports for rabbit colon (Frizzell, 1977). Net secretion of chloride was observed due to both an increase in serosa to mucosa chloride flux and a decrease

in mucosa to serosa flux. There was also an increase in Isc and resistance. Addition of the calcium ionophore had no effect on cAMP levels and removal of calcium from the serosal bathing solution decreased the effects of A23187 but not theophylline. The authors suggested that A23187 elicits secretion by increasing calcium influx into the epithelial cell.

Bolton and Field (1977) also observed that those secretagogues that increase cAMP (vasoactive intestinal peptide, prostaglandin E) did not require external calcium to exhibit a full electrical response in rabbit ileum in vitro, while secretagogues that had no effect on cAMP levels did require extracellular calcium (carbamyl choline, serotonin).

A number of experiments using marine teleost intestine have defined further the coupling of Na^+ and Cl^- transport mechanisms. Field et al. (1978b) reported that net Cl^- flux was almost three times that of net Na^+ flux in in vitro short circuited intestinal mucosa of the winter flounder, Pseudopleuronectes americanus. Studies using Na^+-free and Cl^--free solutions abolished net Cl^- flux and net Na^+ flux respectively. Ouabain added to the serosal medium abolished NaCl transport and potential difference. They interpreted these results to be evidence of a cation selective paracellular pathway made evident under Isc conditions. The pathway selectively permits a back flux of Na^+, thus increasing the apparent Cl^- flux under Isc.

Duffey et al. (1979) measured intracellular Cl^- activities and active Cl^- absorption in the winter flounder and determined that Cl^- accumulated within the cell and that this accumulation was coupled to the entry of Na^+ down its electrochemical gradient. Chloride inside the cell was three

times that predicted by the Nernst equation for passive distribution, while in the absence of Na^+, Cl^- did not differ from the predicted passive diffusion values.

Frizzell et al. (1979b) reported a coupled NaCl influx process at the brush border of the winter flounder. Ion replacement studies demonstrated a mutual requirement for Na^+ and Cl^- as indicated by a decrease in Isc and decrease influx of the opposite ion when either was replaced. Furosemide added to the mucosal solution inhibited the Isc as well as Na^+ and Cl^- influx under control conditions but not when either ion was missing. Reduction of Na^+ or Cl^- influx by furosemide or ion replacement was approximately equal, suggesting a coupled one-for-one influx mechanism from mucosa to serosa which was consistent with inhibiting a coupled influx. Dibutryl cAMP also inhibited the coupled influx similar to its effect in other epithelia (Frizzell et al., 1975; Nellans et al., 1973).

Frizzell et al. (1976) have presented a table, reproduced below, which correlates the relation between coupled NaCl transport mechanisms and the effects of cAMP on various epithelia.

The results listed demonstrate the range of effects due to cAMP in tissues with and without a coupled NaCl influx. Rabbit gall bladder has a neutral coupled NaCl influx mechanism at the mucosal border that is inhibited by cAMP, leading to an inhibition of neutral NaCl absorption. Chloride secretion is not observed in this epithelium (Frizzell et al., 1975).

In contrast, rabbit colon, frog cornea, and seminal vesicles do secrete chloride in response to cAMP, but there is no evidence for coupling of Na^+ and Cl^- absorption, nor is Na^+ absorption affected by cAMP (Zadunaisky et al., 1973; Frizzell et al., 1976).

Table 1. Effects of cAMP on Na^+ and Cl^- movement in epithelia

Epithelia-	Rabbit Gall Bladder	Rabbit Ileum	Rat Colon	Rabbit Colon	Cornea	Seminal Vesicle
Increase Isc	0	+	+	+	+	+
Cl^- secretion elicited	0	+	+	+	+	0
Na^+ absorption decreased	+	+	+	0	0	0
Presence of coupled NaCl transport	+	+	+	0	0	0

Rabbit ileum and rat colon lie between these two groups, sharing characteristics with both. In rabbit ileum, a major part of active Na^+ absorption is coupled to the simultaneous absorption of Cl^- via a neutral influx mechanism at the mucosal border membrane which is inhibited by cAMP (Nellans et al., 1973; Nellans et al., 1974). Binder and Rawlins (1973) have presented evidence for coupled Na^+ and Cl^- absorption in the rat colon. In these tissues, cyclic AMP abolishes Na^+ absorption and promotes Cl^- secretion. Cyclic AMP abolishes the neutral coupled influx of Na^+ and Cl^- (Nellans et al., 1974; Field, 1971b).

Two characteristics stand out. An increase in Isc is associated with Cl$^-$ secretion, and Na$^+$ absorption is only affected in those tissues with a neutral, coupled NaCl influx process.

Field (1978a) and Frizzell et al. (1979a) have proposed a new model of intestinal secretion to explain the observations in mammalian ileum, colon, gall bladder, and teleost intestine (Figure 2).

The primary feature of the model is a coupled NaCl influx process at the serosal border. Frizzell et al. (1980) reported that addition of furosemide to or removal of Na from the serosal bathing solution of rabbit colon in vitro abolished active Cl secretion. Similar results in ion replacement studies have been reported in vitro in rabbit ileum (Nellans et al., 1974; Powell et al., 1974). Furosemide has been shown to inhibit a coupled absorption process at the mucosal border of flounder small intestine (Frizzell et al., 1979b).

Other characteristics of the model are that chloride secretion is dependent on Na$^+$ in the serosal solution and is inhibited by ouabain in the serosal solution. Evidence for this has come from studies in rabbit ileum and colon in vitro (Nellans et al., 1974; Frizzell et al., 1980). The above characteristics are similar to those found in other secretory epithelia including shark rectal gland (Silva et al., 1977), mammalian salivary gland (Young, 1973), and frog cornea (Beitch et al., 1974).

In the model proposed above, cAMP or calcium would mediate secretion by changing the permeability properties of the luminal membrane for chloride. Chloride would normally accumulate in the cell above its electro-

Figure 2. Model of intestinal absorption and secretion (after
Field et al, 1978a and Frizzell et al., 1979a)

◄━━WWW Inhibition

◄━━━ Stimulation

ABSORPTIVE CELL
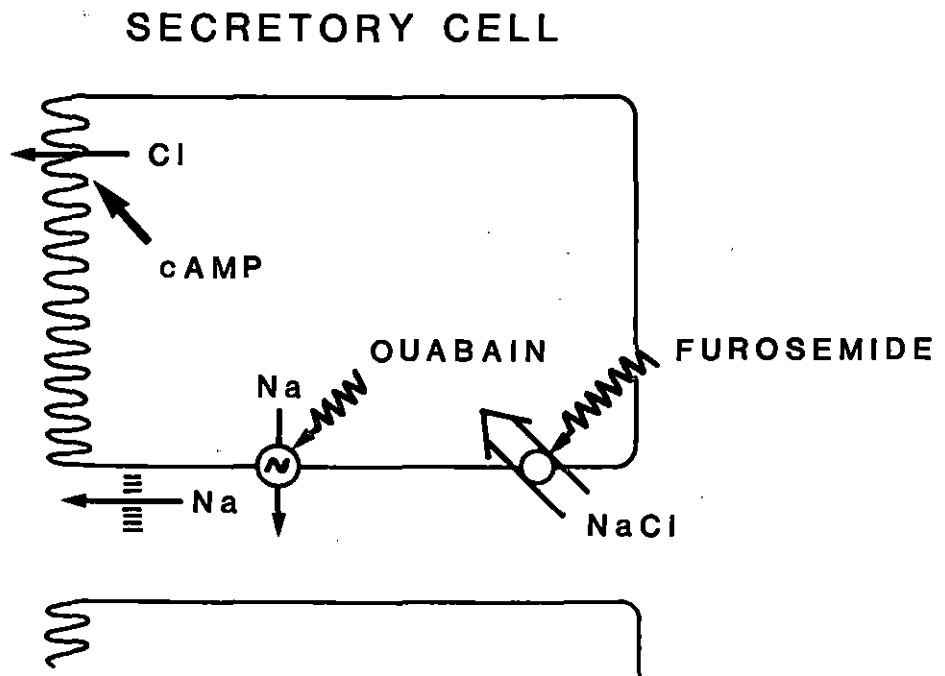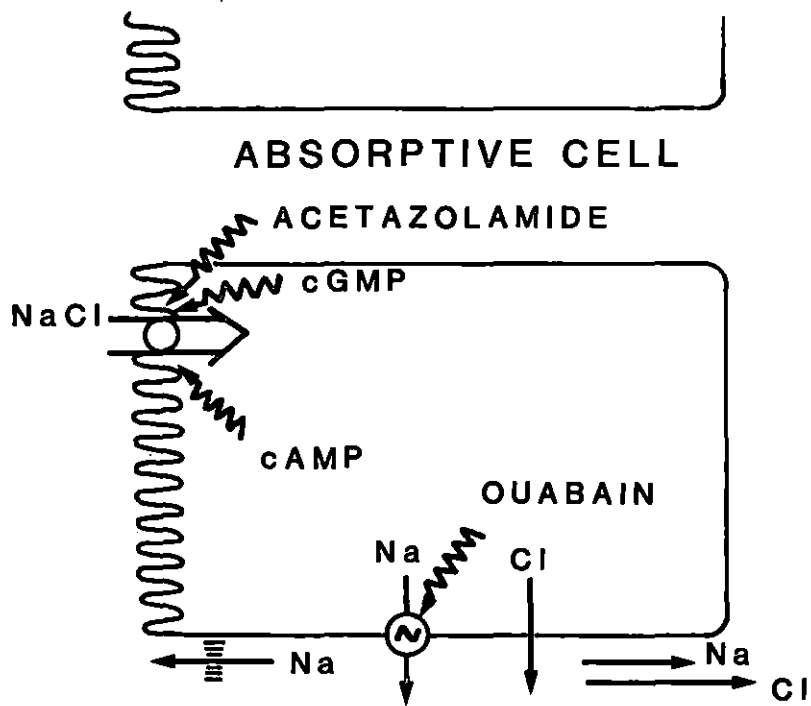
SECRETORY CELL

chemical equilibrium through coupling to sodium entry at the basolateral

border. In the resting secretory cell, chloride conductance across the

luminal membrane would be limited with most chloride recycling to the

serosal medium. A secretory stimulus causing transepithelial chloride

secretion would increase chloride permeability or conductance at the luminal

membrane (Frizzell et al., 1979a; Field, 1978a). Klyce and Wong (1977)

have reported that cAMP increases the luminal chloride conductance in frog

cornea. Similar results have been reported linking changes in chloride

permeability in insect salivary gland in response to intracellular calcium

increase (Berridge et al., 1975). An increase in electrical conductance

accompanying rabbit colonic secretion in vitro has been noted, a change

which is consistent with a permeability change (Frizzell et al., 1976).

Field (1978b) has stated two requirements for this model to be

viable: 1) There must be a quantitatively coupled NaCl influx process at

the basolateral membrane. 2) Chloride must accumulate in the secretory

cell above its electrochemical equilibrium and the uphill and downhill sites

for chloride movement must be at the basolateral and luminal membrane, re-

spectively.

In the small intestine, there are crypt cells and villus cells. Crypt

cells have been shown to be precursor cells of villus cells, with increasing

complexity of enzymes and structural characteristics as the cells mature to

villus cells. The location of these cells may correlate with a secretory

function or absorptive function. Schultz (1980) has pointed out that these

cell types do not have to be exclusive in function. It is possible that

villus cells secrete and crypt cells absorb, depending on appropriate

stimuli. The disruption of function in these separate populations of cells in intestinal disease has not been examined closely.

The mammalian small intestine has both paracellular or shunt pathways and transcellular pathways for solute movement. Any net absorption or secretion of fluid or electrolyte depends on the relative influx and efflux of solute across the mucosal border. A small derangement in solute movement may have relatively dramatic effects in net absorption or secretion. The etiologic agents which bring about intestinal dysfunction are numerous but the main mechanisms involved in diarrhea are limited.

Pathogenesis of Diarrhea

Moon (1978) has listed four mechanisms implicated in the pathogenesis of diarrhea. These are: 1) hypermotility, 2) increased permeability, 3) malabsorption, and 4) hypersecretion.

Hypermotility is discounted as having any primary function in diarrheal disease. If hypermotility is seen it is secondary to the increased volume of intestinal contents which impairs segmentation.

Increased permeability is manifested by changes in the membranes of the small intestine, permitting proteins and other large molecules to pass freely from blood to lumen and vice versa. If the change in permeability is not large, only small molecules can move freely, resulting in transudation into the lumen. If the volume is large enough and the absorptive capacity of the intestine is overwhelmed, diarrhea can occur (Loehry et al., 1970). As permeability increases, pore size increases permitting larger molecules, such as proteins, to pass into the lumen, resulting in exudation.

Hemorrhagic exudates are seen when the intestine is severely inflamed or necrotic, resulting in pores large enough to pass erythrocytes.

Malabsorption covers a wide range of disorders and might be better termed maldigestion-malabsorption. In many cases, the absorptive capacity of the gut is unimpaired, but digestive failure has occurred. If food stuff cannot be digested, products cannot be absorbed. An example of this is lactase deficiency. Young animals have a high concentration of lactase in their intestines while adults do not. If an animal deficient in lactase ingests milk, the lactose sugar cannot be hydrolyzed to glucose and galactose and absorbed. The excessive lactose serves as an excellent media for bacteria, which produce various short chain fatty acids and gases. These products plus the undigested lactose serve to increase the osmolarity of the lumen contents, drawing water into the intestine and causing a loose, watery diarrhea.

A similar pathogenesis is noted in pancreatic insufficiency. The deficit of amylase as well as lipase, may lead to a diarrhea and/or steatorrhea. In both examples, diarrhea occurs because the intestine cannot digest the contents.

A second type of malabsorption occurs when the disorder is in the absorptive capability, as opposed to the digestive capability of the intestine. In certain viral diseases of the small intestine, there is destruction of the mucosal epithelium, loss of villus structure, and a decrease in the absorptive surface of the intestine. Transmissible gastroenteritis (TGE) of swine is such a disease. The virus multiplies in and destroys the villus epithelium, resulting in blunting of the villi. In

addition, the villus cells are replaced by relatively undifferentiated crypt-like cells that have less digestive and transport ability than normal villus cells. This results in decreased digestive and absorptive ability of the intestine. The two dysfunctions combine to produce an osmotic diarrhea. The undigested and fermented products have a high osmotic pressure that draws water into the lumen of the intestine, overwhelming the colon's capacity to absorb fluid (Hooper and Haelterman, 1969; Thake et al., 1973; Kerzner et al., 1977).

Hypersecretion is the fourth mechanism of diarrhea, defined by Moon (1978) as "net intestinal efflux of fluid and electrolyte, occurring independent of changes in permeability, absorptive capacity, or exogeneously generated osmotic gradients". Hypersecretion occurs across a histologically normal mucosa. This mechanism characterizes the diarrhea produced by the enterotoxins of Vibrio cholerae and Escherichia coli. The diarrheal disease caused by Vibrio cholerae has been extensively studied and serves as the classic example of secretory diarrhea.

Cholera Toxin

The disease, cholera, is caused by colonization of the intestine by the organism Vibrio cholerae (Gorbach et al., 1970). The vibrios do not invade the mucosa but produce an enterotoxin that causes a massive fluid loss through the intestine (De and Chatterje, 1953; Watten et al., 1959; Sack and Carpenter, 1969). The disease symptoms of dehydration and collapse are attributed solely to the fluid loss produced by the enterotoxin.

The mechanism of fluid loss has been studied extensively. Exudation, or an increased pore size of the intestine permitting protein-containing fluid to pass from the blood into the intestine, does not occur. Gangarosa et al. (1960) found normal intestinal mucosa on biopsy in clinical cholera. In addition the protein content of the intestinal fluid is too low for an exudative hypothesis (Norris and Majno, 1968). These observations have also been reported with experimentally infected animals (Elliot et al., 1970; Pierce et al., 1971).

Increased filtration into the intestine, either due to increased permeability or an increase in mesenteric hydrostatic pressure, also has been refuted. Studies using radioactive labeled substances have demonstrated that permeability changes are not a cause of fluid loss in cholera. Gordon et al. (1972) injected radiolabeled mannitol intravenously and observed no difference in stool radioactivity between cholera and normal patients. Other investigators used unequal-sized pairs of labeled substances to measure the ratio of intestinal to plasma radioactivity following intravenous injection. No differences were observed between controls, osmotic induced diarrhea, or cholera induced diarrhea (Rohde and Chen, 1972; Scherer et al., 1974).

Carpenter et al. (1969) reported no change in fluid loss in experimental cholera, even when blood flow through the superior mesenteric artery was reduced to 30% of normal. In addition, the osmotic pressure of the blood increases as fluid is lost with cholera, which orients the osmotic pressure in the wrong direction for more fluid loss into the intestine

(Watten et al., 1959). In clinical cases, fluid is still lost into the intestine, even though the pulse may be undetectable (Hendrix, 1975).

The question of impaired absorption in cholera is still somewhat unresolved with some workers reporting no change in unidirectional flux of sodium from mucosa to serosa, in vivo (Banwell et al., 1968; Iber et al., 1969; Love, 1969), while other workers have reported that sodium absorption is decreased (Swallow et al., 1968; Phillips, 1968).

Glucose and glucose-enhanced sodium absorption is normal in cholera (Iber et al., 1969; Pierce et al., 1968; Serebro et al., 1968; Hirschorn et al., 1968). This phenomenon has been taken advantage of in the therapy of clinical cholera by orally replacing fluids and electrolytes lost in diarrhea with glucose and electrolyte solutions.

Work done in vitro has shown that cholera toxin (CT) abolishes active transport of sodium into the mucosal cell (Field et al., 1972; Field, 1971a) and decreases net sodium flux to zero in isolated rabbit ileal mucosa. In normal ileum, there is a net movement of sodium and chloride from mucosa to serosa. Field et al. (1971) concluded earlier that there is an active process for sodium and chloride absorption and an active secretory process for bicarbonate into the lumen in normal gut epithelium in vitro.

Studies using isolated rabbit ileum have been useful in defining the mechanism of cholera toxin induced secretion. Field et al. (1972) observed that CT added to the mucosal side of stripped rabbit epithelium in vitro reduced net sodium flux to zero, reversed net chloride flux to net secretion, and did not change the residual ion flux, i.e., bicarbonate secretion. Glucose added to the mucosal side produced net sodium absorption, but did not alter the CT induced secretory fluxes. Powell et al. (1973) used a

similar technique but reported a reversal of sodium flux as well as chloride flux to net secretion and an increased residual flux.

Powell et al. (1973) and Field et al. (1972) agreed that CT was only active at the mucosal surface and that CT increased potential difference (PD) and short circuit current (Isc). Powell et al. (1973) postulated that CT stimulated a neutral NaCl or $NaHCO_3$ transport from serosa to mucosa without affecting electrogenic, or current producing, sodium absorption. Field et al. (1972) concluded that the increase in Isc was due to electrogenic anion secretion and inhibition of electrogenic sodium absorption. Powell et al. (1973) attributed the increase in Isc to the net change in electrolyte movement caused by stimulation of a neutral NaCl or $NaHCO_3$ secretory mechanism with unaltered electrogenic sodium absorption.

It is difficult to reconcile in vitro findings with those of in vivo findings. Hendrix (1975) points out that what is clearly established is that CT stimulates transport of anion into the lumen against an electro-chemical gradient.

One of the outstanding characteristics of cholera enterotoxin is the apparent mediator role played by cAMP. Work with isolated rabbit ileal mucosa has shown that cAMP alters transport of electrolyte identical to cholera (Field, 1971b; Field et al., 1972). Field (1971b) observed that theophylline and cAMP abolished sodium absorption and elicited chloride chloride secretion across short circuited rabbit ileal mucosa largely through simultaneous reduction of unidirectional mucosa to serosa fluxes of sodium and chloride. Exposure of intestinal mucosa to CT results in an increase in adenylate cyclase activity and an increase in the intracellular concentration of cAMP (Kimberg et al., 1971; Sharp and Hynie, 1971;

Guerrant et al., 1972). The magnitude of the secretory response to CT parallels the increase in adenylate cyclase activity produced by cholera toxin (Guerrant et al., 1972). In addition, the enterotoxin's effect on secretory activity lasts at least 24 hours and possibly leads to activation of adenyl cyclase for the life of the cell. The effect of CT on adenyl cyclase has been demonstrated in a number of cell types and species (Greenough et al., 1970a; Donta et al., 1973; Gill and King, 1975).

It has been suggested that prostaglandins serve as intermediates between the toxin and cAMP mediated secretion (Pierce et al., 1971). In support of this hypothesis, aspirin and indomethacin have been shown to inhibit secretion induced by cholera enterotoxin. (Finch and Katz, 1972; Jacoby and Marshall, 1972). Both of these agents are known to inhibit prostaglandin synthesis (Vane, 1971). Bourne (1973), however, was unable to inhibit CT-induced cAMP elevation in leukocytes using the same drugs. Kimberg et al. (1974) found that CT and prostaglandin effects were additive in intestinal mucosa. They suggested that cAMP is elevated by different pathways by the two agents. Hamilton et al. (1978a) also found only an additive effect of theophylline and CT on cAMP levels. They reasoned that because theophylline is a phosphodiesterase inhibitor, there should be a synergistic effect on cAMP levels with CT as CT activates adenylate cyclase.

Cyclic AMP inhibits a coupled uptake of NaCl across the brush border, which decreases active absorption of sodium and chloride. This action of cAMP seems to be specific and does not affect all other sodium absorptive processes, e.g., glucose coupled sodium transport (Nellans et al., 1973). This antiabsorptive effect of cAMP does not explain net secretion. Either

an underlying secretory process is unmasked or cAMP has a second effect of directly stimulating active secretion (Field, 1979). Desjeux et al. (1974) reported that sodium movement from serosa to mucosa is by two pathways: diffusion through the shunt and the transcellular pathway. Theophylline stimulated the transcellular process of sodium secretion and this effect was blocked by metabolic inhibitors or the absence of Cl^- and HCO_3^-. These results were consistent with a neutral secretory transport of sodium and an anion. Nellans et al. (1975) reported that neutral coupled sodium chloride transport was inhibited by acetazolamide in rabbit ileum in vitro, but secretion was not evident until theophylline was added. Acetazolamide was believed to block coupled NaCl absorption by a receptor phenomenon as it did not increase cAMP concentration.

The location of secretion has been a matter of interest for a number of years. A considerable amount of circumstantial evidence points to the crypts as the location of cAMP-mediated secretion. Elliot et al. (1970) has reported that the crypt lumens of dog intestine are dilated in CT-induced secretion. DeJonge (1975) demonstrated that a brief exposure of rat small intestine to CT increased villus cell cAMP levels, but not crypt cell levels of cAMP. This brief exposure inhibited absorption but did not induce secretion. Prolonged exposure to CT stimulated adenylate cyclase in both crypt and villus cells and produced actual secretion. Selective damage to villus cells by hyperosmotic solution does not inhibit cholera-induced secretion (Roggin et al., 1972). The diuretics, furosemide and ethacrynic acid, which are believed to inhibit coupled NaCl transport across basolateral membranes of Cl^- secreting cells, have been shown to

inhibit secretion induced by cyclic nucleotides (Al-Awqati et al., 1974; Naftalin and Simmons, 1979).

These results indicate that increased cAMP has two actions: 1) inhibiting a coupled absorptive process for NaCl and 2) stimulating active anion secretion.

Cholera toxin has a molecular weight of approximately 84,000 (Finkelstein and LoSpalluto, 1970; Pierce et al., 1971; Finkelstein and LoSpalluto, 1972). Cholera toxin is a protein with three peptide chains, A_1, A_2, and B that have molecular weights of 24,000, 5,000, and 11,000 respectively. (Lai et al., 1976; Lai et al., 1977). Each complete toxin molecule contains one A_1 chain and one A_2 chain, along with five B chains (Lai et al., 1976; Gill, 1977). The B chain aggregation is known as choleragenoid and serves only to bind to cell surface receptors (Finkelstein and LoSpalluto, 1970; Gill, 1977). The cell surface receptor is a ganglioside, G_{M1} [galactosyl-N-acetylgalactos-aminyl (sialosyl) lactosyl ceramide] (Peterson et al., 1972; Fishman and Brady, 1976).

The A subunit $(A_1 + A_2)$ is not toxic to intact cells, but is active in lysates. The toxicity resides in the A_1 segment (Gill and King, 1975). It is hypothesized that in order to activate adenylate cyclase the B subunit must bind to the cell surfact receptors (G_{M1} ganglioside) and the A_1 peptide must be inserted into or through the membrane (Gill, 1977). Binding of the toxin to the cell is rapid and irreversible. A short exposure to the toxin is as effective as continuous exposure in eliciting a maximum intestinal response (Goodgame et al., 1973). There is a characteristic lag time of 15 to 120 minutes before an increase in cAMP or adenyl cyclase activity

is noted after exposure to CT (Pierce et al., 1971; Sharp, 1973). One interpretation of the lag time is that the toxin acts intracellularly and can only reach the interior slowly (Gill, 1977).

The characteristic effects of cholera toxin on various cells and tissues have been used to develop a number of assays for CT. In the adrenal cell tissue culture, CT elicits morphological and metabolic changes. The cells round up and secrete ketosteroids, a trait correlated with increased levels of cAMP (Donta et al., 1973; Donta et al., 1974; Donta, 1974). The skin permeability assay is based upon the ability of CT to produce characteristic induration and erythema (Evans et al., 1973a).

In summary, CT was one of the earliest known enterotoxins implicated in diarrheic disease. Cholera toxin seems to act via a cyclic nucleotide intermediate mechanism, cAMP, and has been shown to increase intracellular cAMP in virtually every tissue tested. Cyclic AMP is believed to act on intestinal mucosa via two mechanisms as observed in vitro: 1) Inhibiting a coupled NaCl absorption at the mucosal border, and 2) eliciting an active anion secretion that may arise from the crypt region.

As the first secretory diarrhea studied, the techniques and concepts used to investigate cholera toxin induced diarrhea were valuable aids in investigating the secretory diarrhea of E. coli, an area which has been as intensively looked at as cholera, particularly in domestic animals.

Escherichia coli Enterotoxins

Pathogenic strains of E. coli are a primary cause of neonatal diarrhea in domestic animals. Diarrhea may be produced by invasion of the mucosa by

some strains, or by elaboration of enterotoxin, or both. Two distinct

forms of enterotoxin are produced by E. coli, a heat labile (LT) form and

a heat stable (ST) form (Smith and Halls, 1967; Smith and Gyles, 1970;

Gyles and Barnum, 1969).

Early investigators noted that fluid accumulating in isolated loops

in response to E. coli enterotoxins had a similar ionic composition to that

of normal intestinal fluid and was low in protein (Pierce and Wallace,

1972; Sherr et al., 1973; Moon, Whipp, and Baetz, 1971). In addition,

fluid loss occurred in a histologically normal mucosa (Moon, Whipp, and

Baetz, 1971) and the enterotoxin did not cause inflammation (Dupont et al.,

1971). The ileal loop assay involves injecting the material to be tested

into ligated segments of ileum and observing the fluid response.

Smith and Halls (1967) proposed an exotoxin-mediated mechanism after

demonstrating that filtrates of bacterial cultures, as well as the cultures

themselves, produced fluid accumulation in the ligated intestinal loops of

several species of animals. From these data, they concluded that E. coli

must elaborate an enterotoxin which was capable of causing diarrhea.

Kohler (1968) demonstrated that broth filtrates from E. coli cultures

enteropathogenic for pigs contained enterotoxic material that was heat-

stable and methanol soluble. Intravenous injection of methanol and ethanol

extracts of these filtrates proved that the material was not endotoxin, as

no response was noticed. Vaccination of gilts with these filtrates did not

give protection to suckling piglets. Later work, using both broth filtrates

and whole cell lysates of E. coli cultures enteropathogenic for pigs, demon-

strated that both preparations were enterotoxic. The broth filtrate was

heat-stable and faster acting than the whole cell lysate. The whole cell lysate was associated with high mortality, marked dehydration, and was heat-labile (Kohler, 1971).

Smith and Gyles (1970) proposed that two types of toxins, heat-stable (ST) and heat-labile (LT), might be elaborated by E. coli. They prepared LT and ST by two different methods of culturing. Heat-labile enterotoxin was prepared by washing off the culture growth and ultrasonically lysing the cells, and then centrifuging and filtering the lysate. This lysate was tested for enterotoxicity before and after heating to 65 degrees Centigrade. Heating destroyed toxin activity. Heat-stable enterotoxin was prepared from soft agar culture fluids, heated to 65 degrees Centrigrade for 15 minutes, and centrifuged to remove bacteria. Toxin activity was not affected by heating. They concluded that the earlier results of Gyles and Barnum (1969) using cell lysates could be attributed to LT.

In their study (Smith and Gyles, 1970), antisera to the crude lysates neutralized LT activity but not ST activity. The strain specific antisera would neutralize the live organism's activity also. Heat-stable enterotoxin did not induce production of antibody to LT nor was the effect of ST neutralized, indicating that LT was antigenic and neutralizable, while ST was neither antigenic nor neutralizable.

Evans et al. (1973b) tested enterotoxin in ligated loops of rabbit using ST and LT from human coliforms. The effect of ST on fluid accumulation was immediate with maximum fluid accumulation at 4-6 hours. The effect of LT was rapid at high doses with a maximum effect at not less than 10 hours. Smaller doses of toxin yielded a slower onset of secretion.

They also demonstrated ST to be acid stable and LT to be acid labile.

Characteristics of Heat-Labile Enterotoxin

Purification and characterization of the two toxins has involved a number of laboratories around the world. Gill et al. (1976) isolated an enterotoxic material with molecular weight of 23,000. A number of single peptides were isolated by Finkelstein et al. (1976) with molecular weight from 35,000 to 100,000. Dorner (1975) isolated an LT with molecular weight of 102,000. Evans et al. (1976) purified a polymixin B-released LT, with a molecular weight of 20,000.

Clements and Finkelstein (1979) demonstrated two subunits (A and B) in LT isolated from three strains of E. coli. These subunits were remarkably similar to the cholera toxin subunits A and B in molecular weight. Heating in sodium dodecyl sulfate (SDS) and subsequent determination of molecular weight demonstrated that the "coli B" subunit dissociated into monomers with molecular weight of 11,500.

Treatment of the isolated LT with trypsin before thiol reduction and electrophoresis dissociated the 28,000 "coli A" subunit such that it comigrated with the 21,000 A_1 peptide of cholera toxin. A proposed molecular weight of 96,000 was given for the holotoxin, with six B subunits as opposed to five B subunits for cholera toxin. Similar activity of LT and CT was noted in the adrenal cell assay and skin permeability assays.

Kunkel and Robertson (1979) isolated a highly purified LT that exhibited biological activity similar to that of cholera toxin in four test systems: 1) Y-1 adrenal tumor cells, 2) Chinese Hamster ovary cells (CHO),

3) pigeon erythrocyte lysate (PEL), and 4) Skin permeability tests. Tests one and four have been mentioned previously. The CHO test is based on the fact that CT and LT alter morphology and adhesion of CHO cells in tissue culture, a cAMP-mediated characteristic (Guerrant et al., 1974). In the PEL test, CT has been shown to activate adenyl cyclase (Gill and King, 1975). Kunkel and Robertson (1979) determined a molecular weight of 73,000 for the holotoxin, which dissociated with SDS treatment into two subunits of molecular weight 44,000 and 30,000. Heating in SDS dissociated the 44,000 component into a species of molecular weight 11,000.

Kunkel and Robertson (1979) have proposed that the A subunit of LT is synthesized in early stages of growth and inserted in the outer membrane of E. coli to facilitate association with a binding component. Polymixin B extraction of toxin in the early lag phase of growth then would yield a species of LT of molecular weight less than 30,000, as observed by Evans et al. (1976). Higher molecular weights of LT may reflect an association of the A subunit with membrane or cytoplasmic proteins.

The LT preparations isolated by Kunkel and Robertson (1979) were stable over a wide range of storage conditions, temperature, and pH. Pronase and proteinase K destroyed the biologic activity, while trypsin digestion increased the activity, an observation confirmed by Clements and Finkelstein (1979). Amino acid analysis by both groups showed some similarities in CT and LT.

Dorner (1975) demonstrated the toxin to be heat-labile at 65 degrees Centigrade for 30 minutes. Kunkel and Robertson (1979) found the purified

toxin was more resistant to heating, and suggested that the purified LT, like the A subunit of cholera toxin, is heat-stable, even though the crude preparation of enterotoxin is heat labile and acid labile. Other investigators have noted the heat lability of E. coli LT toxin (Gyles and Barnum, 1969; Kantor et al., 1974; Schenkein et al., 1976).

LT has immunologic cross reactivity with CT. Using a CHO cell assay, Guerrant et al. (1974) inactivated both CT and LT activity with antiserum to CT. Pierce (1977) immunized rats with CT/toxoid and found significant and equal protection to intestinal challenge with CT or LT. Gill et al. (1976) used a purified LT with molecular weight 23,000 and demonstrated CT antibody inhibited the effect of LT on adenyl cyclase in pigeon erythrocytes. Evans et al. (1976) demonstrated a single precipitin band to polymixin B released LT in the presence of cholera antitoxin and antiserum to choleragenoid. These findings all indicate a similar antigenicity of LT and CT.

Further similarities of CT and LT are indicated by the reaction with G_{M1} ganglioside, the binding site for CT on cells (Holmgren et al., 1973; Van Heynigen, 1974; Fishman and Brady, 1976). G_{M1} ganglioside will inhibit the secretory response of rabbit ileum to CT and LT (Pierce, 1973). G_{M1} also was found to inactivate CT and LT activity in rabbit intradermal or skin permeability and ileal loop assays by Holmgren (1973). The inactivation of LT was not as specific as for CT, because it required several orders of magnitude greater concentration of ganglioside for inhibition. Pierce (1973) hypothesized that the binding of E. coli enterotoxin reflected a similarity in structure of the two toxins rather than a specific binding site for E. coli LT enterotoxin.

Similar to CT, LT initiates its secretory response by activating adenyl cyclase and increasing intracellular cAMP. Kantor et al. (1974) demonstrated an increase in adenylate cyclase in intestinal mucosa without a change in phosphodiesterase activity. Evans et al. (1972) demonstrated increased cAMP and adenyl cyclase activity in rabbit intestinal mucosa and rat adipocytes after exposure to E. coli LT. Guerrant et al. (1973) demonstrated that the onset of secretion and recovery corresponded to changes in adenylate cyclase activity in intestinal mucosa of dogs exposed to LT. Al-Awqati et al. (1972) reported chloride secretion after exposure of rabbit ileal mucosa in vitro to LT. These changes were similar to changes associated with cAMP or theophylline addition to the isolated tissue. These results suggested activation of adenylate cyclase and increased cAMP concentration as a mechanism of action for LT.

Not all investigators acknowledge the link of increased adenylate cyclase and secretory activity as cause and effect. Hamilton et al. (1978a) were unable to show a potentiation of LT, ST, and CT effects with the addition of theophylline in studies on secretion in weanling swine. They pointed out that theophylline added to adenylate cyclase activators causes synergistic responses in other tissues (Butcher and Baird, 1969; Epstein et al., 1971). In their results, they questioned the role of adenylate cyclase in causing secretion in swine small intestine in response to the three toxins.

Hamilton et al. (1978b) reported further dissenting data for cAMP involvement in LT-induced secretion in weanling swine. They reported CT and LT elevated cAMP and stimulated intestinal secretion in ligated rabbit in-

testinal loops, while these toxins induced fluid accumulation but did not raise cAMP levels in the pig. They hypothesized that either adenylate cyclase and cAMP elevation and intestinal secretion do not necessarily have a cause and effect relationship in swine small intestine, or that the proportion of cells involved with secretion may be small relative to the total mucosal population. They pointed out that the latter hypothesis does not explain the results in rabbit intestinal loops.

Summary

In summary, LT has been shown to have many similarities to CT in structure and activity. Both CT and LT seem to act via cAMP, and antisera to one can neutralize the secretory effect of either toxin. Their binding affinity to G_M ganglioside is similar, although CT is more specific. Both toxins are antigenic, heat labile, and have a characteristic lag time before secretory activity is induced. These characteristics are not true for another enterotoxin produced by E. coli, the heat-stable enterotoxin.

Characteristics of Heat-Stable Enterotoxin

Smith and Halls (1967) first demonstrated the heat stability of E. coli ST enterotoxin. They heated a crude toxin to 100 degrees Centigrade without affecting toxicity. They were unable to dialyze the toxin.

Bywater (1972) used Thiry-Vella loops (intact loops of intestine opening to the outside of the body) in calves to test the toxicity of ST. In contrast to Smith and Halls' work (1967), he was able to dialyze the toxin, although it was a very slow process. His results suggested a molecular

weight of 1,000 to 10,000 for the toxin. Smith and Gyles (1970) used
separate methods to prepare ST and LT and reported that ST was non-antigenic
and non-neutralizable with antisera prepared against ST.

Evans et al. (1973b) noted that the effect of ST was immediate in the
ligated rabbit loop, with maximum fluid accumulation at 4-6 hours. The
toxin was also acid stable. Pierce and Wallace (1972), using ST derived
from a human coliform, demonstrated that the effect of ST disappeared as
soon as the toxin was removed. They noted that the maximum effect in the
canine jejunum was observed within the first 90 minutes of exposure to ST.

Jacks and Wu (1974) partially purified ST of human origin using ultra-
filtration and gel chromatography. They reported a molecular weight of
1,000 to 10,000 for ST. The toxin was resistant to acid, trypsin, and pro-
nase. A purified ST from a porcine strain of E. coli recently has been ob-
tained using ultrafiltration, acetone fractionation, preparative gel elec-
trophoresis, ion exchange chromatography, and gel filtration (Alderete and
Robertson, 1978). A molecular weight of 4,400 was determined using SDS elec-
trophoresis and gel filtration. Amino acid analysis yielded 47 residues
with a calculated molecular weight of 5,100. Chemical characteristics in-
cluded heat stability (100 degrees Centrigrade for 30 minutes) and resist-
ance to pronase, trypsin, proteinase K., deoxyribonuclease, ribonuclease,
and phospholipase C. Organic solvents and oxidation with periodic acid had
no effect on biological activity. The ST was stable in acid to pH 1.0, but
pH greater than 9.0 decreased the activity. Using passive hemagglutination
and hemolysis titer assays, antigenicity of the ST was demonstrated. In
addition, antiserum from rabbits immunized with ST neutralized the entero-

toxic activity in suckling mice. Despite this humoral response, ST was considered to be a poor antigen.

Gianella (1978) purified ST from human origin E. coli and reported slightly different results. His data indicated ST to be a small peptide containing 22-27 amino acids with a molecular weight of 2,480-3,220 daltons. A molecular weight of 3,000-3,500 daltons was estimated with gel electrophoresis using organic solvents.

Mullan et al. (1978) partially purified a heat stable toxin and assayed it in infant mice. Their findings reinforced the current knowledge of ST as heat and acid stable, labile to pH greater than 10, and methanol soluble. Ultrafiltration demonstrated a molecular weight of 1,000 to 10,000.

Cyclic nucleotides have been implicated in the secretory effect of E. coli ST enterotoxin. Hughes et al. (1978) used culture filtrates from E. coli producing only ST to inoculate ligated rabbit intestinal loops. Biopsies of these tissues showed a three fold increase in cGMP levels after 20 minutes compared to controls. Cyclic AMP levels were not significantly different from controls. Cyclic GMP analogues were also tested in the same model. Significant fluid secretion was produced by 8BrcGMP without changing cAMP levels. Similar effects were observed in the infant mouse. These results suggested a role for guanyl cyclase in mediating ST induced secretion. Other investigators, (Newsome et al., 1978) used methanol soluble STa in the infant mouse assay. They reported a significant rise in cGMP following exposure to STa, but no increase in cAMP.

Field et al. (1978a) further characterized the cGMP and fluid response to ST in an in vitro system. Using rabbit ileal mucosa in the Ussing

chamber they demonstrated that ST, like theophylline, increased PD and Isc, but to a lesser degree. They reported that ST abolished net chloride absorption by decreasing chloride flux from mucosa to serosa while slightly increasing serosa to mucosa chloride flux. Theophylline had a significantly larger effect on serosa to mucosa flux, leading to net secretion. Theophylline and ST combined did not have a greater effect than theophylline alone. Both agents decreased conductance. They further demonstrated that the effect of ST could be reversed. Serial additions or dilutions of ST caused incremental changes in transepithelial PD in the in vitro mucosa. In addition, calcium concentration had no effect on the electrical response of in vitro rabbit ileum to ST, suggesting ST does not alter calcium permeability of the mucosal membrane. ST did not change the cAMP concentration but did cause a large and persistent increase in cGMP. Theophylline elevated cGMP levels three-fold both in the presence and absence of ST, suggesting ST's effect was not on cyclic GMP phosphodiesterase. In addition guanylate cyclase levels in a crude membrane fraction of intestinal mucosal cells were increased seven-fold over controls by ST. These results suggest that E. coli enterotoxin action is mediated by stimulation of guanylate cyclase. They proposed that ST inhibited a coupled uptake of NaCl at the brush border, pointing out that most of the guanylate cyclase activity in the small intestines is found in villus cells, with high specific activity in the brush border membrane (Quill and Weiser, 1975; DeJonge, 1975).

In later experiments by Guandalini and Field (1979), ST and furosemide, a known inhibitor of coupled NaCl influx, were shown to have equal effects in reducing chloride influx in rabbit ileal mucosa in vitro. The effects

were not additive and were similar to those shown previously for cAMP.

Ende and Gianella (1978) injected suckling mice with ST and recorded fluid secretion and cGMP and cAMP levels in the intestine with time. Heat-stable enterotoxin induced fluid secretion within 15 minutes with secretion reaching a plateau at 2-3 hours. Cyclic AMP levels did not change while cGMP increased within 5 minutes of ST exposure, peaked at 90 minutes, and returned to control levels after 2 hours in spite of continual fluid secretion. Perfusion of ST caused prompt secretion, within 5 minutes, in the jejunum and ileum of rats. In contrast, perfusion of the rat colon with ST inhibited water absorption but did not induce secretion. Glucose absorption in the rat jejunum was unchanged by ST, although a significant increase in glucose and water absorption was noted during the recovery periods of the perfusion. Glucose at a level of 56 mM was able to maintain net absorptive rates similar to those seen with electrolyte solution in the absence of ST.

Gianella and Drake (1979) further tested ST in suckling mice and found a maximum increase in cGMP three minutes after administration, with levels 10 times that of controls. Fluid secretion did not become evident until 15-30 minutes after ST administration, demonstrating that the increase in cGMP preceded fluid secretion. A cGMP analogue, 8BrcGMP, also induced fluid secretion with a time course similar to that of ST.

Rao et al. (1979) studied the effect of ST on guanylate cyclase compared with the effect of ST on cGMP levels and potential difference to determine tissue specificity and relation to ion transport in the rabbit. They found that guanylate cyclase was stimulated by ST throughout the small intestine (4-6 times control) and colon (2-3 times control). The enzyme

was found in both the brush border and non-brush border membrane fragments. Cyclic GMP was increased 10 fold in the small intestine and 2-5 fold in the colon. ST increased PD in the jejunum, ileum, and proximal colon in vitro, but not in the duodenum or distal colon. ST increased PD in the proximal colon only when added to the serosal side. They concluded that ST's action is restricted to the intestinal mucosa and that the discrepancy in PD response (ileum versus proximal colon) is a function of guanylate cyclase distribution (brush border vs. basolateral membrane). They also concluded that a cGMP responsive transport mechanism is not present in the duodenum and distal colon.

In light of the decreasing gradient of guanylate cyclase from villus to crypt (DeJonge, 1975; Quill and Weiser, 1975), Field et al. (1978a) have postulated further that the smaller electrical response of rabbit ileal mucosa in vitro to ST, as opposed to theophylline, may reflect the lesser amount of guanylate cyclase in the crypt region, where it is presumed the electrogenic response in secretion originates, i.e., an increase in Isc and P. Alternatively, he proposed that cGMP may be a less effective activator of crypt cell secretion than cAMP.

Figure 3 demonstrates the proposed sites of action of the cyclic nucleotides believed to be involved with enterotoxin mediated secretory diarrhea.

The understanding of ST has been complicated by the isolation of two types of heat-stable enterotoxin, one methanol soluble (STa) and the other insoluble in methanol (STb), by Burgess et al. (1978), from porcine strains of enteropathogenic E. coli. STa was partially heat stable, active in one

Figure 3. Proposed sites of cyclic nucleotide activity

cAMP INHIBITS COUPLED NaCl INFLUX

cGMP INHIBITS COUPLED NaCl INFLUX
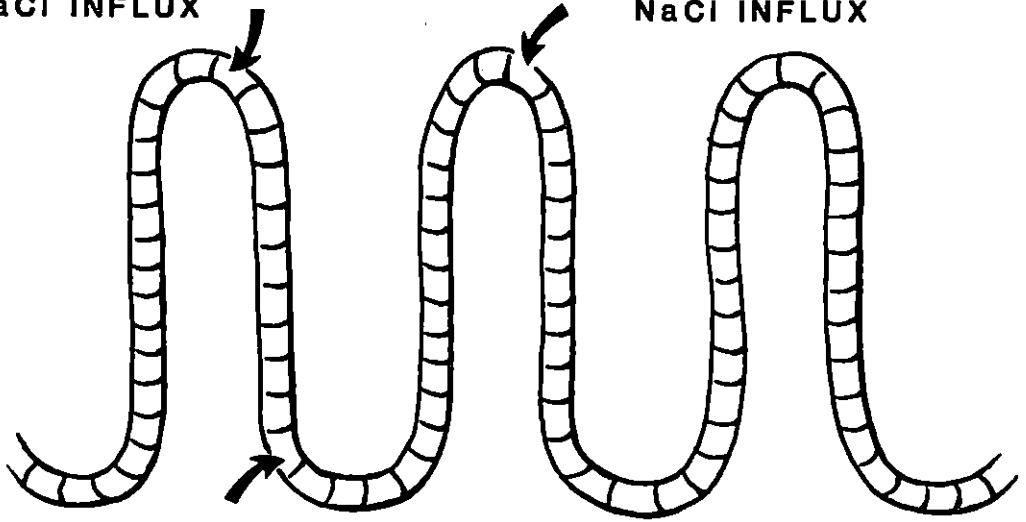
cAMP STIMULATES ANION SECRETION

to three-day-old piglets, and infant mice, but was inactive in weaned pigs seven to nine-weeks-old. STb was active in weaned pigs and rabbit ligated loops, but inactive in infant mice.

More recent experiments have confirmed that STb is not active in the suckling mouse, is active in weanling pig intestinal loops, and does not increase cGMP levels in intestinal cells (Greenberg et al., 1982).

Further studies by Weikel et al. (1983) have shown that STb increases Isc in isolated porcine jejunal mucosa within 5 minutes and that this effect is inhibited by serosal addition of ouabain. Mucosal addition of furosemide does not have an effect on the response and STb has no effect on adenylate cyclase. The mechanism of STb action is unknown.

Summary

In the small intestine, there are crypt cells and villous cells, possibly corresponding to secretory and absorptive cells. Schultz (1980) has pointed out that these cell types do not have to be exclusive in function. It is possible that villus cells secrete and crypt cells absorb depending on appropriate stimuli. Crypt cells have been shown to be precursor cells of villus cells with an increasing complexity of enzymes and structural characteristics as they mature to villus cells.

A number of secretory diarrheas occur in the presence of a histologically normal mucosa and are marked by a primarily anionic secretion. Cation absorption and coupled Na^+ and Cl^- absorption has been shown to be inhibited by agents such as cyclic AMP and cGMP implicated in secretory diarrhea.

E. coli heat-stable enterotoxin has a rapid onset of action and does not produce a secretory change to the degree of LT or CT. Its mechanism of action is still largely unknown. Because of its rapid action, ST can be used in experimental situations such as an isolated cell system, where the possible effects of ST on sodium efflux can be measured. Because villus cells and crypt cells are different histologically and physiologically, the hypothesis for these experiments was that there may be a different effect or degree of effect of ST on villus than on crypt cells. The following experiments tested the hypothesis that ST has a different effect on sodium efflux of isolated cell populations of the intestinal mucosa.

EXPERIMENTAL METHODS

Toxins

Two culture filtrates of E. coli were used in these experiments. One filtrate was a heat-stable enterotoxin from a toxigenic strain of E coli, 1261 designated ST (Class 1, serotype O138:K81; Moon et al., 1978) and the other was from a non-enterotoxic strain, 123, designated CB or control broth (serotype 043:K-:H28) (Moon et al., 1970). The 1261 ST filtrate was produced and assayed at the National Animal Disease Center, as previously described (Moon et al., 1979), and then was diluted in incubation fluid prior to each experiment to give an ST activity of approximately 15 mouse-units/ml. This concentration has been shown to produce a consistent secretory response in porcine intestine in vivo (Ahrens and Zhu, 1982).

Animals

Thirty three-week-old pigs were obtained from the National Animal Disease Center, Ames, Iowa. Feed was withheld 12 hours prior to each experiment, but water was provided ad libitum. Each experiment used two littermates.

The pigs were anesthetized with sodium pentobarbital. The abdomen was quickly opened and approximately 60 centimeters of jejunum, just distal to the ligament of Treitz, were removed and flushed with warm (37° C.) isolation medium (Appendix).

Isolation of Cells

The intestinal segment was everted over a metal spiral previously covered with dialysis tubing, and tied tightly at both ends (Figure 4).

Figure 4. Vibration apparatus with dialysis tubing-covered
          metal spiral
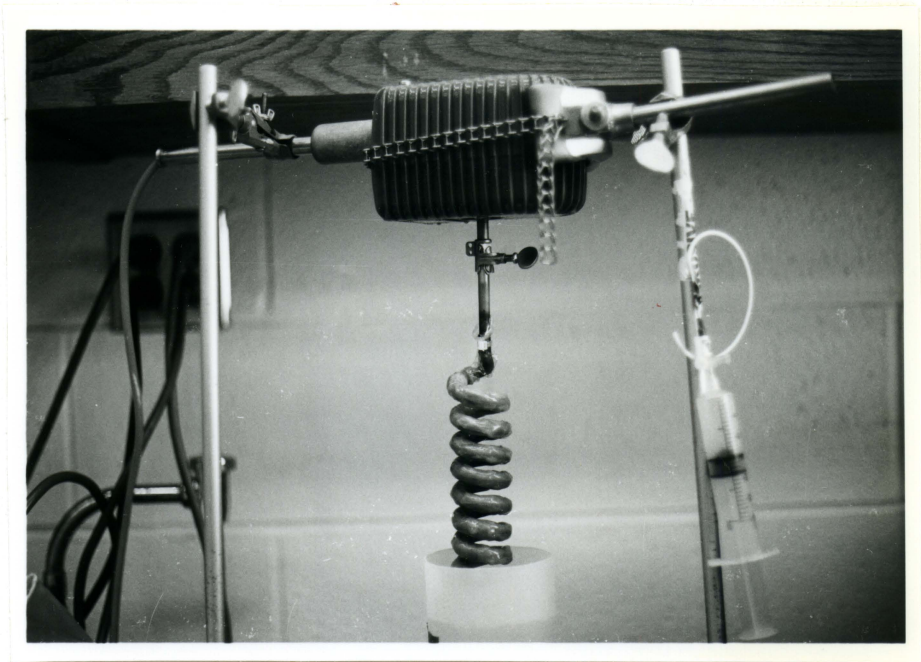
The total length of intestine on the spiral was approximately 45 cm. The intestine then was allowed to soak for 30 minutes at 37° C. in oxygenated isolation medium. Preliminary studies had demonstrated that 30 minutes had to elapse before significant cell removal was noted, with or without vibration.

After 30 minutes, the spiral was attached to a vibration apparatus[1] and vibrated for approximately 60 minutes in 400 ml of isolation medium, sequentially isolating villus and crypt cells. This procedure followed the technique of Harrison and Webster (1969) in which the mature villus cells are shed first and the crypt cells last.

Three collections of cells were used: 1) a villus-rich fraction, 2) a mixed cell fraction, and 3) a crypt cell-rich fraction. The time of vibration for each fraction varied slightly, depending on the rate of cell removal as monitored by a turbidity of the solution. The total vibration time needed for the three fractions was 5-15 minutes, 15-30 minutes, and 45-60 minutes for fractions I, II, and III respectively. For the final fraction, the dialysis tubing was inflated to distend the everted intestine to facilitate the release of crypt cells.

At the appropriate time, the vibration was stopped and the 400 ml of isolation medium from each cell fraction was evenly divided between two 350-ml centrifuge tubes. These were centrifuged at 500 times gravity (g) for 10 minutes and the pellet was washed into a 50-ml centrifuge tube. This was centrifuged, and the pellet was washed into a 12-ml tube and

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[1]Chemapec Inc., Woodbury, N. Y.

centrifuged again. All solutions were kept at 37° C. After the second

washing each 12-ml tube contained half the cells of fraction I, II, or III,
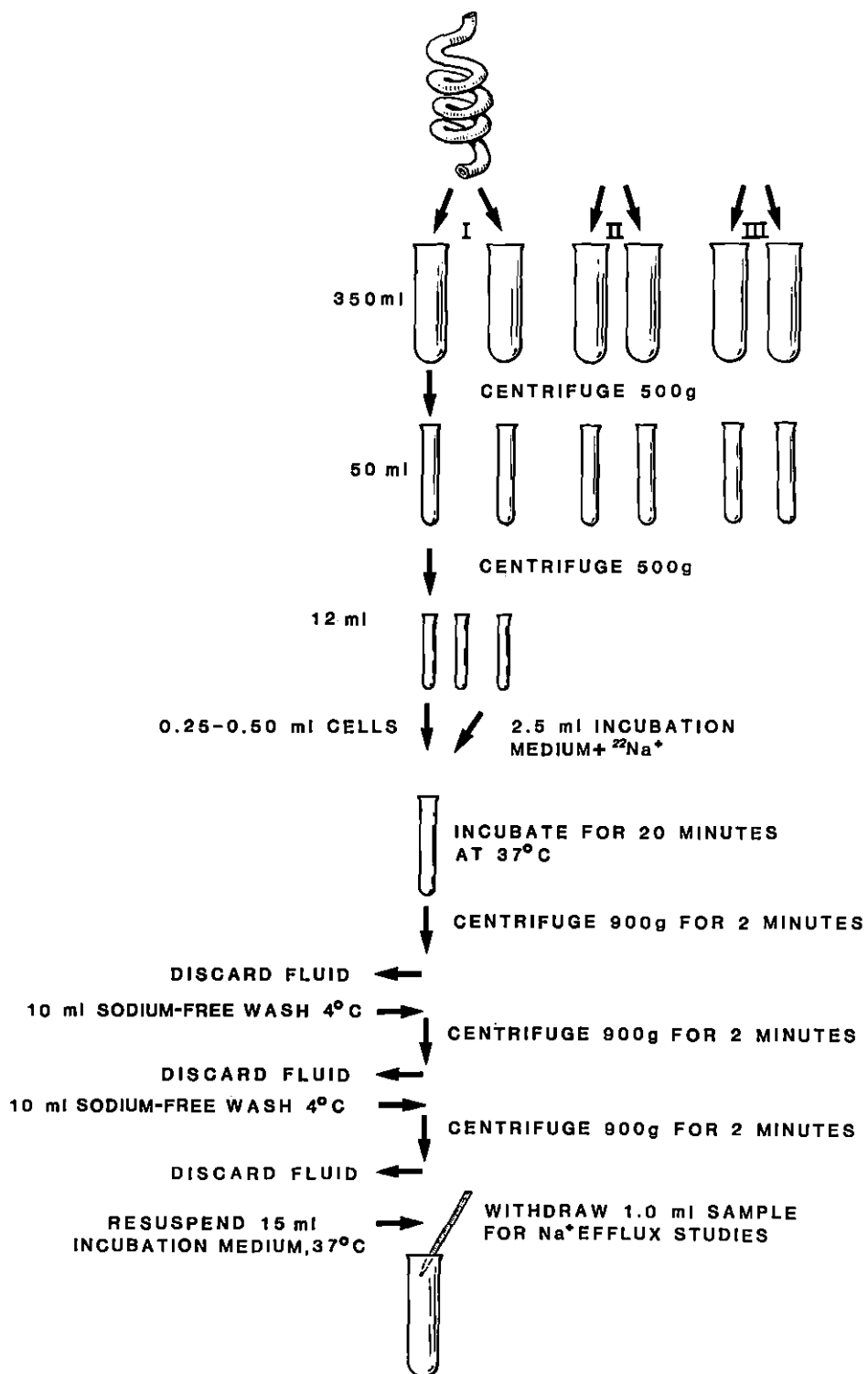
a volume of 0.25 to 0.5 ml of cells. (See Figure 5.)

The sodium transport characteristics of the cells were determined

following the method of Gall et al. (1974). The cell pellet was suspended

in 2.5 ml of incubation medium (Appendix) containing ST, ST+ouabain, CB,

or CB+oubain, and 10 Ci of ^{22}Na (New England Nuclear) was added to the

suspension. The cells were incubated at 37° C. for 20 minutes in a Dubnoff

shaker.

Following incubation, the cells were quickly centrifuged and the

pellet washed twice with cold Mg-Tris buffer (Appendix). Each centrifuga-

tion was for two minutes and was performed at 4° C. The pellet was not

disturbed or resuspended during the two washes to minimize loss of ^{22}Na

from the cells.

The pellet, consisting of approximately 0.5 ml of cells, was resus-

pended in 15 ml of tracer-free incubation medium at 37° C. to give a dilu-

tion of 30:1. Sodium efflux was determined by the rate of ^{22}Na appearance

in the isotope-free incubation medium. Samples of the cell suspension (0.5

ml) were obtained at the beginning and end of the efflux period. These

samples were labeled A and Z and were taken as a measure of total radio-

activity in the suspension. A one ml sample of suspension was obtained at

0, 2, 4, 6, 8, 10, and 12 minutes of the efflux period. Each one ml sample

was placed into a pre-chilled centrifuge tube, centrifuged for 2.5 minutes,

and 0.5 ml of the cell-free supernatant was pipetted into a 5 ml disposable

glass culture tube. The ^{22}Na activity was determined in a Beckman Biogamma

Figure 5. Flow chart for cell isolation and sodium efflux studies

INTESTINAL SEGMENT

350 ml

CENTRIFUGE 500g

50 ml

CENTRIFUGE 500g

12 ml

0.25-0.50 ml CELLS   2.5 ml INCUBATION
                            MEDIUM+ ^{22}Na$^+$

INCUBATE FOR 20 MINUTES
AT 37°C

CENTRIFUGE 900g FOR 2 MINUTES

DISCARD FLUID

10 ml SODIUM-FREE WASH 4°C

CENTRIFUGE 900g FOR 2 MINUTES

DISCARD FLUID

10 ml SODIUM-FREE WASH 4°C

CENTRIFUGE 900g FOR 2 MINUTES

DISCARD FLUID

RESUSPEND 15 ml   WITHDRAW 1.0 ml SAMPLE
INCUBATION MEDIUM,37°C   FOR Na$^+$EFFLUX STUDIES

II gamma Scintillation Counter[1], for each 0.5 ml of supernatant or sus-
pension.

Two series of experiments were done. In the first series of experi-
ments one-half of each fraction was treated with CB and the other half was
treated with ST. The difference in total sodium efflux between ST- and CB-
treated enterocytes was examined for each fraction.

In the second set of experiments, the difference between total Na^+
efflux following exposure to ST or CB was examined in the presence or
absence of ouabain. This set of experiments used only one toxin type (ST
or CB) for each fraction (I, II, or III) of enterocytes, but the cardiac
glycoside, ouabain (Final concentration 1 mM) was added to one-half of each
fraction.

Earlier experiments by Gall et al. (1974) demonstrated a maximum in-
hibition of (Na^+/K^+)-ATPase with 1 mM ouabain. Under these conditions,
passive efflux is defined as the efflux remaining after maximum inhibition
by ouabain (Gall et al., 1974). The difference between total efflux and
passive efflux is then a measure of ouabain-sensitive efflux of Na^+, and
is considered active Na^+ transport. Total Na^+ efflux comprises both
passive and active efflux of sodium. The passive efflux has been inter-
preted to be a measure of the permeability of the cell membrane (Gall and
Chapman, 1976).

Enzymes

Marker enzymes were used to determine the specificity of the isolation
procedure for enterocyte fractions. Sucrase was assayed in each fraction.

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[1]Beckman Instruments, Palo Alto, CA.

Sucrase activity is high in mature villus cells with well-developed brush borders (Nordstrom et al., 1968; Weiser, 1973; Charney et al., 1974). Two littermates were used for each experiment, one in the morning and one in the afternoon. The enzymes were analyzed separately to rule out any diurnal variation in enzyme levels.

The enzymes were assayed using cells collected after the first wash in isolation medium. The cells were washed from the 350-ml centrifuge tubes into 50-ml centrifuge tubes with 15 ml of isolation fluid. Two ml of suspension from each 15 ml quantity were pipetted into a single disposable tube and centrifuged at 900 g. The cell pellet was washed with Na_2HPO_4 buffer (96 mM at 4° C.), pH = 7.2 recentrifuged and resuspended in 2.5 ml of Na_2HPO_4. The cells were homogenized for 20 seconds with an ultrasonicator. The homogenate was frozen until the enzymes were assayed two days later. Protein determinations were performed within 24 hours. Sucrase activity was determined by the method of Dahlquist (1968) for intestinal dissacharidases. The thawed homogenates were diluted to give a baseline protein level of approximately 0.5 mg/ml. One hundred microliters of the diluted homogenate were added to 100 μl of substrate buffer (Appendix) containing sucrose. The mixture was incubated for 60 minutes at 37° C. After 60 minutes, 3.0 ml of TGO reagent (glucose oxidase-Appendix) were added to the mixture and incubated at 37° C. for 60 minutes in a Dubnoff metabolic shaker as color developed. Blanks were prepared using 200 μl of homogenate, 3.0 ml TGO, and 100 μl of substrate buffer added in that order. Standards were prepared using 200 μl of standard solution plus 3.0 ml TGO solution to give 10, 20, 30, and 40 μg of glucose in the final mixture. Standards and

blanks were incubated at 37° C. for 60 minutes. Absorbance was read at 420 nm on a spectrophotometer. Sucrase activity was expressed as units/mg protein.

Protein determinations used in all assays were determined using the method of Lowry et al. (1951).

Histopathology

Tissue samples of the everted gut were taken at the beginning and end of the vibration procedure and fixed in 10% neutral buffered formalin. Section of these tissues were mounted on slides, stained with hematoxylin and eosin, and examined microscopically for completeness of cell removal and normal microanatomy.

Calculation

Calculations of sodium efflux were performed using the following protocol. The 0.5 ml supernatant collections from time 0, 2, 4, 6, 8, 10, and 12 minutes were counted for ^{22}Na radioactivity as were the 0.5 ml suspensions taken at the beginning and end of the 12 minute period. The fraction of sodium remaining within the cells was calculated according to the formula $1 - \dfrac{Na_t}{Na_m}$, where Na_m is the average radioactivity of the A and Z 0.5 ml suspensions, and Na_t is the radioactivity of the 0.5 ml supernatants for time 0, 2, 4, 6, 8, 10, or 12 minutes. This equation yields the actual values for the fraction of sodium remaining in the cells at each time period.

The equation $K_m = \ln(1 - \dfrac{Na_{t=K}}{Na_m})/$ time-unit was used to calculate the slope, K_m, for sodium efflux. This constant, K_m, refers to the amount of

Na^+ exchanged per hour. A standard program for linear regression was used to calculate the slope by using x values of 0.033, 0.067, 0.1, 0.133, 0.167, 0.2 hours and the fraction of sodium remaining in the cells at those times as the y axis.

Statistical Analysis of Sodium Efflux Values

The SAS system was used for statistical analysis of sodium efflux through the Statistical Consulting Laboratory at Iowa State University. The SAS system was used to check for any interaction of animal, toxin, treatment, ouabain treatment, and cell fraction, using analysis of variance.

RESULTS

Histopathology

The histology of normal jejunal mucosa is shown in Figure 6. The villi are long and cylindrical with an intact villus mucosa of tall columnar absorptive cells and goblet cells, and cuboidal to columnar cells in the crypt region. The lamina propria has a rather diffuse cellular makeup and the submucosa is thin and compact.

Figure 7 demonstrates the appearance of jejunal mucosa following dilation and vibration. The villi are shrunken and collapsed and devoid of normal villus cells. The lamina propria is condensed and the submucosa is quite thick and edematous. All but 5-10% of the enterocytes have been removed, these being the very basal crypt cells. These cells appear to be essentially normal. The tenacity of these crypt cells was remarkable, with the majority of sections revealing some crypt cell retention even after 60 minutes of dilation and vibration.

Sucrase Analysis

The results of sucrase analysis are given below in Table 2. The results indicate that there were no differences in sucrase activity between animals killed in the morning or killed in the evening. Consequently, the overall mean of both groups was used to check for differences in sucrase levels between fractions.

The mean sucrase activity for both fractions I and II was more than two standard errors different than fraction III, but there was no difference in activity between fractions I and II. This indicates an actual decrease

Figure 6.   Histological section of jejunum prior to vibration showing
            normal mucosal epithelium, villi, and lamina propria

Figure 7.   Histological section of jejunum following vibration showing
            loss of mucosal epithelial cells, shrunken villi, and a few
            remaining crypt cells
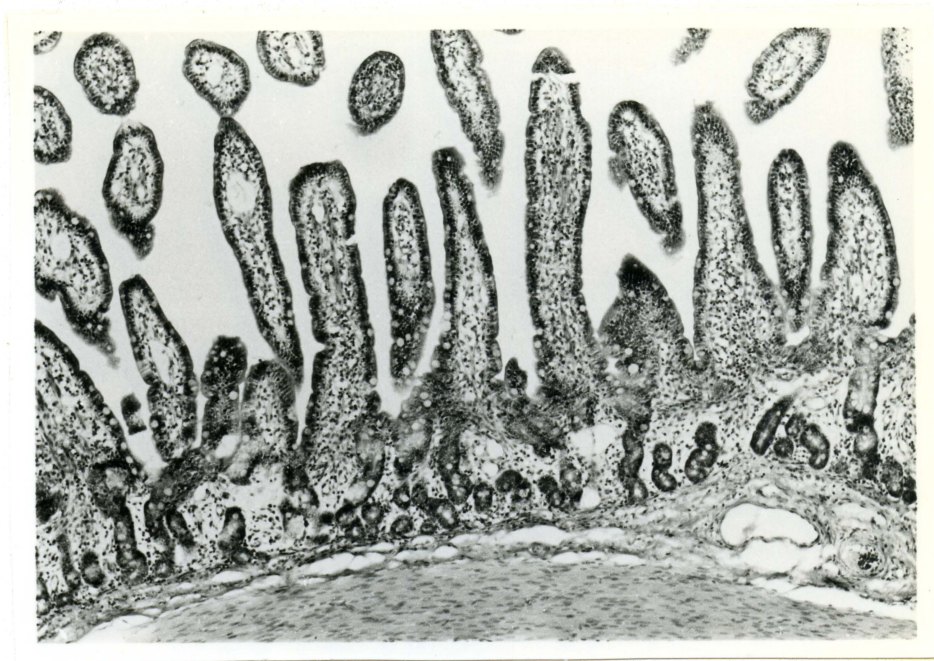
in activity between I and III, and II and III, but not I and II. These results indicate that while fraction III was composed of less differentiated cells than I or II, it was not possible to define a separate set of cells for fractions I and II by use of sucrase levels.

Table 2. Sucrase activity of swine jejunal enterocytes

	Fraction			
Time	I	II	III	Overall
A.M.	54.5 ± 6.4[a,b]	57 ± 8.3	39.2 ± 6.09	49.5 ± 4.13
P.M.	63.1 ± 11.2	55.3 ± 8.1	36.4 ± 6.5	51.6 ± 5.2
Overall	58.9 ± 6.5	56.1 ± 5.7	36.9 ± 4.3	

[a]Each value is the mean $\pm$ standard error of activity.

[b]Each value is expressed as units of sucrase activity/gram protein.

Effects of CB or ST on Sodium Efflux in Mature and Immature Enterocytes

The results of sodium efflux contrasting ST and CB effects are shown in Figure 8. Individual values of sodium efflux for the 16 animals used can be found in Table 3 in the Appendix. Fraction I had a K_m (sodium exchanged per hour) of 4.74 for CB-treated enterocytes and 5.15 for ST-treated enterocytes. Fraction II had a K_m of 4.63 for CB-treated enterocytes and 4.92 for ST-treated enterocytes. Fraction III had a K_m for CB-treated enterocytes of 4.65 and a K_m for ST-treated enterocytes of 4.37. None of these pairs demonstrated any significant difference between treatments. There also was no significant difference of sodium efflux between fractions.

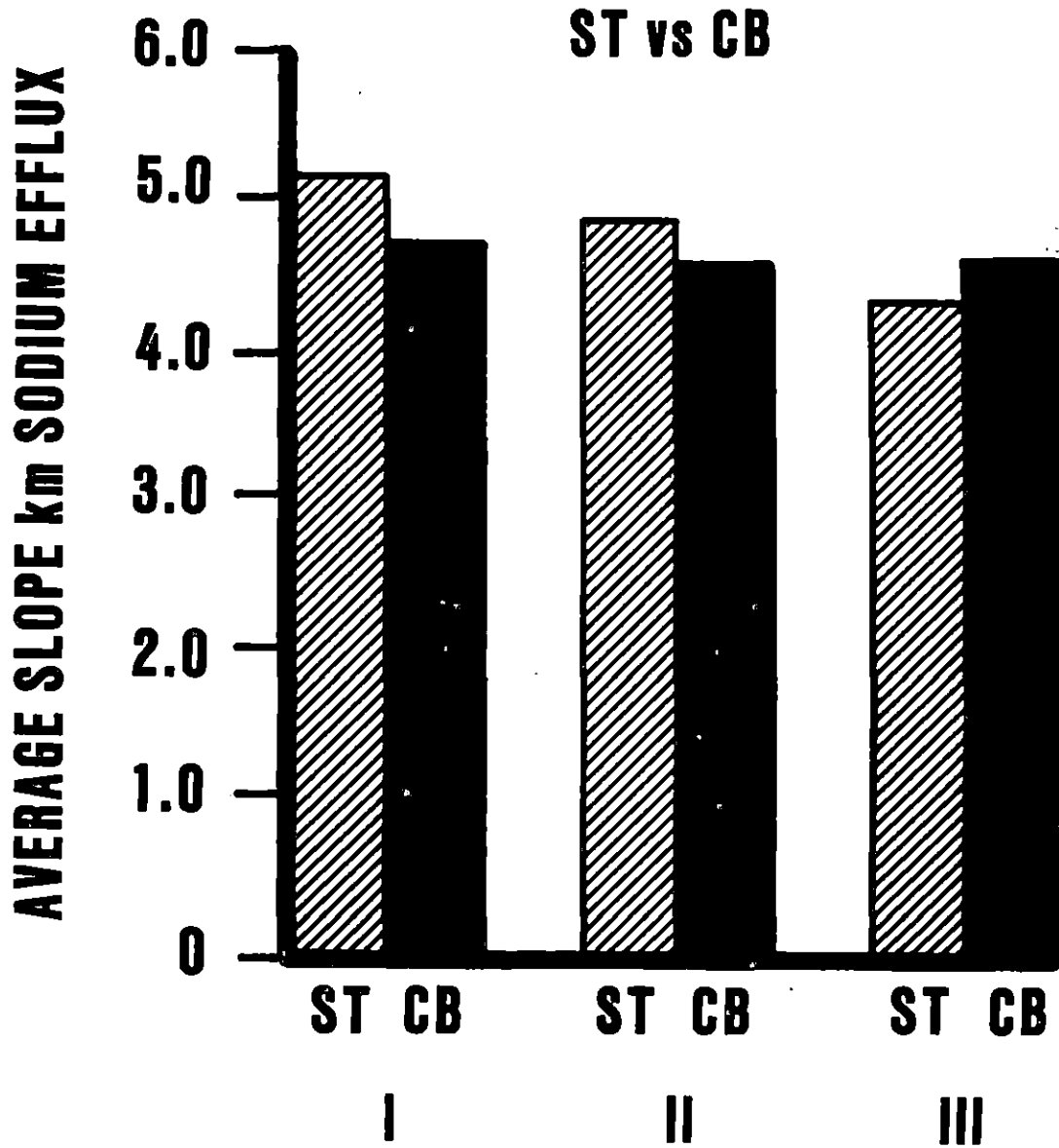Figure 8. Mean sodium efflux for fractions I, II, and III comparing
ST- and CB-treated enterocytes

      ST   (Escherichia coli heat-stable enterotoxin-1261-NADC)

      CB   (Escherichia coli control broth - 123-NADC)

      K_m (Natural log of the slope of ^{22}Na efflux indicating
              sodium exchange/hour)

No significant difference between any fraction or between
ST and CB was noted

ST vs CB

Effect of Ouabain on Sodium Efflux of CB- and ST-treated Enterocytes

The effects of ouabain on sodium efflux for CB- and ST-treated entero-
cytes are shown in Figure 9. Individual values for sodium efflux for the
14 animals used can be found in Table 4 in the Appendix. Fraction I had a
K_m value of 3.79 for CB-treated enterocytes and 3.01 for CB+ouabain-treated
enterocytes. Fraction II had a K_m of 4.47 for CB-treated enterocytes and
3.17 for CB+ouabain-treated enterocytes. Fraction III had a K_m of 3.53
for CB-treated enterocytes and 2.43 for CB+ouabain-treated enterocytes.
There was a significant difference between CB- and CB+ouabain-treated cells
in each fraction, but not between fractions.

The ST-treated enterocytes had similar results. Fraction I entero-
cytes had a K_m of 4.17 for ST treatment and 2.84 for ST+ouabain treatment.
Fraction II enterocytes had a K_m of 4.50 for ST treatment and 2.71 for
ST+ouabain-treated enterocytes. Fraction III had a Km of 4.49 for ST treat-
ment and 2.72 for ST+ouabain treatment. There was a significant difference
between treatment in each fraction, but not between fractions. Overall,
there was no significant difference between ST and CB treatments as well.

Figure 9. Mean sodium efflux for fractions I, II, and III comparing ST-
and CB-treated enterocytes with and without ouabain

ST   (Escherichia coli heat-stable enterotoxin -
            1261-NADC)

SO   (ST + ouabain)

CB   (Escherichia coli control broth - 123-NADC)

CO   (CB + ouabain)

K_m (Natural log of the slope of ^{22}Na efflux
            indicating sodium exchange/hour)

ST vs. CB   No significance in any fraction

ST vs. SO $p < .05$ for all 3 fractions

CB vs. CO $p < .05$ for all 3 fractions

SO vs. CO   No significance in any fraction
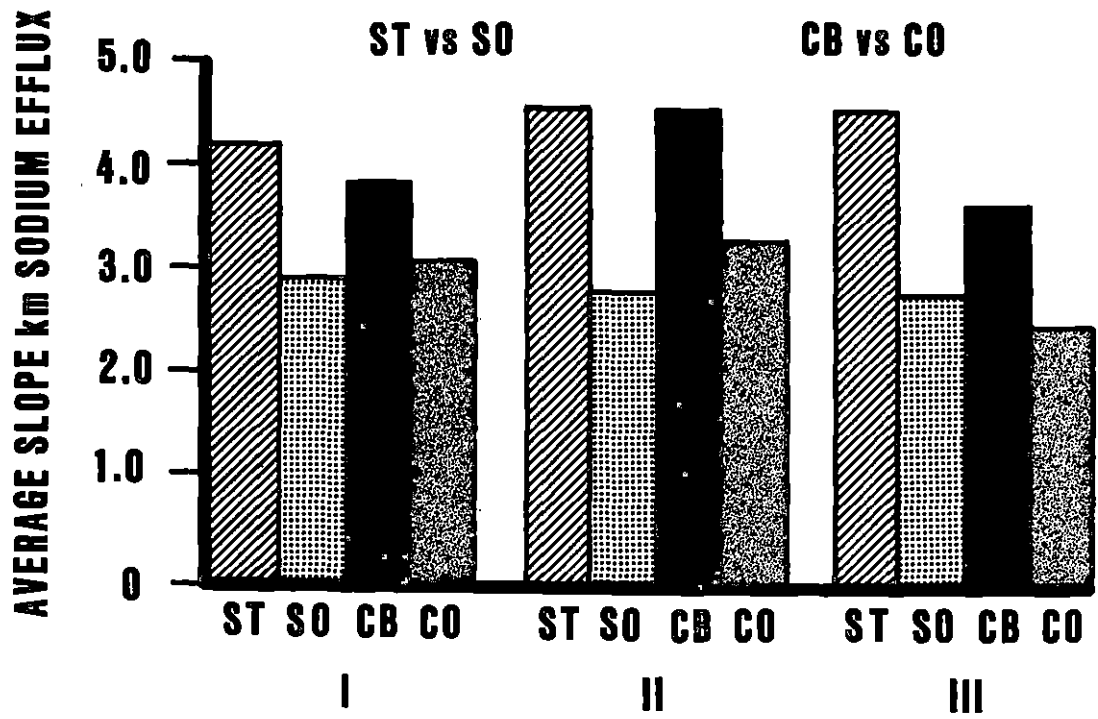
DISCUSSION

Gall et al. (1974) questioned whether the isolated cell could be a legitimate tool in studying intestinal ion transport in view of the polarity of morphology and function of the intestinal epithelial cell. In support of this technique, they reported that ouabain inhibited sodium efflux and phloridzin inhibited glucose-stimulated sodium efflux in the isolated cell. These results correlated well with the known effects of these two agents in intact epithelium. The results of this study indicate that ouabain inhibition of sodium efflux can be observed with the isolated cell technique, but it is not clear whether differences in sodium transport by a particular cell type, villus or crypt, can be determined.

Two observations of these experiments were most noticeable. First, there was no significant difference in sodium efflux between control broth and heat-stable enterotoxin for any given cell fraction. Secondly, no difference in sodium efflux was observed between fractions I, II, and III when using either ST or control broth. The reason for the lack of stimulation of sodium efflux by ST can only be presumed, some of the possibilities being:

1) The cells were incapable of responding to ST stimulation, i.e., the cells were non-functional or dead.

2) ST had no effect on Na^+ efflux in an isolated system, or,

3) The effect was masked by the system.

Viability of the preparation is a major concern in any in vitro system. The trypan blue exclusion test was run on cell samples immediately after the efflux procedure and in the great majority of cases, viability of cells

was estimated at greater than 50% by trypan blue exclusion. In addition, there were differences in efflux noted between ouabain and non-ouabain treated cell fractions, which would also indicate the cells were still functional.

The second possibility is more difficult to resolve. Escherichia coli 1261 ST has been shown to be active in the infant mouse assay and in the baby pig in vivo (Whipp, Moon, and Lyon, 1975) and in the weanling pig in vivo (Hamilton et al., 1978a; Whipp et al., 1981). Scoot et al. (1980) reported 1261 STa caused a net secretory flux in ligated loops in weanling swine, and that the secretory flux was positively correlated with the mucosal cyclic GMP concentration. Argenzio et al. (1984), using both in vivo and in vitro techniques in 6- to 8-week-old swine, reported a net secretory response to 431 STa. The secretory response was greater than the electrical response, suggesting an electrically neutral NaCl or $NaHCO_3$ secretory process. An active HCO_3 secretion in the jejunum was also elicited by ST. While none of these studies used an isolated cell technique, the in vivo studies demonstrate that 1261 ST does have a secretory effect on weanling swine. It is possible that while 15 mouse units/ml has an effect in vivo, that quantity is insufficient in vitro. It would seem more likely that an in vitro system would be more sensitive, but that is not always true.

Sodium efflux is a technically difficult term to interpret, as a number of parameters may influence efflux. Active transport of sodium out of the cell is believed to be primarily by way of (Na^+/K^+)-ATPase. Heat-stable enterotoxin does not affect (Na^+/K^+)-ATPase (Ahrens and Zhu, 1982).

Schultz (1980) has stated that sodium entry into the cell is the rate limiting step in sodium absorption, using the mammalian colon as an example. Field (1978b) has proposed that ST may inhibit a coupled NaCl influx at the mucosal border of enterocytes. This coupled entry of NaCl accounts for only about 20% of sodium entry in the rabbit ileum in vitro (Nellans et al., 1973).

A recent report by Guandalini et al. (1982) reinforces this idea. Using isolated rabbit ileum, they demonstrated an inhibition of chloride influx by ST. Addition of ST to rabbit ileal mucosa in vitro caused an inhibition of sodium flux from mucosa to serosa, but not a net secretory flux of sodium. In contrast, ST caused a reversal of net chloride flux from absorption to secretion. Chloride influx was equally inhibited by furosemide and ST. When glucose was added to the mucosal side 60 minutes after ST, the short circuit current response was not significantly different from control.

All of the solutions in the experiments presented here using isolated swine enterocytes contained glucose. Most of the in vitro work with rabbit ileum has been done without glucose, but work with transportable sugars (Schultz and Zalusky, 1964b; Goldner et al., 1969) has demonstrated that glucose in the bathing media will increase sodium transport. The addition of glucose to the incubation media and concomitant increase in sodium absorption may have been enough to offset the proposed decrease in sodium influx due to ST. Certainly in clinical use with cholera patients, glucose and electrolytes given orally have been shown to reverse net secretion to absorption. Other in vitro studies using isolated swine enterocytes have

demonstrated an increase in sodium efflux when glucose was in the incubation media (Kerzner et al. 1977). Hamilton et al. (1978a), using weanling swine, demonstrated a significant decrease in net fluid and electrolyte secretion in isolated intestinal loops exposed to ST when glucose was present.

If the neutral coupled NaCl absorption is only 20% of total sodium absorption, an intact Na-glucose cotransport may be more than enough to offset an ST block of neutral-coupled NaCl influx.

Most secretory responses appear to be due to anion, and (Na^+/K^+)-ATPase is necessary for such a response. Active sodium efflux may remain relatively constant during a secretory stimulus. In an isolated cell system devoid of cation selective tight junctions and lateral intercellular spaces, little or no change in sodium efflux activity due to ST may be observed.

A further result was that no differences in sodium efflux were observed between fractions I, II, and III using either ST or control broth. Gall et al. (1977) demonstrated a decreased sodium efflux and (Na^+/K^+)-ATPase levels in crypt cells compared to villus cells in vitro using cells isolated from rat jejunum. Glucose had little effect on crypt cell efflux but increased efflux from mature villus cells. No difference was noted in passive efflux of sodium using glucose or sugar-free media. Crypt cell passive efflux was less than passive efflux for villus cells. Kerzner et al. (1977) reported total efflux rate constants of -12.81 and -9.42 using jejunal enterocytes isolated from control pigs and pigs infected with TGE, in which the villi are covered with relatively undifferentiated crypt-like cells. In these experiments, the total sodium efflux was -4.38 for ST

treated animals and -3.93 for control broth treated animals. Individual experiments approached the levels reported by Kerzner et al. (1977) for sodium efflux, but the overall mean was lower. Passive sodium efflux was the same for all groups in the present studies as well, indicating that there was little permeability change for sodium due to ST (Gall and Chapman, 1976).

These results differ from those of Panichkriangkri (1982) who demonstrated that ST increased chloride efflux from isolated swine enterocytes in both villus and crypt cells. Most secretory stimuli result in an anion secretion, but some degree of change in sodium efflux would be expected if there was an actual difference in active sodium efflux or permeability between crypt and villus cells. The lack of response to ST suggests that ST has no effect on active or passive efflux of sodium in isolated cells.

The lack of response to toxin may be explained by the proposed model of intestinal secretion of Field (1978b) and Frizzell et al. (1979a), which incorporates a coupled basolateral NaCl influx and a change in chloride permeability at the luminal border of the cell. Since most secretory responses appear to be due to anions, chloride in particular, and (Na^+/K^+)-ATPase is necessary for such a response, active sodium efflux may continue unchanged by ST in any fraction of cells. In an isolated cell system, each individual cell is in essence short circuited. Any transport of electrolyte must go through the cell and is dependent on the interior electrochemical potential compared to the cell exterior and the enzymes necessary for maintaining that gradient, particularly (Na^+/K^+)-ATPase. If crypt cells have a basolateral coupled NaCl entry mechanism similar to a mucosal

mechanism in villus cells, ST addition will equally inhibit sodium entry in both fractions and will have no observable effect. This explanation does not explain Panichkriangkri's (1982) results. If ST does inhibit a coupled entry mechanism for NaCl, which is a primary pathway for chloride to enter the cell, a decreased amount of chloride would be expected within the cell, and a decrease in the rate of chloride efflux might be expected. The opposite results suggest that chloride is accumulating within the cell and subsequently moving out of the cell.

The observation of Gall et al. (1977) that sodium efflux levels are decreased in crypt cells, coupled with the histological results, may explain some of the efflux data presented here. The presence of the very basal crypt cells in most histological sections indicates the third fraction may not have had the complete complement of crypt cells, but may have been made up primarily of poorly differentiated cells from the base of the villus, where efflux values may have been similar to fractions I and II.

Sucrase levels generally followed the results of other investigators (Gall et al., 1977; Nordstrom et al., 1968), but the actual values did not change as dramatically between fractions. The histopathology results showing remnants of crypt cells explain the relatively high sucrase levels in fraction III compared to fractions I and II. An actual gradient of cells was obtained, as demonstrated by the change in sucrase levels. However, the remnant of crypt cells may indicate that marked differences in populations of cells for fractions I, II, and III, in regard to transport characteristics, was not achieved.

The transport capabilities of the poorly differentiated cells from the base of the villus may have been similar enough to those of fraction II to prohibit observation of any difference in active transport between fraction. It is possible that the few crypt cells remaining after vibration had transport capabilities quite different from the villus tip and mid-villus cells.

It is interesting to note that Whipp et al. (1984) have shown that ST has a significantly smaller effect on fluid accumulation in ligated intestinal loops in swine exposed to Transmissible gastroenteritis virus 24 hours previously compared to noninfected controls. TGE causes necrosis of the villus enterocytes and spares the crypts. These results would appear to implicate the villus cells as the site of ST induced secretion in the pig. This correlates well with the linkage of ST and cGMP and location of guanylate cyclase. Most guanylate cyclase in the small intestine has been localized at the villus cell, with the highest activity in the brush border (Quill and Weiser, 1975; DeJonge, 1975). ST may not have an effect on crypt cells. The results of Panichkriangkri (1982) do not agree with this hypothesis, as she demonstrated increased chloride efflux in all three fractions in ST-treated cells. The results of the sodium efflux studies given here suggest that ST does not have an observable effect on sodium efflux from mature and immature cells isolated from the intestinal villus.

SUMMARY

A gradient of jejunal enterocytes was isolated from 3-week-old swine. Three fractions of cells were isolated and sodium efflux was studied in all three fractions. Cells were treated with heat-stable (ST) enterotoxin, ST plus ouabain, control both, and control broth plus ouabain. Sucrase was used as a marker enzyme to determine location of cell fractions at the villus tip, mid-villus, or crypt.

Heat-stable enterotoxin did not affect sodium efflux in any fraction of cells compared to controls. Ouabain significantly inhibited sodium efflux in all fractions in both ST-treated and control broth-treated cells. The third fraction was probably a mixture of crypt and less differentiated villus-base cells. The results indicate ST does not increase sodium permeability of isolated swine enterocytes, nor does it increase active transport of sodium in isolated enterocytes.

APPENDIX

Isolation Medium

    154 mM NaCl
      2 mM Tris-HCl   310 mOsm
      3 mM K_2HPO_4
     10 mM Sucrose   pH 7.4 at 37° C.
    0.1% Albumin

Incubation Medium

    120 mM NaCl
     20 mM Tris-HCl   280 mOsm
      3 mM K_2HPO_4
      1 mM $CaCl_2$ pH 7.4 at 37° C.
     10 mM Glucose
    0.1% Albumin

Sodium-free Wash

    110 mM $MgCl_2$ 292 mOsm
      2 mM Tris-HCl
    0.1% Albumin   pH 7.4 at 4° C.

TGO Solution

    0.5 M Tris Buffer q.s. 100 ml after following reagents added:

        Glucose oxidase   (2 mg)

        Peroxidase Solution   (0.5 ml) 1 mg/ml

        Detergent Solution   (1.0 ml) 20 gm Triton X-100 in 80 gm
                                         95% ethanol

        O-dianisidine Solution (100 mg) O-dianisidine in 10 ml 95% ethanol
                                       (1.0 ml)

Substrate Buffer

    0.056 M Sucrose in 0.1 M Sodium Maleate

Table 3. Individual sodium efflux constants (K_m)[1] for ST versus CB
treatments

		SODIUM EFFLUX CONSTANTS (K_m)			
EXPERIMENT	FRACTION	CB	ST	CB	ST
7	1	-4.182	-3.938	-5.0326	-6.4035
7	2	-3.830	-4.093	-3.6071	-3.4268
7	3	-3.0256	-2.5740	-4.0669	-4.1097
8	1	-4.8599	-4.1309	-4.6732	-5.1602
8	2	-4.5865	-4.4929	-3.5912	-3.2391
8	3	-3.9598	-3.0357	-3.8109	-4.1083
9	1	-3.6964	-2.9024	-3.6479	-5.0609
9	2	-4.3569	-6.5148	-4.1287	-3.2829
9	3	.	.	-5.0158	-8.6538
10	1	-4.2501	-4.2039	-4.7197	-4.8605
10	2	-4.9415	-5.7329	-4.0929	-5.4022
10	3	-8.8267	.	-3.5881	-1.4294
11	1	-4.4705	-3.9846	-5.1964	-5.4796
11	2	-4.0117	-4.0622	-5.1936	-3.9372
11	3	-4.0103	-4.7020	-4.0957	-6.1606
12	1	-6.194	-5.793	-4.567	-6.414
12	2	-4.947	-5.283	-5.803	-4.744
12	3	-4.984	-5.643	-9.334	-6.628
16	1	-5.701	-5.659	-6.010	-5.534
16	2	-6.808	-5.153	-4.125	-10.038
16	3	-3.392	-4.269	-4.826	.
19	1	-4.388	-5.421	-4.297	-7.505
19	2	-4.309	-3.508	-5.768	-5.759
19	3	-3.882	-3.361	-2.867	-2.136

[1]K_m = Natural log of the slope of ^{22}Na efflux (sodium exchange/hour)

Table 4. Individual sodium efflux constants (K_m)[1] for ST and CB
treatments versus ST+ouabain and CB+ouabain treatments

SODIUM EFFLUX CONSTANTS

EXPERIMENT	FRACTION	Control	Control ouabain	ST	ST ouabain
2	1	-3.9062	-3.0458	-2.0689	-1.4975
2	2	-3.6960	-3.4659	-2.0078	-1.8847
2	3	-3.5988	-1.6636	-5.6506	-2.0182
3	1	-3.7179	-2.2520	-3.6808	-2.6082
3	2	-3.8007	-2.8848	-3.2636	-2.3847
3	3	-4.3951	-3.0023	-3.4905	-3.2034
4	1	-2.8422	-1.8845	-3.3618	-2.4996
4	2	-2.7383	-1.5450	-2.7013	-1.9794
4	3	-2.8154	-2.1470	-1.9973	-1.9928
5	1	-2.6451	-2.4838	-4.1117	-2.1714
5	2	-3.9201	-1.3887	-3.1413	-2.0147
5	3	-3.5722	-2.4293	-3.6132	-3.0934
6	1	-5.1465	-3.2099	-4.3708	-3.1851
6	2	-5.4946	-4.1386	-5.082	-2.773
6	3	-3.9009	-2.5712	-4.747	-3.471
17	1	-3.438	-4.582	-5.990	-4.859
17	2	-6.515	-5.906	-12.119	-5.328
17	3	-1.615	-2.823	-3.271	-3.235
18	1	-4.821	-3.610	-5.635	-3.080
18	2	-5.139	-2.874	-3.212	-2.608
18	3	-4.829	-2.357	-8.434	-1.984

[1]K_m = Natural log of the slope of ^{22}Na efflux (sodium exchange/hour)

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