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Bruce Thomas Volpe
Yale University

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THE EFFECT OF BILE SALTS ON RABBIT JEJUNUM AND RAT COLON: AS PERTAINS TO INTESTINAL SECRETORY PHENOMENA

BRUCE THOMAS VOLPE

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The Effect of Bile Salts On Rabbit Jejunum and Rat Colon: As Pertains To Intestinal Secretory Phenomena

BRUCE THOMAS VOLPE

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To Claudia ("Mrs. Jack"), Bea, and Joanna for their encouragement and help throughout day-to-day frustrations.

To Dr. Binder for prodding and persuading, but most of all teaching. His distaste for dogma, argumentative approach, and patient understanding led me to the excitement of discovery of phenomena, I am sure, he already expected.

To Nancy who had the unenviable task of dealing with me for the remainder of time not spent in the hospital labs, for unceasing presence and perspective without which I surely would have suffered a hypertensive crisis.

Dedication

To all oryctolagus cuniculus,

especially the "New Zealand whites".

....thought, he is inclined to hold, is still secreted by the brain; but then Poetry and Religion (and they are really worth knowing) are a product of the smaller intestine....

Thomas Carlyle.

The gastrointestinal tract is often portrayed as a primitive organ system, functioning merely to digest and transport nutrients into the interior of the body. Unlike other organ systems, whose unconscious functioning, for the most part, leads one to forget their crucial presence, the gastrointestinal tract daily demands attention in one way or another. Because, or perhaps in spite of this quotidian intrusion, myths and romantic notions have always existed, encouraging the simplicity of g.i. function. Simplicity of function notwithstanding, episodic gastrointestinal irregularity occurs in most individuals without underlying chronic intestinal disease, and, based on insurance disability claims this symptom is the leading cause for absence from work.[10]. Clearly the situation is not so simple and there exists a complicated series of events which define absorption, the primary function of the alimentary tract.

Absorption however involves two processes: secretion of enzymes, bile, electrolytes and water; and absorption of the digested food materials and diluting fluids. Intestinal function and dysfunction then can be analyzed in terms of absorptive capacity and secretory capacity of each of the many components which traverse the lumen. The enzymatic and physiochemical events necessary for the absorption of fat, carbohydrate and protein are extensive subjects more properly dealt with in recent reviews[58,43,59]. as well as major texts. Certainly the discussion of the movement of electrolytes and water is just as formidable a task, and has itself been the subject of many

volumes. This experimental work deals with electrolyte movement and focuses on the recently re-discovered electrolyte secretory phenomenon.

To introduce these experiments it seems appropriate to discuss the complex process of water and electrolyte absorption, more specifically movement from mucosa to serosa, or, so called, insorption. Next follows a discussion of intestinal secretion, as mentioned a relatively recent re-discovery that was almost totally neglected by physiologists after Florey and Wright's review in 1941. Even the Handbook of Physiology up to 1968, which contains one complete volume on secretion and another on absorption, had not so much as a section on intestinal secretion. Since net intestinal secretion is not apparent until the volume of fluid moving through the lumen exceeds the normal absorptive capacity, it is not an obvious process and it is pertinent to discuss the possible mechanism of active secretion and its role in diarrheal states. The pathogenesis of two of these diarrheal states, cholera and cholerheic enteropathy, provides the direct experimental and historical basis for the study that follows. The methods, results and discussion will then be presented in traditional fashion.

Experiments elucidating water and electrolyte movement in the intestine began with the development of a variety of ingenious in vitro[91,101,66]. and in vivo[4,33]. techniques. The state of the art had progressed from Sanctorius Sanctorius' first complicated total body weighing experiments demonstrating absorption in the 17th century.[30,78]. Models of the twentieth century studied absorption in vivo by recovering the absorbed

substrate or derivative from the urine. Another model measured the appearance of the absorbed materials in body tissues. Early intragastric instillation models could not control the amount of test dose delivered to the absorbing surface, and this delivery problem was not overcome until the access was direct as through chronic fistula construction (after Thiry and Vella). The first fistula was an isolated segment of bowel with an intact blood supply and with one or both ends brought to the surface. Pavlov, Oni and Heidenhain made interesting major modifications of this basic scheme all attempting to approach the physiologic state as nearly as possible. Even with control of the test dose delivered, there was the problem of measurement. Non-absorbable markers like polyethylene glycol seem to solve that problem for with a knowledge of material introduced at the beginning of the experiment and analysis of the amount remaining at the end of the experiment, taking into account the ratio of non-absorbable marker to total fluid at the start and finish, absorption could be assessed and expressed as a rate referred to a unit mass of tissue. Problems with residual fluid present before starting were overcome by several washes with some diluent, but some investigators protested, and maintained that the intestine was not a pipe and with complicated networks of pores how could one ever rid an experiment of contaminants by mere washing? By taking aliquots of preliminary wash solutions and analyzing them, a correction factor could be calculated so the amount added at the start is corrected by this standardized residuum.

Wilson's everted gut sacs and Ussing's chamber were two important in vitro models. In the sac experiments, the mesentary was stripped manually, and a glass rod was pushed through the ileal end and out the duodenum. The eversion was completed by rolling the proximal half of the intestine onto the rod and then removing the rod. One end was tied with a ligature and the other loosely tied so that the interior could be filled by a syringe and then tightly purse stringed close. The mucosa, now on the outside of the sac, was handled by exposure to large volumes of oxygen in a well stirred bath.[101]. One objection to this method arose when the glucose in the surrounding medium caused great distension of the sac, and the affect of the hydrostatic pressure became difficult to determine. Ussing, in a model that will appear often throughout this discussion, worked first with frog epithelium. He suspended the tissue between two halves of a lucite chamber, and bathed both sides with reservoirs of heated, oxygenated, mixed, buffered solutions. By placing agar bridges connected to electrodes into opposite baths, he was able to monitor potential difference and current generated in the medium. This model was refined later by Clarkson[7]. and Schultz and Zalusky[79,81]. and current measurement came to be interpreted as a reflection of ion movement. So occurred several of the highlights in the development of a more perfect model.

In any case, no matter what model, and many were used, insorption experiments were interpreted as if the observed phenomena were the result of the activity of a homogeneous group of cells all participating equally

and simultaneously in some aspect of transport. More likely, since the intestinal mucosa is a heterogeneous cell population, these classical studies represent the net difference of activities of various cell types. Assuming an ideal homogeneous membrane with classical structural features; that is, a bi-molecular lipid membrane, the presence of aqueous filled pores or channels, and the presence of a membrane carrier system, various transport mechanisms exist. They include: solvent drag, simple passive diffusion (both ionic and nonionic), facilitated diffusion, exchange diffusion and active transport.[31] In the presence of favorable electrical, pH, or activity gradients passive absorption may occur by ways of simple diffusion, or, postulating a carrier mechanism in the outer membrane, by facilitated diffusion. These forms are recognizable in that the latter usually shows substrate and steric specificity, saturation phenomenon and competitive inhibition between related transported substances.[51] Depending on the direction and magnitude of net water flow, absorption of electrolytes may occur via solvent drag, but this assumes solvent flow and ion movement are occurring through the same aqueous filled channels. Exchange diffusion accounts for rapid, synchronous and opposite movement of certain ions across the mucosa, but cannot result in the net absorption of these substances. Finally, active transport is carrier mediated, requires cellular expenditure of energy and accounts for ionic absorption against electrochemical gradients.

Debate raged over the relative importance of active transport and diffusion mechanisms. In 1957 Curran and Solomon[11] designed in vivo

studies which measured the flux ratios for sodium and chloride across the rat ileum. The bidirectional fluxes of each were followed using radioactive Na24 and Cl36, and water movement was monitored using spectrophotometric parameters. Simultaneous determinations of the electrochemical potential gradient across the intestine were also obtained.[41] The observed flux ratios were clearly higher than predictable by the Nernst[92] or Ussing[89,90] equation describing the relationship of the ionic activity of the mucosal and serosal solutions to the transmembrane potential difference. This strongly suggested active transport of the ions, and furthermore, the kinetics of the insorption of sodium in relation to the luminal concentration appeared to be those of a saturatable carrier system. In vitro preparations of rat ileum[12] also demonstrated unequivocal active sodium transport.

Schultz and Zalusky[79] applied the short circuit current of Ussing[90,91] which has been mentioned and will be discussed in some depth later, and demonstrated in rabbit ileum, that most of the current generated was due to active sodium transport. Other authors described this transport process in proximal as well as distal intestine,[31] and established an active transport mechanism for sodium across the mucosa at all levels of the bowel. In addition this transport system seemed at least in part, glucose dependent. Some reported the active transport of sodium closely related to glucose absorption,[13,14] and others went farther and reported amino acids to have a similar stimulating effect.[80] In a well known experiment Zalusky and Schultz examined the effects of alanine on short

circuit current; again with Ussing's technique. They added alanine to solutions containing different concentrations of sodium, and observed that the greatest increase in short circuit current occurred in baths where the concentration of sodium was the highest, and that alanine had no effect on the short circuit current in sodium free solutions. This supported earlier statements, that sodium movement was reflected by the short circuit current, and flux studies with radioactive ions supported this association. Moreover, they observed that once maximal stimulation with one actively transported sugar or amino acid was reached, subsequent addition of another actively transported sugar or amino acid had NO EFFECT on current. They concluded that sodium could be actively transported, that it could be coupled to sugar, or amino acid transport, that a carrier mechanism existed showing saturation phenomena, and that specificity of this mechanism for sugar or amino acid was involved.

The elucidation of water movement followed a similar pattern. Curran[11]. in the perfusion of the rat ileum, substituted mannitol for sodium chloride in order to maintain constant osmolarity with the added dimension of variation of the sodium chloride concentrations. He found that net water movement followed net solute movement. Moreover when solute movement was zero, water movement was also zero. Since net water movement could not occur in the absence of net solute movement, it seemed that water movement was a passive process dependent entirely on the active absorption of dissolved substances. The exact linking of the two had no clear mechanism except the notion that removing solute from the lumen

creates an osmotic pressure and a water activity gradient which favors water absorption. Earlier data, notably of Parsons,[67.] Fischer,[28.] and Visscher[94,95.] favored the presence of active water transport primarily because water absorption could occur against adverse osmotic pressure gradients and water activity gradients. Goldschmidt and Dayton in 1919 showed that dog colon in vivo could continue fluid absorption even when intestinal contents were 20% hypertonic to plasma. Parsons fifty years later, used rat intestine in vitro to confirm fluid absorption from hypertonic sodium chloride solutions. These experiments suggest that absorption can occur from a solution in the lumen which is hypertonic in respect to the fluid in which the segment is bathed. Water appears capable of moving against its activity gradient, a situation which is difficult to reconcile with the conclusion that water absorption is strictly a passive process.

Interpretation of this data is complicated by the fact that water movement involves an interplay of diffusion and bulk flow. The former concept is familiar, the latter depends on permeability characteristics of the membranes and pore size. These concepts make estimation of the effective osmotic pressure impossible to predict simply by knowledge of the solute concentration on both sides of the membrane. In any case these arguments were a potent threat to the notion that water absorption was a passive process. Undaunted, but ready to revise his original theory, Curran performed another series of experiments in the rat colon[15.] and developed his double membrane hypothesis,[12.] which accounts for the passive nature of water absorption in a unique manner. It also profers

an explanation for the linkage of water and solute movement, and the ability to absorb water from a hypertonic medium. In short, it is a complex membrane system, shown in figure 1, the interior of which is a separate compartment (B). The entire system exists in series. Presumably the solute is actively transported from A to B where its respective diffusion is impeded by side walls. This results in an osmotic pressure gradient with water movement from A to B; the water is following its activity gradient. Very little difference in effective osmotic pressure exists between B and C, because of the large pores separating these spaces (based on Staverman's definition of the reflection coefficient as the ratio of effective to theoretic osmotic pressure and as a manifestation of membrane pore size). Water entering B causes an increased hydrostatic pressure which will drive water from B to C, but will have little effect on water flow from B to A. The net result is movement of water from A to C, regardless of the concentration in A, and without active water transport. Nevertheless the intestine probably never normally absorbs from hypertonic medium,[36] the process

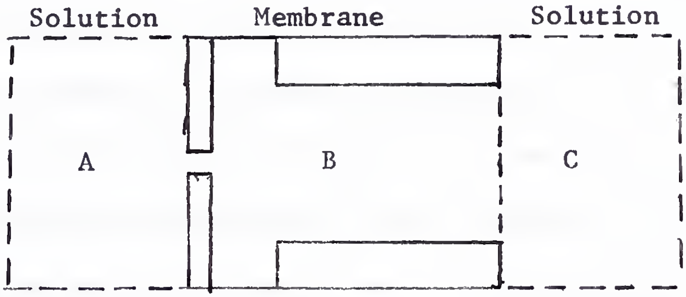


Figure 1. Curran Model of Serial Membrane Hypothesis

of osmotic equilibration begins in the stomach and continues in the duodenum. Diamond has invoked Curran's hypothesis of passive water movement to explain the development of gradients in the lateral intercellular spaces in the intestine and in the gall bladder.[16.] Water malabsorption then may be considered secondary to solute malabsorption or perhaps solute secretion.

So, sodium can be transported across the intestinal mucosa against electro-chemical activity gradients by an energy dependent process which may be related to glucose and amino acid absorption. And water absorption is considered to be passive, secondary to absorption of solute. These general features and development of absorption characteristics will aid in understanding subsequent sections of this discussion. This was not intended to be an exhaustive review of water and electrolyte absorption, but more to develop a framework so that later the postulated secretory effects of bile salts will have some physiological as well as historical experimental documentation.

Net intestinal secretion is not appreciated until the amount discharged exceeds the capacity to absorb. Application to secretory states of concepts and techniques that have been so important in advancing understanding of absorption should be equally productive in defining secretion. The first problem is a satisfactory definition of the term, for in 1924 Vincent noted that "secretion is not used in a very definite or restricted sense." In the broadest sense it came to mean the process

by which material is elaborated through some energy dependent expenditure. The traditional view that intestinal secretion constitutes an important digestive juice responsible for the final intraluminal step before absorption was abandoned when Lunderstrom-Lang in 1939[61.] indicated that most enzymatic activity attributed to the succus entericus was derived from desquamated cells. (This notion, with some minor modifications was confirmed twenty five years later.[43.]) Two years later Florey et. al.[29.] agreed with Lunderstrom-Lang's idea and tried to stimulate interest by suggesting that secretion may have "other functions besides that of contributing enzymes," and that "it may be necessary,... to keep food particles in suspension while they are attacked by pancreatic enzymes." During the present generation three mechanisms, not mutually exclusive; have been proposed to explain water and electrolyte movement into the lumen: first, transfer via filtration generated by increased tissue pressure; second, transfer via electrochemical gradients generated across the mucosa (the mucosa being defined as a semi-permeable membrane); third, via active secretion,[49.] where active secretion signifies similar characteristics to active absorption as previously developed.

Proponents of the filtration hypothesis depended on the early in vitro data of Wells, in which hydrostatic pressure gradients were established across the mucosa by exposing it to sub-atmospheric pressure.[97,98,99.] Passage of fluid under these conditions was concluded to depend on the sum of osmotic and hydrostatic pressure gradients. These experiments produced intensely swollen villi and thickened boggy

mucosa. It later became clear that these histologic changes did not have to be present for secretion to occur. Nevertheless Hakim[46,47.] also with in vitro systems found many years later that increasing the pressure on the mucosa had no effect on fluid movement. But by increasing pressure on the serosal surface a secretion phenomenon was exposed. Similar criticism, as in Wells' work, explained that the situation appeared unphysiologic. Glucose appeared in the mucosal fluid in direct proportion to the induced net fluid movement,[46.] and this reported active secretion of glucose as well as inulin suggests physical damage to the mucosa. In vivo there is no movement of glucose from serosa to mucosa even when the net water movement is changed from absorption to secretion by a hypertonic nonabsorbable toxic solute. And glucose will appear in the lumen only with solute concentrations that will damage the epithelium.[8.] Moreover, filtration seemed a less likely explanation for the secretory state because purging in cholera patients was severe and obvious in spite of severe hypotension and hypovolemia. Recently Carpenter et. al.[6.] manipulated mesenteric arterial pressure and flow by implanting blood flow transducers in dogs. They found that the secretion in response to orogastric instillation of cholera toxin was not affected by reduction of mesenteric artery pressure to well below control values. In spite of this evidence, Hendrix and Bayless[49.] conclude "it is not unlikely that net fluid and electrolyte transport rates may be modified by changes in hydrostatic pressure gradients across the mucosa."

Transfer via electrochemical gradients generated across a semipermeable membrane draw heavily on the theories developed for

absorption of fluid and electrolytes discussed earlier. This mechanism of secretion also depends on the permeability characteristics of the semi-permeable membrane-or intestinal mucosa. The jejunum and ileum have different permeability characteristics. Fordtran[34.] using Staverman's notion, measured reflection coefficients of solutes of different molecular size in hypertonic media. Of mannitol, urea, and erythritol; mannitol, which is not absorbed, exerted its full theoretical osmotic effect. The smaller molecules exerted less than full theoretical effect, since they could be partially absorbed. Assuming an idealized membrane, that is, each section of the intestine is a homogeneous membrane with uniform perforations, it is possible to calculate theoretic pore diameters and quantify some important differences between proximal and distal small bowel. The flow of water induced by osmotic gradient due to hypertonic fluid in the lumen was much greater in the proximal jejunum than in the terminal ileum. The effective pore radii were estimated to be 7.5 and 3.4 Angstroms respectively.[31.] The size of pores seemed to determine the character of the fluid that could be transported, and the direction was determined by the orientation of the gradient. Fordtran continued these "pore" studies,[35.] no pun intended, in patients with celiac disease and calculated greatly diminished pore sizes. And the net secretory state he found in the jejunum, with water, sodium and, potassium being added to the luminal fluid, could not be easily explained decreased pore size, since this fact would serve to inhibit movement in both directions.

Schmidt et. al.[77] also acknowledged jejunal secretion in patients with sprue. To a variety of isotonic perfusing solutions, including a glucose solution (which normally stimulates absorption), continuous outpouring of water and electrolytes occurred from the jejunal tissue of the sprue patients, as opposed to normal volunteers whose response depended on the perfusate. This study was unable to define a mechanism. Schmidt felt that the development of a new secretory mechanism was "unlikely to result from disease," and the findings were explained by invoking impairment of a normal absorptive mechanism revealing a functionally opposed secretory pump for sodium. In a similar light, Binder,[3] studying inflammatory bowel disease, observed jejunal diminution of non-glucose stimulated sodium, chloride and water absorption, but glucose stimulated absorption intact. Although ulcerative colitis and regional enteritis are very different entities than celiac disease, he suggested that deranged lumen-to-plasma movement in the latter offered a basis for speculation about the responsible mechanism in inflammatory bowel disease; more specifically, the active secretory pump becomes functionally unopposed. Neither study completely ruled out decreased pore size as an explanation for the severe limitation to absorption, but they did encourage the possibility that an active secretory state may be normal, but masked by absorption parameters. And this active secretion may not depend totally on filtration pressure, membrane characteristics, or electrochemical gradients. Finally it is of interest to note the area of

biological membranes occupied by pores is thought to be very small; Paganelli[65.] using the red cell, calculated the range to be 0.01 to 1.0% of the human red cell. In the intestine the relative calculated sparcity of pores and their small size preclude their identification by electron microscopy or x-ray diffraction.[31.]

Turning from membrane characteristics to electrochemical gradients and their influence on the secretory process, two recent sets of data are relevant. Taylor et. al.,[87.] attempted to support the concept that short circuit current (this is an externally applied current which reduces the potential difference across mucosal tissue suspended between two halves of a lucite chamber to zero, and will be more fully discussed later) can be accounted for by sodium movement.[7.] He showed that the discrepancy observed between sodium flux and short circuit current, in the presence of a poorly metabolized sugar, was due to an "active secretion of chloride into the lumen."[87.] This observation led them to suggest the activation of a neutral NaCl pump, or an anion secretory pump. The data may also be interpreted as a nontoxic, poorly metabolized hypertonic luminal solution establishing some electrochemical gradient favoring secretion. In a similar mode Halsted et. al.[48.] studied the intestinal fluid production induced by an intraluminal hypertonic glucose solution in rabbit jejunum in vivo. He was able to induce net secretion with the hypertonic solutions, but further found he could reduce the volume of this secretory response by adding cycloheximide, an inhibitor of protein synthesis. This suggested that fluid secretion could result

from osmotic forces and may be mediated through protein synthesis. Filtration pressure, electrochemical gradients, pore quantity and quality all undoubtedly play some role in secretory processes. Interest again turned to clinical medicine and pathological states for explanation of this active secretory process.

Men of research medicine in an earlier generation had no hesitancy in ascribing a secretory function to the intestines, even more specifically to the crypts of Lieberkuhn. Connheim in 1895 observed the natural history of cholera, examined the autopsy findings and concluded that "the process of cholera may be interpreted by supposing that first, under the influence of the virus... there takes place an extra-ordinary profuse secretion from the glands of the small intestine".[9.] Other experimenters developed this farther and these early suggestions, regarding cholera, led to a functional separation of absorption and secretion. Active secretion, in a broad sense, became that process by which material moves from the serosa to the mucosa. The process requires some expenditure of energy and has the potential to move against electrochemical gradients. Serebro et. al.[82.] looking to support this concept, used cycloheximide to poison the epithelial cell of the crypts of Lieberkuhn. Postulating that cholera toxin stimulates secretion by these crypts without altering the absorptive function of the villi, preferential damage to the crypts should modify the secretory response. Cycloheximide remember, is an inhibitor of protein synthesis. The crypt cells have a higher protein synthetic rate and are affected by the poison earlier

and at a lower dose than the absorptive columnar cells of the villi. This inhibition is reversible and the earliest morphological evidence is the disappearance of mitotic figures.[93.] At a level causing only disappearance of mitotic figures from the crypts, for higher levels will cause irreversible damage with accompanying morphological and functional change, cycloheximide inhibits the secretion of fluid that normally follows exposure of the intestinal mucosa to cholera toxin. Furthermore this inhibition of secretion had NO EFFECT on glucose absorption. The suggestions are strong indeed that some active secretory process plays a leading role in this pathologic state. Whether the direct mechanism is related to protein synthesis is undetermined at this time.

The possibility also remains as Florey and Wright[29.] suggested in the 40's and Schmid[77.] and others revived in the 60's, it may be an integral component of normal intestinal processes. Certainly the work of Powell et. al.[71.] might support this notion, for they found that the small intestine of the guinea pig spontaneously secretes into the lumen a fluid rich in sodium bicarbonate. This secretion had no effect on glucose absorption, and was present even with an unfavorable concentration gradient. The basic process of secretion could be studied without the necessity of inducing a pathological state or using pharmacological stimulants. In a subsequent work[72.] these investigators tried to establish in vitro models for this process. And calculating electrolyte fluxes, after the method of Schultz and Zalusky,[79.] the in vitro secretory state was due to net serosal to mucosal transport of sodium and bicarbonate

and net mucosal to serosal transport of chloride; similar to in vivo responses.

Certainly filtration, membrane characteristics and electrochemical gradients as stated before, contribute to the secretion process, but active secretion, perhaps from the crypts of Lieberkuhn, has stimulated a great revival. A mounting body of evidence suggests that certain humoral and pharmacological agents are responsible for net secretion of water and electrolytes. For example, Stein and Dennis in the 40's suggested that adrenalectomy decreased net NaCl and water absorption in the rat and dog. More recently Soergel et. al.[86.] demonstrated that vasopressin decreased the net rate of water and sodium movement out of the lumen of the jejunum and ileum of man, and in some instances produced net secretion. They obtained similar results by increased endogenous vasopressin induced by dehydration. Shields et. al.[83.] found that mineralocorticoids augmented sodium absorption and stimulated potassium secretion in the human colon. Secretion has been demonstrated in a variety of other models by a variety of agents including prostaglandins,[45.] especially the F2alpha fraction,[75.] gastrin,[40.] secretin,[40.] cholecystokinen,[40.] a combination of glucagon and gastrin,[1.] and most recently two new polypeptide substances isolated from the gut mucosa - vasoactive intestinal peptide and gastric inhibitory peptide.[2.] Many substances seem to have dramatic effects on intestinal secretion. Other recent data show that bile salts[62,63,88,84.] and hydroylated fatty acids; for example, ricinoleic acid from castor oil and hydroxystearic acid,

and cathartics of the antraquinone group can stimulate secretory phenomenon.[68a.] Bacterial toxins long known to induce diarrheal states are now being examined more closely regarding the mechanism of the profuse discharge. Staphlococcus aureus, clostridium perfringens, certain shigella, escherichia coli and vibrio cholera have each been the subject of investigation. Even without considering the possible secretory states in patients with mucosal disease - nontropical sprue,[77.] regional enteritis,[3.] intestinal scleroderma[68.] - the field of study is suddenly overwhelming and the list keeps growing. But two sets of data, the cholera toxin experiments and the bile salt studies, have developed into the most potent models with which active secretion may be studied. These approaches form the direct basis for the experiments that follow.

In 1885 John Snow made one of the earliest observations of cholera. He explained the fluid loss by postulating a secretory phenomenon, and this postulate was later adhered to by Connheim[9.] and Goodpasture.[42.] Not to indicate that first impressions are always the truest, but more to indicate a recurrent theme in medicine, it turns out that this early explanation was remarkably accurate. The dogma of the late 19th century however, adopted Virchow's notion that fluid loss in cholera resulted from exudation secondary to denudation of the intestinal epithelium.[44.] It remained for Watten et. al.[96.] some decades later, during the Bangkok epidemic of 1958, to describe the precise nature of fluid and electrolyte loss in 17 patients with culture proven cholera. He showed that

electrolyte and volume losses could be replaced by intravenous therapy, and that there was no evidence of protein depletion or denudation of the intestinal epithelium. Virchow's theory finally fell when the integrity of the gut mucosa was demonstrated in biopsy studies of cholera patients,[39.] (confirmed most recently with electron microscopy[21.]) and also the failure to show active protein loss into the gut during the most active phase of the illness.[44.]

In the last several years intubation studies of human cholera patients attempted to answer such questions as the site and the mechanism of fluid loss. Banwell and Pierce[44.] by means of inserting triple lumen catheters into the intestines of actively diseased patients were able to localize the fluid loss to the entire small intestine. The colon continued to exhibit net absorption of intraluminal fluid. They persued these studies infusing non-absorbable markers and radioactive Na22 and Na24 attempting to define some mechanism. There was an increase in movement of sodium from blood to lumen with no detectable change in the opposite direction. Volume seemed to follow this active solute translocation. Another important observation in man, which led to practical clinical application, but which finds its basis in the animal models describing intestinal absorption discussed earlier, is the demonstration that the addition of glucose to the intestinal perfusion solutions could produce a positive net sodium and water balance in an actively purging patient.[50,69.] The clinical management has more than pure empirical foundations, and once shock has been adequately treated by intravenous infusion, normal

hydration can be maintained by oral administration of glucose and electrolyte cocktails. Moreover these studies indicated that sodium absorption coupled to glucose is in tact during cholera, and the fluid elaboration must be due to some active secretory process.

Many approaches to cholera were being undertaken simultaneously, and as some investigators were gathering data from affected men others were developing experimental models. This is a common pattern in which the uncontrolled, destructive experiments of nature provide the initial basis for study. Certain aspects of the disease are separated and observed under controlled conditions, and experimental models define the pathophysiology. In addition a more accurate and complete understanding of normal function is derived. (This is, of course, the ideal and quite far from reality. The New York Times of January 20, 1973 reported 80,000 cases of cholera with 20,000 deaths in the last two years on the African continent.) The first successful experimental cholera model was developed in mongrel dogs by Sack and Carpenter.[73.] Eight to twenty kilogram dogs were fasted and then challenged by orogastric intubation of a broth of v. cholera organisms. The results produced a state similar to the human state; from clinical signs to site of fluid loss, to total fluid loss, to biopsy histology to lack of sequella following adequate therapy during the active illness, and finally to mortality. There was a shorter incubation period and duration,[74.] but this model predictably induced canine cholera quite similar to the human disease. Much experimenting followed, the prototype dog models were extended to rabbits, and orogastric instillation of the organism led

to open and closed loop intestinal perfusion. Of course the spin-offs from this basic model sent the immunologist to ponder toxin-gut interactions and to try to develop vaccines, while the gastroenterologist still puzzled over the secretion mechanism. Success in extracting a highly purified biologically active exotoxin from a crude culture filtrate of v. cholera provided another crucial step in the development of a more refined model. After exposure of canine or rabbit mucosa to this exotoxin, fluid absorption gradually diminishes and secretion begins. The delay of peak secretion is some three to four hours, but some change is apparent within thirty minutes. Now the maintenance of an intact animal was not necessary and more precise electrolyte flux studies could be attempted.

A convenient method for studying ion transport processes is the Ussing flux chamber, developed by H.H. Ussing in the late 1940's,[89,90.] and earlier cited with reference to Schultz's work on absorption of sodium and current measurements. Frog skin was first studied[91.] and then the technique was expanded to include small intestine where preparations were kept viable for five to six hours. Since this is the technique employed in our experiments, the detailed discussion is deferred to the methods section. Field suspended isolated sections of rabbit ileum in the Ussing flux chamber and added purified cholera exotoxin.[26.] In this manner he could monitor electrical parameters which others had shown reflected ion movement.[7,79.] More directly, he could determine the net flux of a particular ion species by introducing a radio-isotope

of the ion into one resevoir of the chamber and measure its rate of transfer to the other. This procedure was repeated in the opposite direction with either a second radioisotope of the ion in the same tissue, or on a different, but carefully paired, tissue.[24.] He found active chloride secretion toward the luminal side, and in an enlarged subsequent study[23.] he found the net absorptive flux of sodium reduced to zero. Furthermore, as the canine and human model would suggest, the addition of glucose to the luminal side of toxin treated mucosa produced a large net absorptive flux of sodium without altering the net chloride flux. There was an additional component to this series of experiments, for a few years earlier Field et. al. had examined the effects of theophylline and cyclic AMP (c-AMP) on the short circuit current across isolated rabbit ileal mucosa.[27.] He found that c-AMP added to the mucosal surface caused a large increase in short circuit current. A peak effect was reached in a few minutes, and was followed by a gradual decline toward base-line levels. The same electrical changes occurred after the addition of theophylline, a known inhibitor of the enzymatic breakdown of c-AMP. To corroborate the fact that these electrical changes measured ion movement, fluxes of radioactive ions measured thirty to sixty minutes after the addition of either c-AMP or theophylline revealed the net sodium flux reduced to zero, and the chloride flux directed from serosa to mucosa. And although reduced to zero by c-AMP and theophylline, the net absorptive flux of sodium could be restored by addition of an actively transported sugar

or amino acid.[25] The net fluxes and currents with these pharmacological mediators produced results strikingly similar to the cholera experiments. These alterations and similarities offered an explanation and a possible mechanism for the intestinal secretion in cholera.

The important test would match the exotoxin and theophylline or c-AMP in the same tissue to see if the response to one or the other were ablated. Cholera exotoxin was added to the solution bathing the mucosal side of rabbit ileal tissue and an effect was noted within thirty minutes. At ninety minutes a plateau was reached, as monitored by no further increases in the short circuit current which by now far exceeded control tissue values.[23] Theophylline or c-AMP added after the peak short circuit current had been reached caused only a small subsequent increase, whereas similar addition to control tissues (not treated with exotoxin) caused significantly larger increases. The marked reduction in short circuit current response to theophylline and c-AMP together with the similar effect on fluxes suggest that the exotoxin and the pharmacological mediators stimulate the same secretory process. And, in terms of a mechanism, the exotoxin may effect increases in adenyl cyclase activity, the catalyst for the synthesis of c-AMP from ATP.[24]

The cholera studies convincingly suggest a secretory process involving active anion secretion and mediated by c-AMP. Simultaneously investigators found that bile salts could induce secretory phenomena in addition to well documented emulsification activity. This story has a diffuse early history, that perhaps begins in 1936 with Verzar's classic monograph

on absorption from the intestine. He had worked extensively on the function of bile salts in fat absorption and was able to conclude that bile salts brought fatty acids, resulting from pancreatic lipolysis, into a diffusable form by the formation of polymolecular aggregates.[52.] This view has remarkable validity based on recent work.[56,57,53.] Also in 1936 Hartley observed the detergent like properties of bile salts and coined the term "amphipath," to indicate that "feelings" of both sympathy and antipathy to water were present. This observation has also lasted three decades and has been confirmed and expanded in recent reviews.[53,5.] Lipids can be defined as a heterogeneous group of organic compounds that include bile salts. As Hartley correctly observed there is a polar region and a non polar region conferring water and/or lipid solubility respectively. Bile salts are a special class of polar lipids called soluble swelling amphipaths which in bulk phase will aggregate above certain concentrations to form micelles. The critical concentration for the formation of micelles, the CMC, is that value at which spontaneous association of monomers to form dimers, tetramers and larger structures occurs. Increasing the concentration above this level results in an increased concentration of micelles while the concentration of monomers remains fixed.[17.] This property allows interaction with a group of insoluble non-swelling amphipaths better known as di- and triglycerides and cholesterol. It also allows interaction with insoluble swelling amphipaths better known as phospholipids and lecithin. The former group represented by cholesterol is insoluble in bulk phase and

does not interact with water. The latter group represented by lecithin interacts with water and forms "liquid-crystals," liquid because of hydrodynamic and macroscopic properties, crystal because of orderly associated arrangements and x-ray properties. Bile salts interact with both groups, solubilizing them and forming mixed micelles. And in order to accomplish the first step in a very complicated absorption scheme they incorporate lecithin, cholesterol, and fatty acids into solution for action by pancreatic lipase.[18,100,76.]

Hofmann the principle author of several cited classic references, was able to apply this physical chemical data to a clinical entity. Tappenheimer's observations made in 1878, that the terminal ileum provides active transport sites for reabsorption of bile salts, and that absorption in the proximal bowel is a passive phenomenon, had been recently confirmed.[19.] In conjunction with this observation, he proposed that the diarrhea associated with ileal disease or resection was related to loss of active absorption sites for bile salts and their subsequent activity in the colon.[52.] Since bile salts were absorbed only minimally in the colon and most were excreted, the enterohepatic circulation of this material was broken and the liver output of bile increased; hence the name cholerheic enteropathy. Hofmann and associates have since expanded the pathophysiology and suggested therapy for this situation.[55.]

This cathartic action of bile salts in the colon had been known,[64.] but the precise mechanism remained a mystery. Forth, Rummel and Glasner, in the German literature,[37.] provided the first experimental clues to the

mechanism of this transport process. They demonstrated a decrease in water and sodium absorption when bile salts were placed in isolated loops of rat large intestine. Furthermore they showed that dihydroxy bile salts (deoxycholic and chenodeoxycholic acid) were more active than trihydroxy bile (cholic acid) and that unconjugated bile salts but not conjugated forms (taurine or glycine are added at the C21 of the basic steroid configuration) produced this effect. Once again, similar to the development of the cholera story, basic biochemistry and a pathologic process combined to form a potent model of investigation of normal and abnormal physiology. Forth's interpretation of a decrease in absorption could include active secretory phenomena as well.

Several excellent in vivo and in vitro studies followed (and will be dealt with in detail in the discussion section) which observed in the hamster jejunum,[88.] rat jejunum,[84.] dog colon,[62.] and human colon[63.] net secretion of water and electrolytes during perfusion with bile salts. The results indicated that dihydroxy bile salts inhibited water and electrolyte absorption, and in some cases induced accumulation in the lumen. Trihydroxy bile salts had no significant effect. Conjugated bile salts caused no significant structural changes in the tissue whereas unconjugated bile salts produced striking morphological change. Taking this information in conjunction with the cholera experiment data, studies were attempted to elucidate the mechanism of bile salt activity. With these two threads of information we examined the relationship of the secretory activity of the cyclic AMP system to the effect of dihydroxy

and trihydroxy bile salts in the isolated rabbit jejunum and rat colon.

METHODS

All the experiments were performed under in vitro conditions in Ussing chambers. This technique was first applied to frog skin,[89,90,91]. and then to other epithelia including the small intestine. We used rabbit jejunum and rat colon. The rabbit (1-1.5 kilogram male, non-fasting) or rat (350 gram male) was anesthetized with 1.5 mg of sodium nembutal; and in the case of the rabbit 30 cm of jejunum was removed; in the case of the rat 20 cm of colon was removed. The animals were sacrificed with an overdose of nembutal. Jejunum was identified by locating the pylorus, quite prominent in the rabbit, and freeing 45 cm of bowel, presumably duodenum and discarding this section. Of the next 45 cm of bowel, the distal 30 cm was carefully removed and flushed with buffered electrolyte solution. The rat colon is easy to identify by locating the ileocecal junction, and of the next 30 cm of tissue we removed the proximal 20 cm of colon and flushed with buffered electrolyte solution. The steps that follow are identical for both species. By carefully sliding the bowel over a glass rod, the serosa could be manually stripped away, and the remaining mucosal layers were cut into squares to be suspended in the chambers. The exposed surface was 1.13 cm^2. All this stripping and cutting took place in iced buffered solutions, and the total time involved from surgical removal of the tissue to final suspension in the chambers is approximately 12-15 minutes. At all times the tissue is bathed in cold buffered solutions.

The membrane was clamped as a flat oriented sheet between two half

lucite chambers, each of which is connected to its own reservoir. The reservoirs are non-communicating and are separated by this tissue. Some interesting glass blowing assures that each reservoir is temperature controlled at 37°C, well mixed, and oxygenated. In all experiments the opposing reservoirs were of identical ionic composition and volume; the volumes being sufficiently large so that active transport activities of the membrane will cause only minimal changes in the total ionic concentration. Chemical and osmotic gradients were absent.

The electrical parameters are most important. The potential difference (PD) across the tissue was monitored through agar bridges made with the particular solution present in the reservoir, and placed nearby each membrane surface. These were connected to balanced calomel electrodes which transferred electrical parameters to a direct reading potentiometer. The initial balancing procedure measures the resistance of the fluid and the electrodes without the tissue in place. Intestinal tissue has a low resistance, so resistance of the perfusion fluid between the electrode tips and the tissue contribute significantly to total resistance. To obtain true transmembrane potential differences this extra-membrane resistance is measured and balanced. It remains essentially constant throughout the experiment. Then through more distal ports the chamber is connected to an external electric circuit, and a direct current is introduced, through Ag-AgCl electrodes, that is just sufficient to nullify the electric potential difference across the membrane. This procedure is known as short circuiting, and the amount

of current introduced is termed the short circuit current (SCC). This is really an automatic voltage-clamp technique where fast feedback electronics apply a step of potential to the membrane and then record the current necessary to keep the potential difference at a desired level. In these experiments the tissue was continuously short circuited except for periods of less than 30 seconds to record the PD. The jejunal or colonic membrane is now suspended between two solutions that are at equal electrochemical potential. Any net ion fluxes across this membrane are likely to be the specific result of an active transport process, and will be reflected by a change in the SCC.

The composition of the Ringer electrolyte bathing solution was in mM, Na-140; Cl-119.8; HCO_3-25; HPO_4-2.4; H_2PO_4-0.4; K-5.2; Ca-1.2; Mg-1.2. In the bicarbonate-free, chloride-free experiments sodium isethionate, calcium sulfate and magnesium sulfate were employed. The oxygenation was accomplished by continuous bubbling with 95% O_2 5% CO_2 in the Ringer solutions, and with 100% O_2 in the bicarbonate-free, chloride-free solution. The pH of these solutions was 7.4.

Four tissues from the same animal were studied simultaneously. The tissues were incubated in the appropriate solution, either Ringers or HCO_3, Cl free, in the absence of any other substance and then short circuited. In the rabbit jejunum, after 40 minutes when the SCC and PD had stabilized, an individual bile salt (taurochenodeoxycholic acid TCDC taurocholic acid TC) a mixed micelle (TCDC:Lecithin:2:1, or TC:Lecithin: 6:3) or theophylline was added simultaneously to both mucosal and serosal

bathing solutions. SCC was monitored continuously and PD was recorded every five minutes. Thirty five minutes later, theophylline was added to bile salt or micelle treated tissue, and bile salt was added to theophylline treated tissue. The SCC and PD were recorded for an additional 15 minutes. In the experiments with rat colon, the steps were identical except the incubation time which allows the SCC and PD to stabilize was 20 minutes instead of 40 minutes. That is, at 20 minutes bile salts, mixed micelles (TCDC:Lecithin:2:1, TCDC:Lecithin:1:1) or theophylline was added simultaneously to both mucosal and serosal bathing solutions. Thirty five minutes later theophylline was added to the micelle pre-treated tissue, and the SCC and PD were recorded through this run. The change in SCC and PD was determined by the difference between the recorded peak value after the addition of the substance in question and the value observed immediately prior to this addition. These values are listed in the table in the following section.

TCDC and TC were obtained from Calbiochem and were greater than 99% chromatographically homogeneous when tested by thin layer chromatography. Lecithin was obtained from Mann-Schwarz and was made into mixed micelles with TCDC and TC. This method required solubilizing appropriate concentrations of lecithin, TCDC or TC in methanol and then by vacuum evaporation the resulting mixture of lecithin and TCDC or TC could be re-solubilized without difficulty in Ringer solution, and made up to known molarity so to facilitate the addition process.

All results are expressed as mean ± SEM. Standard statistical calculations were performed.[85]

Finally histological studies were carried out on the tissues in order to rule out mucosal damage secondary to bile salt addition. Since it was technically difficult to obtain good sections of tissue incubated in the chambers, simultaneously with the running of an experiment in the chambers pieces of tissue were incubated in Erlenmeyer flasks. Here the tissue was subjected to the same conditions of oxygenation, heat, mixing, and bile salt addition as the tissue suspended in the chambers.

RESULTS

There are several parts to these experiments, and they can best be reviewed by examining first the response in rabbit jejunum, and then in rat colon.

In the rabbit jejunum a stable SCC and PD were observed for 75-85 minutes while mounted in the Ringer solution. The mean SCC observed (± SEM) during the initial forty minutes was 36 ± 2 $\mu A/cm^2$, 1.5 ± .08 mV/cm^2. Similar to observations in the literature[27,25]. concerning the effect of theophylline of rat and rabbit ileum, the addition of theophylline alone to both mucosal and serosal bathing solutions resulted in a prompt increase in SCC and PD. The mean increase in SCC and PD after 10 mM theophylline was added to thirty different tissues was 36 ± 2.6 $\mu amp/cm^2$ and 1.9 ± 0.17 mV/cm^2 respectively (Table IIA).

Next we examined the effect of bile salt on rabbit jejunum. Experimenting with various concentrations it soon became apparent that concentrations approaching the CMC of the respective bile salt were most effective. The addition of 2 mM TCDC (dihydroxy bile salt with a CMC value between 2-4 mM) to both mucosal and serosal solutions resulted in a mean increase of SCC and PD of 18 ± 2.6 $\mu Amp/cm^2$ and 0.5 ± 0.08 mV/cm^2 (Table IA). Further, we observed that 2 mM TC (trihydroxy bile salt) had no effect on rabbit jejunum, but 6 mM TC (the CMC for TC is 3-5 mM) caused a mean increase in SCC and PD of 19 ± 3.5 $\mu Amp/cm^2$ and 0.7 ± 0.17 mV/cm^2 (Table IA). This varied

response to different concentrations of the same bile salt, witness 2 mM TC does not have any effect but 6 mM does; and to different concentrations of different bile salts, witness 2 mM TCDC has an effect which parallels the 6 mM TC response, suggests that the basic physical chemical parameters of bile salt diffusion play an integral role in determining the magnitude of the response. Also, based on earlier discussion of current and ion movement relationships we know an increase in SCC may reflect cation movement from mucosa to serosa, or anion movement from serosa to mucosa. In this system the mucosa is negatively charged with respect to the serosa. Experiments were carried out in which the commonly transported ions bicarbonate and chloride were absent from the bathing solutions. (Table IA and IIA). Ample substitution, as explained in the methods section, insures electrochemical and osmotic equality on both sides of the tissue. In these experiments TCDC, TC and theophylline had no effect on the SCC response of the jejunum. We are suggesting that the bile salt and theophylline induced increase represents anion secretion.

In another aspect of the experiments we added 10 mM theophylline, in the usual manner, to jejunum that had been pre-treated with bile salts. (Table IIA). The theophylline response in untreated tissue had been an increase in the SCC and PD, in the order of 36 μAmp/cm^2 and 1.9 mV/cm^2; now, in bile salt pre-treated tissue the response to theophylline was cut in half. Pre-treatment with 2 mM TCDC had a post theophylline increase of 19 ± 2.6 μAmp/cm^2, 0.35 ± .08 mV/cm^2, pre-treatment with 6 mM TC had a post theophylline increase 18 ± 4.4 μAmp/cm^2,

0.6 ± 0.17 mV/cm^2. (Table IIA). We are suggesting here that the two secretagogues may be operating through similar mechanisms. And to test this hypothesis, we reversed the order of treatment. (Table IIA, part B). To rabbit jejunum pretreated with 10 mM theophylline, we added 2 mM TCDC and observed no further increment in SCC or PD. Theophylline is a phosphodiesterase inhibitor known to increase the activity of cyclic AMP (c-AMP). The increase in SCC and PD that theophylline produces is thought to be mediated by the c-AMP system. The action of bile salts in attenuating jejunal response to theophylline, as well as the inability of bile salts to effect increases in SCC and PD after pretreatment with theophylline suggests similar modes of mediation. The action of bile salts, both dihydroxy and trihydroxy may be mediated by c-AMP in the jejunum.

The next set of experiments attempts to manipulate this bile salt phenomenon. Using lecithin in one to two proportions with bile salts at the concentration already shown to be most effective, mixed micelles were added to the chambers. At 2 mM TCDC:1 mM Lecithin a small rise in SCC and PD were recorded 4 ± 1.7 $\mu A/cm^2$, 0.17 ± 0.08 mV/cm^2. (Table IA). These increases are not considered biologically or statistically significant with respect to control values. Again, a quantitatively similar response occurred with 6 mM TC:3 mM Lecithin, and a small rise in SCC and PD were recorded, 6 ± 2.6 $\mu A/cm^2$, 0.4 ± 0.17 mV/cm^2. (Table IA). The response to bile salts, as recorded, was ablated by the formation of mixed micelles. To further test this ablation, we added

10 mM theophylline to mixed micelle pretreated tissue. (Table IIA). The increase in SCC and PD post theophylline addition with 2:1:TCDC: Lecithin pretreatment was 37 ± 3.5 μA/cm^2, 1.6 ± 0.26 mV/cm^2. The response after 10 mM theophylline addition with 6:3:TC:Lecithin pretreatment was 28 ± 7.9 μA/cm^2, 1.2 ± 0.26 mV/cm^2. Both pieces of data are remarkably similar to the values received in tissues treated with theophylline alone. The suggestion is that mixed micelles in some manner ablate the effect of bile salts in the rabbit jejunum.

Our final studies were in the rat colon, and were undertaken to investigate the possibility of a similar response pattern in the colon. The presence of bile salts alone in the lumen of both jejunum and colon is unlikely in normal physiologic states, but may be present in diseased states. In any case, as with the rabbit jejunum, all additions of materials were equally delivered to solutions bathing both mucosal and serosal sides. Adding 10 mM theophylline confirmed earlier work in this laboratory by Binder (in press) in that a rise in SCC and PD was immediately perceived, 123 ± 23 μA/cm^2, 3.6 ± 0.8 mV/cm^2. (Table IIB). Addition of 2 mM TCDC, again confirming Binder's observations, caused an increase in SCC and PD, 121 ± 14 μA/cm^2, 3.6 ± 1.1 mV/cm^2. (Table IB). Prior treatment of colon with 2 mM TCDC effectively ablated the subsequent response to theophylline, (Table IIB), a qualitatively similar observation to the rabbit jejunum results. This supports our previous suggestion that theophylline and bile salts may work through similar mechanisms. It agrees with earlier work from this laboratory which demonstrated bile

salt stimulation of the rat colon through active anion secretion, presumably mediated by c-AMP.

To attempt to confirm the observation that mixed micelles effectively ablate the effect of bile salts in the jejunum, we added mixed micelles to the rat colon. With 2:1:TCDC:Lecithin, a reduction of the usual bile salt induced increase in SCC and PD occurred, 76 ± 15 $\mu A/cm^2$, 3.1 ± 0.6 mV/cm^2. But when the lecithin concentration was increased so the ratio was 2:2:TCDC:Lecithin a biologically and statistically significant ($p < 0.001$) ablation of the bile salt induced increase resulted, 22 ± 6.0 $\mu A/cm^2$, 0.88 ± 0.6 mV/cm^2. (Table IB). Qualitatively similar responses to rabbit jejunum data were observed when 10 mM theophylline was added to tissue pretreated with mixed micelles. In tissues pretreated with 2:1:TCDC:Lecithin subsequent theophylline addition caused an increase of 8.8 ± 2.6 $\mu A/cm^2$, .08 ± .08 mV/cm^2. In tissues pretreated with 2:2:TCDC:Lecithin, subsequent theophylline addition caused an increase statistically similar to tissues treated with theophylline alone, 73 ± 19 $\mu A/cm^2$, 1.9 ± .44 mV/cm^2. (Table IIB). This data supports an earlier suggestion that the mixed micellar form of the bile salts is unable to increase SCC and effect water and electrolyte movement that accompany these electrical changes.

In order to crystallize the phenomena that these data portray, we have compared the increments in SCC to control tissue SCC increments in each individual experiment, and then repeated standard statistical calculations to verify this mode of presentation. (See Table III). The

graphs for the jejunum and the colon, expressed in this manner, (Figure II) are remarkably similar and suggest a generalized intestinal response to bile salts modified by lecithin.

In the 40" Incubation Period, (Figure II) (Table IIIA), the control (clear bar) represents the increase in SCC in rabbit jejunum and rat colon upon addition of 2 mM TCDC or 6 mM TC, and these values (Table IA and IB) are taken as the 100% response. The horizontal cross hatched bars represent jejunal and colonic response to bile salts modified by lecithin, expressed as a percentage of the response to pure bile salt addition.

In the 75" Incubation Period (Figure II) (Table IIIB), the control (clear bar) represents the increase in SCC in rabbit jejunum and rat colon upon addition of 10 mM theophylline, and these values (Table IIA and IIB) are taken as the 100% response. The horizontal cross hatched bars represent the theophylline response of tissue <u>pretreated</u> with bile salts modified by lecithin, and expressed as a percentage of the theophylline response with no pretreatment. The diagonal cross hatched bars represent the theophylline response of tissue <u>pretreated</u> with bile salts only, and expressed again as a percentage of the theophylline response with no pretreatment. The differences between the effects of bile salts alone and in mixed micellar form is statistically significant, and it is apparent that the effects are similar in jejunum and colon.

Finally, histologic studies, as explained in the methods section, were undertaken. When examined under light microscope there was no signi-

ficant histologic change among tissues treated with bile salts, theophylline, mixed micelles and untreated tissues.

TABLE IA

Effect of bile salts on SCC and PD in rabbit jejunum (μA/cm^2, mV/cm^2 ± SEM); using standard Ringer's solution, and HCO_3-free, Na isethionate solution.

Treatment Agent (N)	Ringer's Solution		HCO_3, Cl-free Solution	
	SCC	PD	SCC	PD
2 mM TCDC (17)	18 ± 2.6	0.5 ± .08	(6) 2 ± 1	0
2:1::TCDC:Lec (10)	4 ± 1.7	0.17 ± .08		
6 mM TC (13)	19 ± 3.5	0.7 ± .17	(4) 1 ± .5	0
2:1::TC:Lec (11)	6 ± 2.6	0.4 ± .17		
6 mM TC (13)	19 ± 3.5	0.7 ± .17		
2 mM TC (12)	0	0		

TABLE IB

Effect of bile salts on SCC and PD in rat colon (μA/cm^2, mV/cm^2 ± SEM).

Treatment Agent (N)	Ringer's Solution	
	SCC	PD
2 mM TCDC (6)	121 ± 14	3.6 ± 1.1
2:1::TCDC:Lec (5)	76 ± 15	3.1 ± 0.6
1:1::TCDC:Lec (5)	22 ± 6	0.9 ± 0.6

TABLE IIA

Effect of pretreatment of rabbit jejunum alternately with bile salts and with theophylline (μA/cm^2, mV/cm^2 ± SEM), in Ringer's Solution, and HCO$_3$, Cl-free, Na isethionate solution.

	A. Theophylline Addition				
Pretreatment Agent (N)	Ringer's Solution		HCO$_3$, Cl-free Solution		
	SCC	PD		SCC	PD
None (30)	36 ± 2.6	1.9 ± .17	(6)	6 ± 4.3	.12 ± .05
2 mM TCDC (17)	19 ± 2.6	.35 ± .08	(6)	4 ± 1.5	.12 ± .05
2:1::TCDC:Lec (10)	37 ± 3.5	1.6 ± .26			
6 mMTC (13)	18 ± 4.4	0.6 ± .17	(4)	10 ± 4	.25 ± .1
2:1::TC:Lec (11)	28 ± 7.9	1.2 ± .26			
6 mM TC (13)	18 ± 4.4	0.6 ± .17			
2 mM TC (12)	20 ± 1.8	1.2 ± .04			
	B. Bile Salt Addition-2 mM TCDC				
10 mM Theophylline (8)	0	0			

TABLE IIB

Effect of pretreatment of rat colon with bile salts (μA/cm^2, mV/cm^2 ± SEM).

	A. Theophylline Addition	
Pretreatment Agent (N)	Ringer's Solution	
	SCC	PD
None (6)	123 ± 23	3.6 ± 0.8
2 mM TCDC (6)	4 ± 2.6	.02 ± .02
2:1::TCDC:Lec (5)	9 ± 2.6	.08 ± .08
1:1::TCDC:Lec (5)	73 ± 18	1.9 ± 0.4

TABLE IIIA

SCC values expressed as a percentage of each individual experimental control in rabbit jejunum and rat colon (% ± SEM).

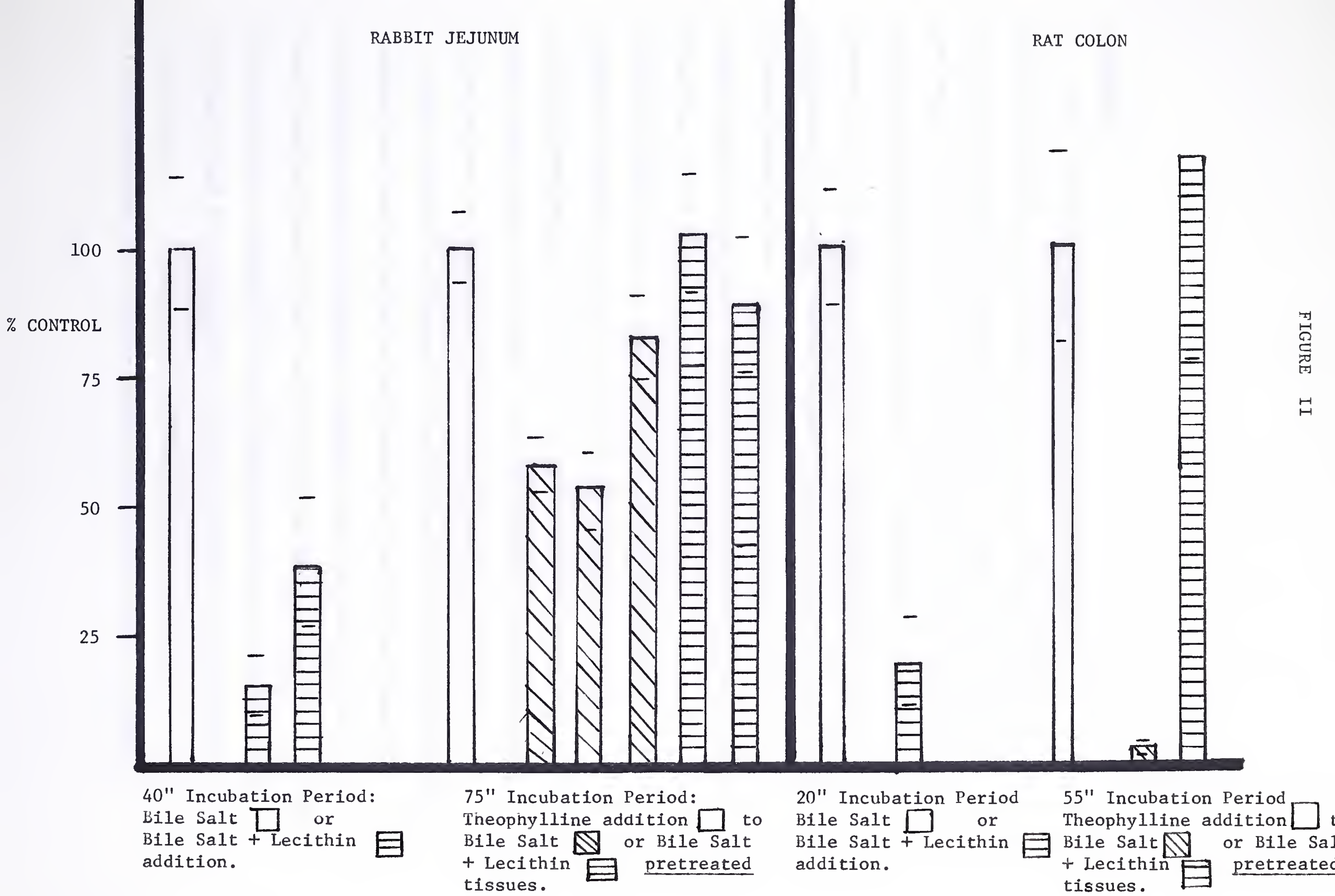
Treatment Agent (N)	Control	% Control
	Rabbit Jejunum	
2 mM TCDC (17)	100 ± 14	
6 mM TC (13)	100 ± 18	
2:1::TCDC:Lec (10)		17 ± 6
2:1::TC:Lec (11)		38 ± 14
	Rat Colon	
2 mM TCDC (6)	100 ± 11	
2:1::TCDC:Lec (5)		69 ± 8
1:1::TCDC:Lec (5)		18 ± 9

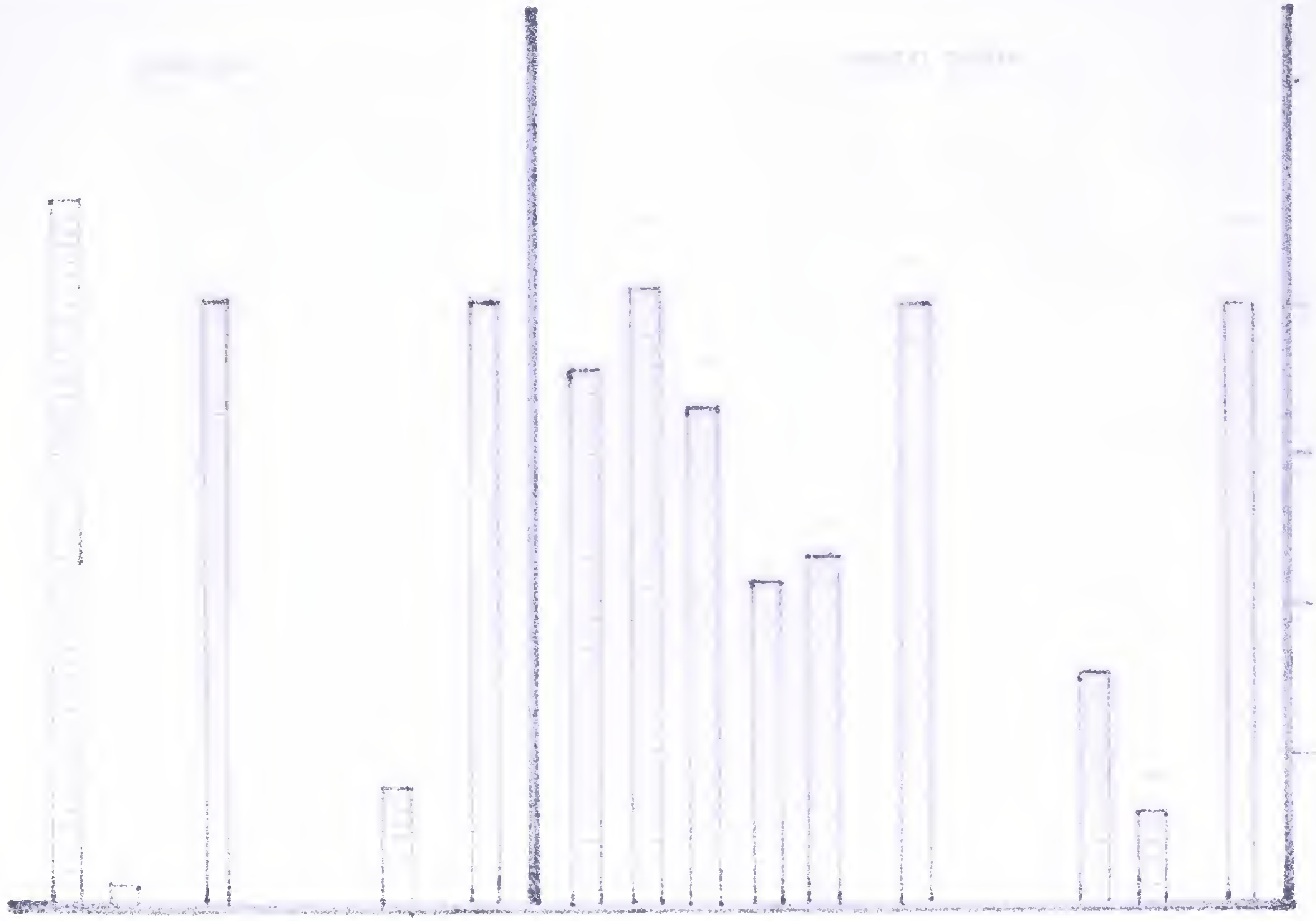
TABLE IIIB

Effect of pretreatment of rabbit jejunum and rat colon with bile salts, and bile salt:lecithin solutions. SCC values again expressed as a percentage of each individual experimental control (% ± SEM).

Pretreatment Agent (N)	Control	% Control
	Rabbit Jejunum After Theophylline Addition	
None (30)	100 ± 7	
2 mM TCDC (17)		57 ± 6
6 mM TCDC (10)		53 ± 8
2:1::TCDC:Lec (10)		101 ± 12
2:1::TC:Lec (11)		89 ± 13
2 mM TC (12)		82 ± 8
	Rat Colon After Theophylline Addition	
None (6)	100 ± 19	
2 mM TCDC (6)		2 ± 1
2:1::TCDC:Lec (5)		8 ± 4
1:1::TCDC:Lec (5)		116 ± 40

FIGURE II


RABBIT JEJUNUM
RAT COLON
% CONTROL
100
75
50
25
40" Incubation Period:
Bile Salt □ or
Bile Salt + Lecithin ▤
addition.
75" Incubation Period:
Theophylline addition □ to
Bile Salt ▨ or Bile Salt
+ Lecithin ▤ pretreated
tissues.
20" Incubation Period
Bile Salt □ or
Bile Salt + Lecithin ▤
addition.
55" Incubation Period
Theophylline addition □ t
Bile Salt ▨ or Bile Sal
+ Lecithin ▤ pretreatec
tissues.



DISCUSSION

Field's highly successful series of investigations[23,25,26,27.] with cholera enterotoxin in vivo and in vitro establish active secretion as a real and important potential in itself. Powell,[71,72.] Schmid,[77.] and Binder[3.] might extend this observation and include active secretion as a normal function which is masked by the extraordinary absorptive powers of all the intestinal segments. During the course of Field's experiments, the Ussing chamber became a potent in vitro tool with which to study these systems. With the absence of electrical, chemical and osmotic gradients and with the modifications of Schultz[79.] and Clarkson[7.] intestinal tissue can be incubated and maintained. Substances of known activity (theophylline for example) may be added and compared to substances of presumed activity (bile salts for example), and unknown activity (mixed micelles for example). Using this method we set out to clarify the action of bile salts, both dihydroxy and trihydroxy forms, in the jejunum and colon.

Forth, Rummel, and Glasner[37.] provided the first direct experimental evidence that bile salts may affect intestinal electrolyte transport. They demonstrated that bile salts placed in isolated loops of the rat large intestine caused a decrease in sodium and water absorption. Further they showed that dihydroxy bile salts were more active than trihydroxy bile salts, and that unconjugated but not conjugated forms produced this effect.

A short time later Hofmann combined data from several labs with

Forth's description of bile salt action in the colon, and proposed that the diarrhea associated with ileal disease and/or resection was related to increased quantities of bile salts entering the colon. He took the older studies of Frolicher and Laker which had been elegantly confirmed by Lack and Weiner, Lundh and Hofmann[54.] and saw the results agreed that fat was completely absorbed in the jejunum, but little demonstrable bile salt absorption occurred there. Bile salt was absorbed in the ileum, and without this absorptive segment bile salts are dumped into the colon and excreted, stimulating liver production; and hence the name cholerheic enteropathy. Hofmann has since delineated the pathophysiology and proposed a therapeutic regimen while showing that there are two separate and well defined clinical entities among patients with ileal resection and bile salt malabsorption.[55.]

These data prompted Mekhjian, Phillips and Hofmann to perfuse canine colon in vivo with unconjugated bile salts.[62.] They perfused the colon in 18 unanesthetized dogs with 3,5,10 mM deoxycholic acid, 10 mM chenodeoxycholic acid, 10 mM cholic acid, and added radioactive Na24 to all media. They found that 5 and 10 mM deoxycholic and 10 mM chenodeoxycholic inhibited water and electrolyte absorption; 3 mM deoxycholic and 10 mM cholic had no effect. There was a discharge of PAS positive material from the goblet cells, but one can ascribe this depletion to the effect of unconjugated bile salt actions. It is unlikely that the depletion is related to inhibition of absorption since similar histological changes were noted after the cholic acid perfusions, and these had no influence

on absorption. They also found that the bile salt effects were reversible. It is interesting to note that the trihydroxy had no effect, and that the dihydroxy had effects within a concentration range many have defined as its CMC. These experiments made no attempt to define a mechanism for, although the flux studies showed decreased absorption, flux studies in general with in vivo perfusions must be interpreted with care. A "decrease in absorption of Na24" may reflect any one of several possibilities, including active secretion. Other questions raised deal with the effective bile salt fraction; that is, are the potent effects in the colon mediated by micellar bile salts as concentration and hydroxylation results suggest, or are the presence of micelles irrelevant to the membrane response of these surface active compounds?

The next step by the same group was to perfuse human colon in vivo.[63]. These studies confirmed the results of the canine perfusions. Dihydroxy bile salts, conjugated or unconjugated, induced continuous but reversible secretion of water and electrolytes. These effects were most prominent when the concentration of deoxycholic increased to 6 mM and when chenodeoxycholic increased to 5 mM. Again cholic acid had no effect, this differential effect may be used to suggest a mechanism. Since they also found that bile salts were absorbed to some extent in the colon, and since there was no significant differential absorption between dihydroxy and trihydroxy, then the response of secretion must be related to intraluminal concentration perhaps micelle formation, and not to the amount absorbed. In point of fact the absorption rate of CDC at 3 mM was

24.3 μm/min and at 5 mM was 9.0 μm/min, yet water and electrolyte secretion occurred only at the higher concentration. These are important pieces of information but a mechanism is still not established.

Experimenters were also examining this bile salt phenomena in the jejunum. With in vivo studies, Feldman[22.] demonstrated in the rat jejunum that bile salts produced changes in electrical parameters similar to the effects produced by anionic detergents, and that conjugated and unconjugated bile salts produced similar results. This, in conjunction with the human colon perfusion suggests the nuclear configuration of the bile salt rather than the ionic side chain as the structural determinant of secretory activity. Sladen and Harris also studied the small intestine and they perfused rat jejunum and ileum in vivo with unconjugated bile salts and radioactive Na22.[84.] The Na flux studies suggest that Na movement from serosa to mucosa is stimulated without inhibition of movement in the opposite direction. Similar observations were made by Mekhjian but not commented upon further. Sladen perfused with glucose free as well as glucose solutions, and observed differences in PD between them, presumably reflecting the potential generated by the glucose dependent component. This added component of PD, present in the glucose containing perfusions, was abolished by bile salts, but the bile salts had no effect on the glucose independent PD, even when the Na absorption was inhibited. This coupled with evidence of increased Na flux into the lumen, suggests that bile salts may directly inhibit glucose dependent absorption and may stimulate glucose independent electrolyte transport

into the lumen. In sum however, this evidence could not define a mechanism. And on histologic study they found definite damage to most of the villous tips in those loops perfused with higher concentrations (5 mM) of unconjugated dihydroxy bile salt. There was no damage in those loops perfused with 1 mM unconjugated dihydroxy bile salt or in the controls. Cells at the tip were swollen, misshapen or lost completely, whereas the crypts appeared normal and the villous architecture was preserved. It is unlikely however that these effects on fluid and electrolyte movement are mediated by tissue damage.

Teem and Phillips with similar perfusion methodology proved this point.[88.] They perfused hamster jejunum in vivo and found dihydroxy bile salts (deoxy- and chenodeoxycholic acid) inhibited water absorption, and at highest concentrations (4 mM) induced fluid accumulation in the bowel. The conjugates of the dihydroxy bile salt had a similar effect, but cholic acid, a trihydroxy bile salt, and its conjugates had no effect. Histological observations demonstrated that unconjugated bile salts produced gross morphological damage of the hamster jejunum in vivo. These changes were in contrast to conjugated bile salts which did not alter the villous structure. This confirms in vitro data, especially from Pope et.al.[70.] which demonstrated severe histological damage associated with irreversible functional impairment upon exposure to unconjugated bile salts. However, Teem and Phillips maintain that structural damage and secretion can be dissociated with in vivo systems, since secretion of water was rapidly reversed when control solutions were

substituted for the unconjugated bile salt solutions.

Regardless of return of normal function, Frizzell[38]. would have supported this concept of a tissue damage mechanism. He used rabbit ileum in vitro in an influx apparatus that permits exposure of eight defined areas of the mucosal surface to solutions of desired composition. He observed influx of several substances after incubation with taurodeoxycholic acid, and proposed, on the basis of altered fluxes, that the bile salt may irreversibly damage the membrane structure. More probably since light and electron microscopy revealed minimal alterations, the bile salt may remove components from the membrane structure through its "detergent-like" action. But others have shown that ATPase activity of intestinal homogenates is reduced following exposure to bile salts, and, in a similar situation that bile salts, c-AMP and theophylline each inhibited Na entry into the epithelial cell of the rabbit ileum. Finally Binder, (publication in press) working in vitro with the rat colon was able to propose a mechanism. He examined the relationship between bile salts and certain aspects of the c-AMP system. On the basis of Ussing chamber work and concomitant flux studies he suggested that bile salts affect the colon by stimulating active anion secretion possibly mediated by c-AMP.

Based on this wealth of data we attempted to clarify the effects of bile salts in the rabbit jejunum and to more precisely define, and perhaps explain the differences between dihydroxy and trihydroxy bile salts. Our results show; first, conjugated dihydroxy bile salts (2 mM TCDC)

and conjugated trihydroxy bile salts (6 mM TC) increase the SCC in rabbit jejunum. Second, theophylline also increases the SCC. Third, bicarbonate and chloride are required for both the first and second to take place. Fourth, the effect of 2 mM TCDC and 6 mM TC attenuates tissue response to subsequent theophylline addition, and, similarly, pretreatment with theophylline ablates the response to subsequent bile salt addition. Fifth, mixed micelles ablate the bile salt response but not the theophylline response in both rabbit jejunum and rat colon. These data are parallel to the effect of cholera enterotoxin, theophylline and c-AMP that have been observed in the rabbit ileum when identical methodology was employed. And we are suggesting that the effect of bile salts on ion transport in the jejunum as well as the colon may be secondary to anion secretion and mediated by c-AMP.

Furthermore, the fact that 2 mM TC had no effect (Table IA, IIA, Figure II) but 2 mM TCDC and 6 mM TC had significant effects, suggests the physical chemical properties of each entity are crucial. Dietschy, in a series of papers and with various colleagues[17,18,19.] has attempted to characterize the kinetics of bile salt absorption in the rat small intestine and colon, with both in vivo and in vitro techniques. His extensive observations define an entity called the apparent permeability coefficient upon which rate of uptake is ultimately based. These relative coefficients suggest that the unconjugated bile salts were always much more permeable than the glycine conjugates which in turn were more permeable than the taurine conjugates. In addition, removal of a hydroxyl

group from the steroid nucleus markedly enhanced permeability; that is, dihydroxy was more permeable than trihydroxy. And finally he demonstrated that mixed micelles had the lowest permeability and correspondingly the lowest permeability coefficients. It becomes clear that the relative differences in vitro between dihydroxy, trihydroxy and mixed micelles are identical to the responses produced by these various bile salts in vivo[62,63,88,84]. and their respective apparent permeability coefficients parallel the electrical responses observed in these experiments. We are suggesting that the rate of bile salt penetration determines the magnitude of their effect on ion movement.

But these experiments pose more questions than explanations, perhaps because the study of bile salt effects is more complex than the study of many other water soluble compounds that exist in solution as a single species, and where molecular activity can be reflected by simple concentration. With reference to an earlier discussion, bile salts may exist as ionized and protonated monomers, or in complex micelles of various sizes and shape, and it is difficult, if not impossible, to pin-point the effective moiety. The contradictory nature of part of these results reflects this knotty problem. We have demonstrated that both dihydroxy and trihydroxy are most effective at a concentration approximating their respective CMC, the presumption has been that micelles are necessary for bile salts to effect electrical and ion changes. Yet when we add lecithin, a swelling amphipath to greatly expand the mixed micelles, and, as is generally accepted lower the CMC, the effect of the bile salts alone is

abolished. (See Table IA and IB). Not only does lecithin lower the CMC value, making micelle formation possible at a reduced concentration, but also it should enhance lipid solubility. At the same time micelle formation appears to be crucial, the experiments demonstrate they are irrelevant. The explanation probably lies in between these extremes, and depends on absorption characteristics for each species in each particular phase. First however, we must note the existence of an unstirred water layer adjacent to the membrane structure which may function as a significant barrier. With this in mind one could speculate that the highly polar trihydroxy would penetrate this lipid membrane less well than the dihydroxy, and that the mixed micelle should penetrate least well of all, on the basis of dimensions and enhanced lipid solubility. This alternative proposal establishes the micellar forms as convenient carrier systems that primarily function in fat emulsification and pancreatic lipolysis. And the final membrane penetration for the products of lipolysis and the bile salts themselves probably depends on the phase of the species. The phase in turn would reflect a multi-variable equilibrium describing the state of each component between the micellar form and the monomer form.

Wilson and Dietschy have recently studied this problem, and lend support to our speculation. Due to the polarity, the trihydroxy permeates the unstirred water layer more rapidly than the cell membrane, and this latter barrier becomes the rate limiting stage. With less polar bile salts, dihydroxys, the cell membrane is permeated more rapidly, but

the unstirred water layer exerts some significant resistance. That the unstirred water layer is even more rate limiting for expanded mixed micelles is indicated by the facts that rate of bile salt absorption from such solutions is much lower than from corresponding monomer solutions.[100.] Furthermore it seems unlikely that micelles are absorbed across the aqueous-lipid interface intact. Since the rate of mucosal uptake plateaus at a constant value when the CMC is reached, and since different components of the mixed micelle are taken up at different rates then bile salt absorption must be explained in terms of monomers in equilibrium with micelles. It then becomes very difficult to know what stage of bile salt-lecithin-cholesterol aggregation is present at the aqueous-lipid interface. Do micelles carry their products and themselves all the way to the cell membrane surface, or does the water layer sufficiently retard its complete passage? Answers to questions like these are important if we are ever to know the mechanism by which intestinal epithelial cells handle dietary lipid, water and electrolytes.

In spite of these problems and contradictions, these studies have some clinical relevance, for diarrhea is a historic and ubiquitous expression of absorption-secretion imbalance.

> Diarrhea consists of the discharge of undigested food in a fluid state... when the heat does not digest the food, nor convert it into its proper chyme, but leaves its work half finished. For it is liquid and wants consistence from not being completely elaborated, and from no part of the digestive process having been properly done, except the commencement.
>
> Areteus the Cappadocian
> (2nd to 3rd century A.D.)[68a]

A century after Hippocrates named the malfunction diarrhea - to flow through, Areteus' description needs little further modification. But the pathophysiology underlying this symptom has grown after two millennia and is a function of multiple variables which are reflected in multiple disease states. The differential diagnosis of this symptom range from infectious, hormonal, and malignant processes to inflammatory, structural and neurological malfunctions and finally to iatrogenic including surgery as well as cathartic abuse and idiopathic conditions. In our study, aside from the problems of luminal transport, we have observed the potent secretory effects of bile salts in the jejunum. These effects stimulate speculation about a specific diarrheal process-malabsorption of fats with attendant water and electrolyte secretion-and the role of bile salts in this process.

Clinical situations with extensive intestinal resection (including total gastric resection, but especially terminal small bowel resection), with intestinal bacterial overgrowth (including "blind loop syndrome", strictures, jejunal diverticulosis, and intestinal scleroderma), with pancreatic insufficiency (including relative pancreatic insufficiency in the face of gastric hypersection), with Laennec's cirrhosis, with inflammatory disease (including ulcerative colitis and regional enteritis), and with mucosal disease (including adult celiac disease and Whipple's disease) - all show impaired fat digestion, probably poor micelle formation and potential direct bile salt effects on jejunal tissue. We have already discussed Hofmann's description of the pathophysiology of ileal

resection, and how bile salts have a direct cathartic effect in the colon. But concentrations of concentrated bile salts below the CMC in the jejunum, consistent with a decrease in total bile salt pool due to enterohepatic interruption, may be the major factor which reduces mixed micelle formation, and permits a direct secretory effect of the bile salts. Acid hypersecretion has been described in patients following massive intestinal resection. A relatively acid pH in the proximal jejunum would certainly be associated with impaired lipolysis and decreased amounts of fatty acids available for mixed micelle formation. This pH factor may be important in cases of pancreatic insufficiency also. Lipolysis in these cases is reduced regardless of the pH, due to a decrease in enzyme production, but decreases in enzyme poor, bicarbonate rich secretion would establish acid pH values in the jejunum and subsequent impaired micelle formation. Obviously gastric acid hypersecretion secondary to any number of problems, may present the same picture; the pancreatic insufficiency in this case is of a relative nature.

There have been associations between pancreatic insufficiency and liver disease. The attendant malabsorption here may be due to decreases in hepatic synthesis or hepatic excretion of conjugated bile salt. Certainly this may be the etiology of diarrhea in obstructive biliary tract disease, or obstructive liver disease.

The bacterial population of the upper small intestine in man is normally less than 10^3 organisms per ml. The variety of intestinal

problems causing bacterial overgrowth are legion. Bacteria can deconjugate bile salts, they are also able to convert chenodeoxycholic to lithocholic, and cholic to deoxycholic. There is a great controversy over the hepatotoxicity and possible cholestatic effects of lithocholic acid, and whether this is relevant to human disease. But, in view of the earlier discussion, the unconjugated ionic bile salt was always much more permeable than conjugated forms. Penetration in this manner being greater, we might expect a direct secretory effect of water and electrolytes, in addition to the documented fat malabsorption.

Speculation about inflammatory disease and mucosal disease is most difficult. Investigators have demonstrated a continuous net outpouring of water and electrolytes perhaps secondary to impaired lumen to plasma movement, but it is improbable that bile salts have a direct role in these states. One may suggest impaired cellular transport of lipid, or an abnormality in luminal lipid distribution, or bacterial overgrowth but these possibilities are difficult to support.

In sum, bile salts have potent intraluminal effects. They emulsify fats and insoluble lipids for action by digestive enzymes, and they facilitate absorption. In order to accomplish this, they form micelles with a swelling amphipath - lecithin - and shuttle components through unstirred water layers to the cell membrane. Their ability to move through different resistive barriers and effect absorption or turn on a potent secretory phenomenon is based on multiple physical chemical parameters, including, and perhaps more important, their own equilibrium

phase. The proximal small bowel absorbs very small quantities of bile salts, and those that it does absorb move passively and obey physical chemical laws of polarity, solubility and partitioning. They can exert a secretory effect at the jejunal level, similar to their effect in the colon, which is manifested by anion movement from serosa to mucosa and presumably mediated by cyclic AMP. This secretory phenomenon may be present normally and may reflect intestinal salt and water regulation which in many cases would only be made evident when the intestine decreased its ability for absorption. Or it may be the direct effect of bile salts unmodified by lecithin and other amphipaths and therefore a possible etiological mechanism for several pathological clinical entities.

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