Prospects for Effective Non-antimicrobial Antidiarrheal Agents

Conference on Non-antimicrobial Antidiarrheal Agents

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INTRODUCTION

Prospects for Effective Non-antimicrobial Antidiarrheal Agents

MARK DONOWITZ, MD, MICHAEL FIELD, MD, and DON W. POWELL, MD

This meeting, organized by the Division of Digestive Diseases and Nutrition of the National Institute of Diabetes, and Digestive and Kidney Diseases (NIDDK), sought to determine the current status of research into the pathophysiology and therapy of diarrheal diseases and whether it would be appropriate for NIDDK to sponsor new specific areas of research. The conference was held on May 8 and 9, 1986, on the NIH campus in Bethesda, Maryland. On the first day, an overview of diarrheal diseases in both developed and developing nations was presented, as well as a summary of advances in the understanding of epithelial transport processes and their regulation, with an emphasis on identification of transport proteins and transport-regulating proteins. The second day consisted of a summary of previous drug trials in diarrheal disease in humans and of studies on the effects of drugs on intestinal water and electrolyte transport. These presentations reviewed the use of animal models to predict which drugs might be useful in the treatment of human diseases.

The overview presentations on the first day and the discussions that followed established that the treatment for acute diarrheal diseases in humans is not being studied in a systematic manner in the United States or in Western Europe. The prevalence of diarrheal diseases in developed nations is not known. Furthermore, use of drug therapy for severe, chronic secretory diarrheas has not been evaluated in an organized, controlled manner. In the developing countries, multiple drug trials for acute diarrhea are being conducted with an emphasis on the use of various forms of oral rehydration solutions. However, even in these countries, the true prevalence of diarrheal diseases has not been clearly defined.

The major message to come from the transport process-regulation part of the meeting was that it appears that investigators have made significant progress towards identifying important transport proteins and understanding how they are regulated in various epithelial tissues. However, to date, no intestinal transport protein has been defined adequately enough to be certain of its size or molecular structure. Regulation of the transport proteins via phosphorylation–dephosphorylation, a theme common to channel proteins, is probably involved in regulation of other epithelial electrolyte carrier proteins as well.

In contrast to these advances in basic science, the second day of the meeting revealed less progress in developing effective therapies for diarrhea. The major advance in drug therapy of diarrheal diseases at the patient level continues to be in the realm of oral rehydration. It was concluded that more research was needed to clarify whether adding several types of transported solutes to a single solution, as may be accomplished by adding amino acids to the classic glucose-containing oral rehydration solution or by making such solutions with broths of cereals or grain, may result in solutions that are not only useful for oral rehydration, but that might actually decrease stool output as well. It has become clear that, thus far, drugs which appear to be potentially useful for the reduction of intestinal secretion, as determined by studies which use current in vivo or in vitro animal models, have not provided a major clinical advance. Although such agents do often decrease stool volume in humans, the reduction has
seldom been of clinical significance or else the side effects of the drug have proven to be limiting factors. The most exciting approach to drug therapy which evolved from this part of the meeting was the concept of using chloride channel blockers to inhibit the secretory process. To date, however, none have been tried, even in animal models.

At the request of Drs. Vay Liang W. Go and Dr. G.G. Roussos of NIDDK, the participants spent some time at the end of the conference discussing what they thought the NIH should support in the area of intestinal epithelial transport research and diarrhea therapy development. It was not surprising that most of the conference participants felt that their area of research was especially relevant and should be given special priority! Therefore, it was left up to the three organizers (Drs. Donowitz, Field, and Powell) to come up with recommendations to be submitted to the NIH as prospective program announcements, requests for applications, or contracts. Copies of these are included at the end of this conference proceedings. It should be emphasized that the views presented in these initiatives represent the opinions of the three meeting organizers, albeit based on the suggestions and input of all the participants at the meeting. At the time of this publication, the NIH has not yet committed itself to supporting any of these initiatives.

Abstracts from the Conference on Non-antimicrobial Antidiarrheal Agents

DIARRHEA IN DEVELOPING COUNTRIES. W.B. Greenough, III, Department of Medicine, Francis Scott Key Hospital, Baltimore, Maryland 21224.

Diarrhea is not reportable as a disease or a group of diseases to governments or world bodies. Even individual categories of the most severe of diarrheal diseases such as cholera, which are reportable, do not have effective surveillance mechanisms or incentives to ensure that what is reported reflects what actually exists in areas or populations of interest. Thus, when discussing diarrhea in either developing or developed countries, the data base is extraordinarily poor and our level of ignorance high.

In the last decade there has been an increasing interest and awareness that diarrhea is one of the largest and most destructive of global health problems. Since 1978 with the establishment of the Cholera Research Laboratory as the International Center for Diarrheal Disease Research, Bangladesh (ICDDR.B), the institution of the Control Program for Diarrheal Diseases of the World Health Organization, and the emphasis and impetus given to attacking the problem of diarrhea in children by UNICEF, there is at least an awareness of the need for better information.

From the best available estimates, diarrhea kills more individuals, mainly children, than any other disease or group of diseases, excepting perhaps acute respiratory illnesses. Since both groups are seasonal in occurrence and varying in etiologies from year to year, it is fruitless to try to say which is number one. From one third to one half of all childhood deaths are due to diarrhea. If the complication of malabsorption or malnutrition is included, the toll is greater. Now growing from well-focused basic research efforts, however, are not only highly effective low-cost curative measures available, but also preventive methods.

At this conference we shall be considering improvements to methods that are principally curative. It should be noted that rapid effective treatment even with hydration alone has tended to reduce not only immediate complications related to dehydration but also the later even more devastating chronic problem of poor nutrition and growth. Thus it is probable that, as it is possible to combine effective antidiarrheal drugs with oral rehydration therapy (ORT), we will enter an era when the requirement for high-cost, high-technology health care setting will not be required for a very common illness. This will greatly reduce the costs to developing countries which can ill afford to expend limited health resources on replicating costly hospitals and clinics that would be needed to provide intravenous fluids to large numbers of patients.

In my mind the goal of research on antidiarrheal agents is first to discover the potentially most effective drugs, then test them in clinical settings first alone and then in conjunction with the best ORT methods which maximize absorptive capacities of the gut. Such goals will ensure that when applied, morbidity will be minimized for both short- and long-term complications. In developing countries this will save many lives and reduce costs. In developed countries, since hospitals are very accessible, there will be principally reduction in the requirement for hospitalization with its attendant costs and morbidity.

A final caveat, however, is that should the agents alone or in combination with ORT be totally efficient in stopping diarrhea, we will need to be alert for the possibility that some infections which are now noninvasive and expelled from the body might become resident and entrenched in the gut and enter the blood stream and lymphatics. In the case of diseases already known to be invasive, this is of particular concern.

DISABLING DIARRHEA IN DEVELOPED COUNTRIES. H.J. Binder, Department of Internal Medicine, Yale University, New Haven, Connecticut 06610.

Both acute and chronic diarrhea in developed countries including the United States causes significant morbidity, but the exact magnitude of this problem is unknown. The inability to provide a quantitative assessment, with any degree of certainty, of disability(s) that various diarrheal diseases produce is a result of
at least three factors: (1) Diarrhea is a symptom and sign (not a disease) and occurs in a large number of diverse circumstances; (2) there is a lack of adequate epidemiology of various diarrheal illnesses; and (3) diarrhea is frequently treated by both patients and physicians as a "closset" condition, ie, a subject that is not discussed by polite (or not so polite) people. Despite the considerable lack of accurate information, it is evident that the disabilities that result from diarrheal illness include (1) a decrease in productivity (since travelers' diarrhea affects individuals both on business and on vacation, is it possible to measure in productivity terms a holiday spoiled by "tourista"?), and (2) a change in life-style. It is readily evident that it is extremely difficult to measure such changes which include an individual's diminished expectations or achievement that may occur as a result of his or her disease.

There are four general approaches to attempt to decrease the disabilities induced by diarrhea: One, we must improve the ability of physicians to make specific diagnoses; second, we must develop specific therapy for as many diarrheal disorders as possible; third, we must increase medical understanding of the pathogenesis and pathophysiology of diarrheal disorders so that even if a specific therapy for the underlying disease is not available, therapy could still be directed toward the pathogenic mechanism(s); and fourth, we must develop improved empiric therapy so that effective treatment of diarrhea would be available even in the absence of a specific diagnosis or in the presence of a disorder without a specific therapy or a treatable pathogenic process.

Although the intensity of symptoms in various diarrheal disorders varies greatly, the magnitude of the total disability on society resulting from diarrheal disease will be the product of three important variables: duration and severity of symptoms as well as the number of individuals affected. Thus, although acute nonbloody diarrhea in both travelers and nontravelers occurs in a relatively large number of individuals (approximately 11 million U.S. citizens traveled abroad in 1985 including 3 million to Mexico) and can totally interrupt an individual's activities, most of the symptoms completely resolve within three to six days. In contrast, chronic idiopathic diarrhea, which is often labeled as "functional," frequently affects individuals only "modestly" but usually over a very prolonged period of time. At the other end of the spectrum, there are, fortunately, relatively few individuals who are profoundly affected with symptoms produced by the short-bowel syndrome or hormonally induced secretory diarrhea.

The consequences of the disabilities produced by diarrhea in developed countries will be diminished both by improved patient and physician understanding and awareness of the diseases and disabilities and by improved symptomatic therapy. A significant increase in the study of the epidemiology of diarrheal disorders is required to provide a more complete understanding of the dimensions of the problem(s) induced by acute and chronic diarrheal disorders in the United States and other Western developed countries.

MECHANISM OF ACTION OF SOME BACTERIAL ENTEROTOXINS. D.M. Gill, Department of Molecular Biology and Microbiology, Tufts University, Boston, Massachusetts 02111.

Cholera toxin (CT) and E. coli heat-labile toxin (LT). We still know little about how CT and LT are internalized. Unlike most toxins, which must be endocytosed before entry, CT and LT do not pass through an acidic cellular compartment. While these toxins must contact phospholipids, no specific lipid-associating regions have been identified. Results of experiments using lipophilic photoaffinity reagents have been unclear. The peptide bond which links subunits A1 and A2 must be cleaved before entry; however, it is not known when the disulfide bond which connects these subunits is reduced.

Inside the cell, ADP-riboylation is carried out by the reduced A1 subunit. This has limited activity on its own but is greatly activated by interacting with a cellular GTP-binding protein membrane protein known as G. It seems likely that a series of events occur:

1. Inactive S + GTP → active S (this step requires the protein CF, described below)
2. Active S + inactive A1 → active A1
3. Active A1 catalyzes NAD + G, (composed of subunits αβγ) → ADPR-αβγ
4. ADPR-αβγ plus GTP results in high-level activation of adenylate cyclase

CF is a recently purified soluble protein which greatly enhances S activity. It might act by helping to load S with GTP by displacing bound GDP. By analogy with other known GTP-binding proteins of the membrane (Gs, Gt, transducin, p21ras, etc), we guess that the physiologic role of S is to transduce some external signal to an unknown internal system and that cholera toxin has paralyzed this mechanism. It is not clear why an extra activation step is advantageous for the toxin.

This activation explains why GTP is required for high toxin activity in vitro. GTP binding to the toxin substrate is not required; indeed, the major target (Gt) is a better substrate if it is not bound to a guanine nucleotide.

How does ADPR-Gt activate adenylate cyclase? Studies with isolated components suggest that the α, β, and γ subunits of Gt, dissociate on activation: Gt + GTP → αt(GTP) + βγ. The bound GDP is slowly hydrolyzed to GDP, resulting in reassociation of the subunits and loss of cyclase activity. Thus, adenylate cyclase is irreversibly activated by nonhydrolyzable analogs of GTP such as GppNHp. ADP-riboylation substantially reduces hydrolysis of GTP, thus GTP and GppNHp become equally good activators of cyclase CT-treated membranes. ADP-riboylation of Gt appears to be an irreversible modification, thus pharmacological intervention is unlikely to correct the cholera toxin effect to activate Gt and thus activate adenylate cyclase.

Pertussis toxin (PT)

PT catalyzes a similar reaction to CT, but it has as a substrate a different membrane-bound GTP-binding protein, Gt: αγ + NAD → αγ-ADPR (Gt, like Gs is made up of a complex αβγ).

As with CT, the αβγ complex is a good substrate for pertussis toxin while the dissociated form, αt (GTP), is a poor one. PT does not require S and CF, and, consequently, GTP is inhibitory to the toxin reaction. PT does, however, bind and require ATP.

Is PT an enterotoxin? Certainly not in the disease in which it is not present in the gut. The ADP-riboylation of Gt could potentially alter intestinal transport by a number of routes when the toxin is used as a chemical tool.

PATHOPHYSIOLOGY OF ENTEROINVASIVE BACTERIAL DIARRHEAS. Thomas L. Hale, Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20012.
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The role of these toxins in the disease process is unclear because only enteroinvasive strains of Salmonella or Shigella can induce diarrhea regardless of the toxin produced. In S. typhimurium-infected rhesus monkeys, for example, net secretion of fluid from the ileum and jejunum is correlated with bacterial invasion of the intestinal mucosa and with the accompanying inflammation. Transport abnormalities probably arise from the combined action of the cholera-like Salmonella enterotoxin and prostaglandins released from infected host tissue. Both these effectors cause elevation of cyclic nucleotide levels in enterocytes.

In contrast to salmonellosis, diarrhea caused by shigellosis is a symptom of net jejunal secretion in the absence of bacterial invasion or inflammation. In addition, both diarrhea and dysentery are associated with the colonic malabsorption which accompanies localized Shigella invasion in the large bowel. It is possible that Shiga-like toxin released during the transit of organisms through the small intestine causes fluid secretion. On the other hand, ingestion of noninvasive, but fully toxigenic, S. dysenteriae does not induce diarrhea, so that the role of exogenous Shiga-like toxin remains a matter of conjecture. Regardless of the mechanism, it appears that the invasion of the colonic mucosa is a critical step in the induction of transport abnormalities, so the most effective method of preventing invasive diarrheas involves interdiction of the process of bacterial invasion.

INTERACTION OF REOVIRUSES WITH THE GASTROINTESTINAL TRACT. B.N. Fields, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115.

The mammalian reoviruses (serotypes 1, 2, 3) have been a useful group of viruses for studying the genetic and molecular determinants of viral-host and viral-cell interactions. In this report, I will describe the role of certain viral genes in interacting with the gastrointestinal tract.

The reoviruses are enteric viruses. Following peroral inoculation, reovirus travels to the small intestine. It crosses Peyer's patches through M cells where primary growth takes place. The most likely site is in macrophages. Following primary replication, there are serotype-specific differences. Reovirus T1 travels in association with lymphoid tissues and spreads in the blood. Reovirus T3 is neurotropic and travels through nerves to neural cells. After a latent period of several days, there is destruction of surface epithelial cells. Reovirus is released in the stool and transmitted via fecal-oral spread to a susceptible host.

Three viral genes have been shown to play distinct roles in interacting with the host. The S1 gene (encoding the crl protein—the viral hemagglutinin) determines tropism and the pathway of spread. It binds to a protein on cell surfaces that is chemically similar to the β-adrenergic receptor. The L2 gene, encoding the L2 spike protein, determines transmission between hosts and release from the gastrointestinal tract. It may also play a role in intestinal growth. The M2 gene, encoding the γC protein, determines sensitivity to proteases (chymotrypsin) and plays a role in growth in intestinal tissue.

These studies indicate that distinct viral components are responsible for different stages of the interaction of the reovirus with the gastrointestinal tract.

INTESTINAL TRANSPORT IN VIRAL ENTERITIS. J. Richard Hamilton, Division of Gastroenterology, Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada.

Our concepts of intestinal transport in viral enteritis are based mainly on studies of a reproducible experimental model in the piglet, transmissible gastroenteritis (TGE). A coronavirus, TGE virus invades the villus epithelium of the small intestine causing a proliferative response to the crypts and rapid migration of enterocytes onto villi as the infected cells are shed into the lumen. Watery diarrhea begins as the TGE-infected cells are shed. Available evidence indicates that rotaviruses (human rotavirus in the pig, sapovirus in the rabbit) interact similarly with the small intestine, although viral shedding may persist for a longer period after the onset of diarrhea.

The impact of TGE virus on 3-week-old weaned piglets studied 40–96 hr after experimental infection can be summarized as follows:

1. Clinical Course: Vomiting, then watery diarrhea beginning at 18-24 hr. Vomiting ceases by 48 hr and diarrhea persists for 96–120 hr. Virus-infected cells are shed by 24 hr.

2. Mucosal Microscopy: If a lesion occurs, it is characterized by shortened villi, deepened crypts, and a relative increase in mitotic figures in the crypts. The infection always hits the upper small bowel, but it can involve the entire small bowel. The severity and extent of the lesion probably are related to virus load and to the youth and immune status of the host. As with rotaviruses there is no proof of direct infection of the stomach or colon. It is important to emphasize that diarrhea and transport disorders can be severe in the absence of disturbed villi-crypt architecture (ie, apparent normal surface area).

3. Mucosal Enzymes: In jejunal homogenates typical findings are a severe decrease in activities of the disaccharidases and alkaline phosphatase (5-15% of normal) and (Na+K)-ATPase (50% of normal) and enriched thymidine kinase activity (500% of normal). This profile of enzyme activities is seen also in small-intestinal normal crypt cells. We have not evaluated the relative importance of disaccharide intolerance as a determinant of acute viral diarrhea, but most clinical studies of viral diarrhea do not suggest disaccharidase deficiency is a major determinant.

4. Solute Transport: Preliminary studies established the sta-
bility and validity of application of marker perfusion, Ussing chamber and isolated brush border membrane vesicles (BBMV) techniques to TGE-infected and control piglet jejunum.

Our findings in piglets during the acute phase of TGE diarrhea are as follows:

In marker perfusion experiments, we showed a blunted Na absorptive response and impaired glucose absorption in response to increasing perfusate glucose concentrations.

In Ussing chamber studies on stripped jejunal mucosa, Na absorptive response to glucose (30 mM) was present but blunted. The antiabsorptive responses of NaCl absorption to cAMP (theophylline) and furosemide were absent. The Cl secretory response to cAMP was accentuated. This secretion was blocked by ouabain and serosal furosemide. Responses to amino acid and peptide are described below by Dr. Rhoads.

In brush border membrane vesicles (BBMV), a Na gradient-dependent d-glucose overshoot occurred, but it was blunted. We found evidence for two glucose carriers in normal piglet BBMV but loss of a high-affinity glucose carrier in TGE membranes in equilibrium kinetic studies with gramicidin. Our studies of amino acid transport will be described by Dr. Rhoads in a later presentation.

As demonstrated by the TGE model, the small intestinal epithelium during acute viral diarrhea is composed of epithelial cells resembling those normally found in crypts. These crypt-type cells have reduced luminal membrane capacity for neutral NaCl absorption and glucose Na cotransport and reduced basolateral membrane (Na+/K)-ATPase activity. Decreased surface area and disaccharidase activities probably are not major determinants of acute viral diarrhea.

OVERVIEW OF INTESTINAL SECRETORY MECHANISMS

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The gravity of diarrheal illnesses arises from the fact that the intestinal tract has an immense secretory capacity, greater than that of any other organ system in the human body. This secretory capacity is distributed over the entire intestinal tract from duodenum to distal colon. Secretory and absorptive functions in the intestine appear to be spatially separated in that secretion arises largely from cells in crypts and absorption from cells on villi in the small intestine and on the luminal surface of the colon. The largest rate of secretion develops when cells in the crypts are prevented from reabsorbing. Both effects are seen with cyclic nucleotides and calcium, the effects of which are thus synergistic, maximizing net secretion. It should be noted that the antiabsorptive effects of intracellular mediators are selective and do not apply to nutrient absorption and nutrient-dependent salt and water absorption.

The mechanism for active chloride secretion has the following components: (1) A carrier in the basolateral membrane that simultaneously translocates 1 Na and 1 Cl or 1 Na, 1 K, and 2 Cl. When the epithelium is actively secreting Cl, intracellular ion concentrations are such that the carrier facilitates the net flow of these ions into the cell whereas, when secretion ceases, the net flux through this carrier also ceases. This is due at least in part to an increase in cell Cl concentration, promoting a back-flux of Na, K, and Cl through the carrier. Possibly there is also a decrease in cotransport due to endocytosis of carriers or to inactivation of the carrier.

The Na, K, Cl cotransporter (or, in some systems, a Na, Cl cotransporter) is a ubiquitous membrane carrier found in a variety of cells, both epithelial and non-epithelial. Examples of absorptive epithelia in which it plays a prominent role are the thick ascending limb of Henle's loop in mammalian kidney and in the brush border membrane of teleost intestine. It has also been identified in the basolateral membrane of secretory cells in mammalian trachea and shark rectal gland. Its affinity for Na and K is higher than that for Cl. It is also inhibited by the so-called "loop" diuretics such as furosemide and bumetanide. By virtue of its stoichiometry (1 Na and 1 Cl or 1 Na, 1 K, and 2 Cl), the translocation process is chemically neutral, which means that the movement of these ions by way of the cotransporter is effectively uncoupled from the electric potential of the cell. In this way an intracellular Cl concentration, above electrochemical equilibrium, need not interfere with the inward movement of ions through the cotransporter.

In cases where K is involved, the 1-for-2 NaCl stoichiometry is also useful in that less turnover of the sodium pump, and therefore less ATP, is required to transport a given amount of Cl. The Na that moves in with Cl is recycled through the basolateral membrane by the Na pump, the K that moves in with Cl recycles through K channels in the basolateral membrane. Cl accumulates above electrochemical equilibrium and then can exit the cell to the lumen via Cl-selective channels in the apical membrane. The Cl channel in the apical membrane appears to be opened through the action of one or more intracellular mediators such as cAMP and Ca. In addition to opening existing channels, these mediators may promote fusion or intracellular vesicles containing these channels with the apical membrane, thereby increasing the number of Cl channels in the apical membrane and permitting a greater efflux of Cl. The Na that is secreted with Cl moves paracellularly, driven by the transepithelial electric potential difference generated by Cl secretion.

Electrolyte absorption in the intestine appears also to involve electroneutral transport processes for Na and Cl although, in the distal colon, apical Na channels are present. In intestinal absorptive cells there are Na/H exchangers which can be inhibited through the action of Cs and cyclic nucleotides. There appears also to be a Na-dependent Cl entry process which may represent a Cl/HCO3 exchange coupled by cell pH to Na/H exchange or a NaCl symport or another as yet undefined Cl entry process. Cl then tends to accumulate in these cells above electrochemical equilibrium and to exit across the basolateral membrane largely through Cl channels there. The pathways for CI absorption and secretion in the intestine share the general feature of an electrically neutral Na-coupled Cl entry process on one membrane and a Cl channel on the opposite membrane. The cellular processes involved and subject to regulation by mediators are opening channels, activation of carriers, and membrane amplification.

[3H]BUMETANIDE BINDING TO MEMBRANES FROM DOG KIDNEY OUTER MEDULLA AND TO AVIAN RED CELLS, AND ITS RELATIONSHIP TO Na,K,Cl COTRANSPORT. Bliss Forbush, III and Mark Haas, Departments of Physiology and Pathology, Yale University, School of Medicine, New Haven, Connecticut 06510.

The studies described here are a starting point in our attempt to identify, isolate, and characterize the transport protein re-
responsible for Na⁺, K⁺, Cl⁻ cotransport. Our approach is to study the binding of radiolabeled loop diuretics such as [³H]bumetanide to isolated plasma membranes; these compounds are known to be potent inhibitors of Na⁺, K⁺, Cl⁻ cotransport. We started with a preparation of membranes from dog kidney outer medulla, which is presumed to include apical membranes from the thick ascending limb of the loop of Henle, a locus of known high cotransport activity. We found high-affinity [³H]bumetanide binding to a small number of sites (2 pmol/mg) and were able to purify binding activity up to 15-fold on a sucrose gradient. The requirements for binding were striking in that Na⁺, K⁺, and Cl⁻ were required for optimal binding, and the affinities of these ions were similar to the previously observed affinities for the cotransport process. High concentrations of Cl⁻ were found to inhibit binding, so that the binding could be modeled by [³H]bumetanide interaction at one of the two Cl⁻ sites. Furthermore, [³H]bumetanide was found to be displaced from its binding sites by various unlabelled loop diuretics at concentrations that were known to inhibit transport. However, to prove that [³H]bumetanide binding is an accurate marker, it is necessary to be able to determine binding and inhibition of transport in the same preparation. Since our attempts to measure transport in renal membrane vesicles have been unsuccessful, we examined [³H]bumetanide binding in duck red cells where shrinkage (or norepinephrine)-induced Na⁺ + K⁺ + 2Cl⁻ cotransport has been thoroughly characterized. Using a glass-fiber filtration assay, we have found that [³H]bumetanide binds with affinity to approximately 1000 sites per red cell. As with the kidney membranes, the binding was prevented by other loop diuretics, required Na⁺, K⁺, Cl⁻, and was decreased at high [Cl⁻]. Na⁺, K⁺, Cl⁻ cotransport was inhibited in close parallel with [³H]bumetanide binding, demonstrating that the binding sites are indeed inhibitory sites on the transporter. A turnover number of 4000 Na⁺ ions/sec/transport is obtained from the ratio of bumetanide-sensitive ion flux to the number of [³H]bumetanide binding sites. Importantly, binding of [³H]bumetanide to duck red cells is stimulated 17-fold by norepinephrine or eightfold by cell shrinkage, conditions under which cotransport is stimulated to a similar extent. This shows that whatever the mechanism of regulation of the cotransport process, it is also reflected by modulation of the number of available loop diuretic binding sites. Preliminary investigations are in progress to photoaffinity label the transport protein and to purify it after detergent solubilization.

Na⁺, K⁺, Cl⁻ COTRANSPORT AND THE PROCESSES OF Cl⁻ ABSORPTION AND SECRETION IN EPITHELIAL TISSUES.

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Electroneutral Na⁺, K⁺, Cl⁻ cotransport systems have been described in several epithelial and nonepithelial tissues. It is distinguished from other transport processes on the basis of interdependence of ion fluxes, sensitivity to loop diuretics (bumetanide and furosemide), and insensitivity to amiloride, ouabain, and thiazide diuretics.

Two different stoichiometries for Na⁺, K⁺, Cl⁻ cotransport have been suggested for nonepithelial tissues and cells. In catecholamine-stimulated duck red cells and Ehrlich ascites cells a 1 Na⁺, 1 K⁺, 2 Cl⁻ stoichiometry was reported to be 2 Na⁺, 1 K⁺, 3 Cl⁻. These values were obtained from direct measurements of diureticsensitive unidirectional influxes. In epithelial tissues studied to date, only one stoichiometry (1 Na⁺, 1 K⁺, 2 Cl⁻) has been found for Na⁺, K⁺, Cl⁻ cotransport. In our studies with winter flounder intestine, the stoichiometry was determined by simultaneous, bumetanide-inhibitable NaCl and KCl influxes. Studies of Na⁺ and Cl⁻ concentration effects on short-circuit current (Iₛ) resulted in a hyperbolic relationship for Na⁺ (Kᵣ = 7 mM; Hill coefficient = 0.9) and a sigmoidal relationship for Cl⁻ (Kᵣ = 7 mM; Hill coefficient = 1.9). Similar results with Rb⁺ influx as a function of Na⁺, K⁺, or Cl⁻ concentration were also observed (Hill coefficients = 0.9, 1.2, and 2.0, respectively, and Kᵣ = 5, 4.5, and 20 mM, respectively). Based on the influx stoichiometry, Cl⁻ binding to the carrier is highly cooperative. Ion affinities (as indicated from Kᵣ values) were at least four to seven times greater for Na⁺ and K⁺ influx than Cl⁻ (depending on whether Iₛ or Rb⁺ influx was measured), suggesting that under physiological conditions transport across the carrier will be limited on the outside by Cl⁻ and K⁺ availability and, on the inside, by Cl⁻ and Na⁺ availability.

Recent studies in our laboratory of [³H]bumetanide binding in microsomal membranes in bovine kidney outer medulla and winter flounder intestine resulted in nearly identical kinetic constants with Kᵣ of 1.6 × 10⁻⁷ M (kidney) and 1.2 × 10⁻⁷ M (flounder intestine), and number of binding sites = 10.5 and 7.3 pmol/mg protein, respectively. Binding of bumetanide to brush border membranes from flounder intestine resulted in a threefold increase in the number of binding sites and a Kᵣ of 5.3 × 10⁻⁷ M. Measurements of bumetanide inhibition of tissue Iₛ gave an IC₅₀ value of 3 × 10⁻⁷ M and agreed well, therefore, with the bumetanide binding studies. The similarity between IC₅₀ and Kᵣ values suggest that the observed specific binding of bumetanide is to the cotransporter. Measurement of bumetanide binding at various Na⁺, K⁺, and Cl⁻ concentrations showed that optimal binding required all three ions to be present at about 5 mM concentration. Higher Na⁺ and K⁺ concentrations did not affect binding, but higher Cl⁻ concentrations (up to 100 mM) inhibited specific bumetanide binding up to 50%. Concentrations of Cl⁻ above 100 mM did not further inhibit; however, analysis of bumetanide binding at 5 and 100 mM Cl⁻ concentration showed decreases in both Kᵣ and number of binding sites at 100 mM Cl⁻, suggesting that Cl⁻ and bumetanide bind to separate sites.

We have also undertaken recently to evaluate the properties of Na⁺-coupled Cl⁻ transport in the basolateral membrane (BLM) of secretory cells, using a preparation of bovine tracheal cells moderately enriched for BLM. [³H]Bumetanide binding to these membranes shows maximal binding of 7.6 pmol/mg protein and Kᵣ of 1.3 × 10⁻⁷ M. Binding is not dependent, however, on Na⁺, K⁺, and Cl⁻ in the bathing medium. Inhibition of specific bumetanide binding at high Cl⁻ concentrations was also seen.

Time-dependent photoincorporation of unmodified [³H]bumetanide was measured in bovine kidney outer medulla microsomal membranes and flounder intestine brush border membranes. After 30 min exposure (50 W high pressure Hg-arc lamp with 340 nm cutoff filter) photoincorporation of bumetanide into specific sites was complete. Addition of 2-mercaptoethanol (100 μM) as a scavenger doubled the specific binding signal by decreasing the magnitude of nonspecific photoincorporation. Control experiments using a wide range of bumetanide concentrations (1μM to 1mM at 5 × 10⁻⁷ M and 1 × 10⁻⁷ M [³H]bumetanide) indicated that significant (>10%) quenching of incident UV light occurs at concentrations above 100 μM, thus a 50 μM excess unlabeled bumetanide concentration was used in these studies to eliminate
this potential artifact. To establish that specific photoincorporation of bumetanide occurs at the same site as that determined from equilibrium binding experiments, kidney microsomal membranes were exposed to UV light (±50 μM unlabeled bumetanide) for 30 min. These membranes were then used in equilibrium binding experiments to determine if the amount of specific bumetanide binding had diminished as a result of specific sites being occupied with photoincorporated unlabeled bumetanide. Our results show about a 50% decrease in the magnitude of specific binding in the presence of photoincorporated unlabeled bumetanide as compared to specific binding observed in UV-treated control membranes.

We are now analyzing bumetanide binding proteins by PAGE (10-17%). A 4-5 Kp peptide is the only peak to show bumetanide concentration-specific and ion-dependent activity.

RECONSTITUTION OF A REGULATED CI TRANSPORTER. W.P. Dubinsky, Department of Physiology and Cell Biology, University of Texas, School of Medicine, P.O. Box 20708, Houston, Texas 77225.

The tracheal epithelium is a useful model for CI secretion. Secretagogue action on this tissue results in an increase in the apical membrane CI conductance which is mediated by the second messengers Ca 2+ and cAMP. We are developing a model to study the regulation of this CI channel in a completely reconstituted system. Thus, in addition to identifying individual proteins involved in the transport complex and elucidating their mechanism of control, we also have a potentially useful assay system for agents which might intervene at any point in the overall process. Bovine trachea is used as the source of a highly enriched apical membrane fraction. The apical membrane is solubilized by detergent extraction and the soluble fraction obtained by ultracentrifugation. The soluble extract may then be further resolved or otherwise biochemically manipulated. Reconstitution of soluble fractions into artificial phospholipid vesicles is accomplished by the freeze–thaw sonication procedure. Preliminary studies of the effects of putative regulators in reconstituted vesicles reveal the following: (1) the transporter is inactivated by incubation at 37°C (t½ = 5 min); (2) Ca 2+ at concentrations greater than 2 x 10^-7 M activates CI transport; (3) a protein fraction can be resolved from the soluble extract by calmodulin affinity chromatography; calmodulin is required for maximal activation of the reconstituted transporter; (4) the transporter exhibits a selectivity for CI > Br > gluconate (1:0:2:0.06). Future studies will examine the effects of cyclic nucleotides and exogenous protein kinases on this CI conductance.

DEVELOPMENT OF LIGANDS FOR THE EPITHELIAL CHLORIDE CHANNEL IN BOVINE RENAL CORTEX. D.W. Landry, M. Reitman, E.J. Cragoe, Jr., and Q. Al-Awqati, Department of Medicine, Columbia University, New York, New York 10025.

Two assays were developed for the chloride channel in microsomal vesicles from bovine renal cortex. KCl-loaded vesicles were stripped of external chloride by a gluconate buffer containing tracer 36Cl in order to generate chemical and electrical gradients favoring tracer uptake. The time course of uptake was followed with a filter assay. Valinomycin (K+ carrier expected to collapse any membrane potential), ethacrynic acid, or external chloride inhibited uptake. A second assay employed 36Cl transport into sucrose-loaded vesicles in the presence of external potassium. Valinomycin accelerated transport, but ethacrynic acid inhibited it. We have now determined the IC50 and Ki values for the above agents. The IC50 value for valinomycin is 100 nM, and the Ki value is 250 nM. The IC50 value for ethacrynic acid is 100 μM. The Ki value for external chloride is 100 μM.
acid inhibited it. These data are consistent with an electrogenic chloride transport process, most probably a channel.

The anion preference of the channel was characterized in two ways. 36Cl uptake into 30 mM KCl-loaded vesicles was measured in the presence of 50 mM external anion. The order of inhibition of tracer uptake was Cl, Br, I, SCN (90+%) > NO3 (70%) > F (40%) > HCO3, CH3CO2 (25%) > gluconate (0%). In a second set of experiments, the uptake of tracer anions into the vesicles was measured in the presence of a constant 0.5 mM external chloride concentration. The relative order of tracer uptake was: 99mTcO4 > [14C]SCN > 36Cl = 82Br > 32SO4 > [14C]HCO3, [32p]HPO4.

Various compounds were assayed for inhibition of 36Cl uptake into KCl loaded vesicles. Bumetanide (10 μM) failed to inhibit tracer uptake. The Ki for 4,4'-diisothiocyanato stilbene-2,2'-disulfuric acid was 20 μM and the effect was completely reversible despite heating to 25°C for 25 min. Valum (10 μM), picrotoxin (10 μM), and GABA (100 μM) had no effect on uptake. Three groups of potent inhibitors were identified and studied. The first two, indanyloxyacetic acids and tetrahydrofluorotaryloxyacetie acids, were based on the ethacrynic acid structure. The third were derivatives of N-benzylanthranilic acid. The most potent members of the first and third classes was Ki < 1 μM and these have been radioiodinated in preparation for binding assays.

CHLORIDE CHANNELS IN THE THICK ASCENDING LIMB OF THE LOOP OF HENLE AND IN THE RECTAL GLAND OF THE SHARK (Squalus acanthias). R. Greger, Physiologisches Institut der Universität, Herman-Herder-Str. 7, 7800 Freiburg, F.R.G.

This contribution will cover only two issues: (1) the properties of chloride channels, identified by patch-clamp techniques in the thick ascending limb of the loop of Henle (TAL) and in the rectal gland of the shark (RGT); and (2) the properties of a new class of compounds which are able to block the chloride conductance in both preparations.

The chloride channels identified in the TAL segment and in the RGT segment have unitary conductances of 10-50 pS. In the TAL segment, one such channel was identified in the basolateral membrane. In the RGT segment, two types of chloride channels were observed: one with ~10 pS and another and more frequent one with 35-45 pS. The larger channel was examined with respect to its kinetics. The channel does not show rectification of its current amplitude as a function of voltage. However, the probability of the channel being open is increased with depolarization and reduced with hyperpolarization. Two open-state time constants and two closed-state time constants were identified and ranged between 1 and 20 msec. This channel is selective for chloride over bromide. Like the corresponding channel in the TAL segment, this channel is blocked reversibly by a new class of blocker derived from diphenylamine-2-carboxylate (DPC).

A survey of 225 derivatives of DPC was performed in isolated perfused TAL segments. The substances were added to the peritubular or luminal perfusate. The equivalent short-circuit current, as a measure of secondary active chloride reabsorption, and the basolateral membrane voltage were measured. Previously, we have shown that DPC acts by blocking chloride channels present in the basolateral membrane of this nephron segment. The present survey reveals that the most potent compounds of this series (Ki < 10^-7 mol/liter) comprises molecules with the general formula:

\[ R_2 - (CH_2)_n - N - X - COO^- \]

All active compounds are secondary amines. The carboxylate moiety has an optimal distance to the amino nitrogen of 2-3 C atoms. R₂ is preferentially a hydrogen atom. R₁ is an apolar residue: phenyl, cyclonexyl, etc. This moiety is separated from the amino group by a (CH₂)ₙ spacer, where n optimally is 2-4.

Our studies reveal that chloride channel blockers in the preparations used conform to a highly restricted configuration. These compounds are related to blockers of the Na⁺, 2Cl⁻, K⁺ cotransporter and to the band 3 protein. Minor modifications of the molecule convert chloride channel blockers to those of the other two systems. This may indicate that all three membrane proteins have some structural similarities.

CA²⁺/CALMODULIN-DEPENDENT PHOSPHORYLATION AND REGULATION OF RABBIT ILEAL Na AND Cl ABSORPTION. M. Donowitz, M. Cohen, M. El-Sabban, E. Emmer, J. McCullen, H. Murer, and G. W. G. Sharp, Departments of Medicine and Physiology, Tufts University School of Medicine and New England Medical Center, Boston, Massachusetts 02111; and Department of Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853.

In rabbit ileum, Ca²⁺/calmodulin (CaM) appears to be involved in the regulation of the basal-linked neutral Na and Cl absorption. Attempts to demonstrate a role for protein kinase C in the regulation of rabbit ileal basal Na and Cl absorption have not been successful, although similar techniques have shown a role for protein kinase C in regulating ion transport in other intestinal tissues. Ca²⁺/CaM regulates phosphorylation of rabbit ileal brush border peptides, which indicates that the Ca²⁺/CaM-dependent protein kinases, substrates, and phosphatases are present in this membrane. In whole-cell experiments in which rabbit ileal mucosa is initially incubated with the Ca²⁺ ionophore A23187 in the presence or absence of indomethacin, the ionophore caused an increase in phosphorylation of five microvillus membrane peptides with Mr 116,000, 110,000, 77,000, 53,000, and 32,000. In broken-cell experiments with purified microvillus membranes or brush borders exposed to Ca²⁺/CaM, there was increased phosphorylation of seven peptides with Mr 137,000, 116,000, 77,000, 58,000, 53,000, 50,000, and 32,000. Ca²⁺/CaM caused a concentration-dependent phosphorylation of these peptides, with the Ca²⁺ concentration causing maximal phosphorylation of all being 0.3 μM, except for the Mr 77,000 peptide, in which maximum phosphorylation occurred at 0.8 μM. The Mr 116,000, 77,000, 53,000, and 32,000 peptides identified as substrates for the Ca²⁺/CaM kinase had the same size and isoelectric point in broken- and whole-cell studies. Promethazine inhibited all brush border membrane Ca²⁺/CaM-dependent phosphorylations, with the concentration of promethazine required to cause a 50% inhibition being between 2 and 12 μM. The concentration of promethazine required to stimulate Na and Cl absorption (50% effective, 9 μM) was very similar to the concentration inhibiting the Ca²⁺/CaM-dependent phosphorylation. To evaluate whether phosphorylation was involved in regulation of Na and Cl transport, freeze-thawing was used to permeabilize brush border membrane vesicles to macromolecules to allow incorporation of ATP plus an ATP-regenerating system. Preliminary data indicate...
that although Ca\(^{2+}\) (1 \(\mu\)M free) plus CaM alone and ATP alone did not affect Na/H exchange, the combination inhibited Na/H exchange by 40\%. These results indicate that Ca\(^{2+}\)/CaM-dependent phosphorylation of brush border peptides is involved in the regulation of ileal Na/H exchange.

**SIGNAL TRANSDUCTION IN MAMMALIAN ENTEROCYTES: PROTEIN PHOSPHORYLATION AND POLYPHOSPHONOSITIDE CYCLING IN THE BRUSH BORDER MEMBRANE.** Hugo R. de Jonge and A. Bas Vanstraelen, Department of Biochemistry I, Medical Faculty, Erasmus University, Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

The opening of anion-selective channels in the apical membrane of the intestinal crypt cell (allowing Cl\(-\) secretion) and the inhibition of cation (Na\(^+\)/H\(^+\)) or anion (Cl\(^-\)/HCO\(_3\)\(^-\)) exchangers in the brush border membrane (BBM) of the mature villus cell (blocking NaCl absorption) is provoked by at least three intracellular signals: cAMP, cGMP and Ca\(^{2+}\)-ions. Specific high-affinity receptors for each of these messengers have been identified in isolated BBM by isotope binding, photoaffinity labeling, and immunoblotting techniques. Cyclic AMP binds to the type II isoenzyme of cAMP-dependent protein kinase enriched in BBM and provokes phosphorylation of several BBM proteins, including a minor 25-kD component. Cyclic GMP binds to a unique isoenzyme–enzyme of cGMP kinase found so far exclusively in intestinal brush borders and provokes phosphorylation of two BBM proteins, i.e., the kinase itself (86 kD; autophosphorylation) and the 25-kD protein (cophosphorylation). In contrast to the kinases and most membranal phosphoproteins, the 25-kD proteolipid might, itself, function as a regulatory subunit of a channel or carrier protein but may also play a role in the regulation of the phosphatidylinositol (PI) cycle in BBM. Isoenzyme-bound enzymes of the PI cycle, including PI kinase, PK kinase and a PL\(_7\)-specific phospholipase C activatable by both Ca\(^{2+}\) and a GTP-binding protein. Hydrolysis of the PI\(_7\) pool in BBM generates PI\(_3\) and diacylglycerol which may provoke intracellular Ca\(^{2+}\) mobilization and activation of protein kinase C (PK-C), respectively. Prephosphorylation of BBM in the presence of cGMP (but not in its absence) led to a two- to threefold increase in PI-P formation apparently due to stimulation of the PI kinase. A possible link between cGMP, PI-P formation, PI cycling, and Ca\(^{2+}\) signaling in intact enterocytes is presently under investigation.

By monitoring changes of quin-2 fluorescence in monolayers of the human colon carcinoma cell line HT-29 (Glc\(^-\)) we have also obtained direct evidence for a Ca\(^{2+}\)-mobilizing (carbachol, epinephrine) action of intestinal secretagogues coupled to Cl\(^-\) channel opening. Epinephrine-provoked Ca\(^{2+}\) signaling was mediated through \(\alpha_1\)-adrenergic receptors and was inhibited completely and selectively by phorbol ester-induced PK-C activation. Resting Ca\(^{2+}\) levels appeared unchanged in the presence of forskolin, 8-Br-cAMP, 8-Br-cGMP and heat-stable *E. coli* toxin ST\(_2\)). Therefore, Ca\(^{2+}\) is unlikely to play a role as a third messenger in Cl\(^-\) secreting intestinal epithelial cells.

**SODIUM ABSORPTIVE RESPONSE TO ALANINE AND GLUCOSE IN PIGLET VIRAL DIARRHEA.** Marc Rhoads, Division of Gastroenterology, Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario MS 1X8, Canada.

We measured the response of jejunal sodium absorption to neutral amino acid (L-alanine) and to dipeptides (L-alanyl-L-alanine, glycylsarcosine) in normal piglets and in piglets with acute viral diarrhea 40 hr after experimental infection with an invasive coronavirus, transmissible gastroenteritis (TGE) virus. Stripped jejunal mucosa was mounted in conventional Ussing chambers. In preliminary studies we established that control jejenum responded maximally to D-glucose at 30 mM and L-alanine at 20 mM. Response of jejunal Na absorption to L-alanine (20 mM) was blunted in TGE compared with control jejunum (\(\Delta F_{Na} = 1.1 \pm 0.3\) vs 2.5 \pm 0.4 \(\mu\)eq/cm\(^2\)/hr, \(P < 0.01\); similarly, the response to D-glucose (30 mM) was reduced (\(\Delta F_{Na} = 1.5 \pm 0.4\) vs 6.1 \pm 0.7 \(\mu\)eq/cm\(^2\)/hr, \(P < 0.01\)). L-Alanine added together with D-glucose caused a significantly greater increment in Na absorption than either L-alanine or D-glucose alone in control (8.0 \pm 0.3 \(\mu\)eq/cm\(^2\)/hr) and in TGE tissue (4.1 \pm 0.3 \(\mu\)eq/cm\(^2\)/hr). Response to L-alanine plus D-glucose did not differ from that to L-alanine plus 3-O-methylglucose in either control or TGE jejunum, demonstrating that the response to D-glucose with L-alanine cannot be attributed to a nonspecific effect of glucose on tissue metabolism.

In small intestinal brush border membrane vesicles (BBMV) from control and TGE-infected piglets, enrichment of sucrose activity in control (6 \pm 1\(\times\)) and TGE BBMV (16 \pm 3\(\times\)) was significantly higher than the sodium gradient (23 \pm 8\(\times\)) and in TGE tissue (25 \pm 8\(\times\)). By equilibrium (2 hr) uptake in a NaCl gradient were equivalent to control (4 \pm 1\(\times\)) and TGE (7 \pm 2\(\times\)) BBMV were comparable. Therefore, L-alanine absorption was not significantly different between control and TGE jejunum,

**NON-ANTIMICROBIAL ANTI DIARRHEAL AGENTS**

*GLYCINE-BASED ORAL REHYDRATION SOLUTION REASSESSMENT OF SAFETY AND EFFICACY.* Mathuram Santosh, Department of International Health, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland 21205.

Previous studies have suggested that adding glycine to standard oral rehydration solutions results in the therapeutic advantages of decreasing stool volume and shortening the diarrheal
MECHANISMS OF ACTION OF ANTIDIARRHEAL DRUGS IN 
HUMANS IN VIVO. L.R. Schiller and J.S. Fordtran, 
Department of Internal Medicine, Baylor University Medical Center, 3500 
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Diarrhea can be viewed as resulting from reduced net absorption 
of water by the intestine. Conversely, antidiarrheal drugs can be 
viewed as acting by restoring net absorption of water toward normal. 
Under usual conditions in which boluses of fluid move through the intestine, this might be accomplished by increasing the rate of water absorption by intestinal mucosal cells or by increasing the time available for absorption to occur. We have used steady-state total gut perfusion to examine the effect of several antidiarrheal drugs on the rate of water absorption by intestinal mucosal cells in normal subjects. An absorbable balanced electrolyte solution was infused at a rate of 30 ml/min into the stomach. Polyethylene glycol (PEG) was added to the solution as a nonabsorbable marker and the rate of net water absorption or secretion was calculated by measuring the PEG concentration in rectal effluent. As shown in Table 1, net water absorption rate was increased by intraluminal glucose and to a lesser extent by clonidine. Codeine, loperamide, and somatostatin analog had no significant effect on the rate of net water absorption. Since these drugs were given in doses that were shown in other studies to have an antidiarrheal effect, these results suggest by elimination that the antidiarrheal effects of codeine, loperamide, and somatostatin analog, and much of the antidiarrheal effect of clonidine must have been due to effects on the time available for absorption, i.e., a motility effect.

Techniques for measuring the relevant intestinal motor function (contact time) are rudimentary at present. We have estimated total gut volume (volume of fluid retained within the gut) by perfusion studies by marker techniques. By monitoring the amount of PEG infused into the intestine and the amount recovered in rectal effluent, the amount of PEG remaining within the gut can be calculated by subtraction. Assuming that the average concentration of PEG in the intestine is the mean of the concentrations infused and recovered, the volume of fluid retained in the gut can be calculated by dividing the amount of PEG by the average concentration of PEG. Estimation of total gut volume gives an idea about changes in intestinal capacity which could influence the transit of fluid through the gut. Results of these calculations are shown in Table 1. Glucose had no effect on total gut volume, but the other four agents had substantial effects on increasing the capacity of the gastrointestinal tract. This suggests that these four agents could alter the transit of fluid through the intestine under non-steady-state conditions, thus allowing more time for absorption to take place.

These studies indicate that an effect on motility rather than an effect on the rate of absorption by mucosal cells is the major mechanism by which the opiates, codeine and loperamide, and (probably) somatostatin analog act. Motility effects also contribute importantly to the effect of clonidine. Glucose, on the other hand, seems to work exclusively by accelerating the rate of water absorption by the mucosa.

| Antidiarrheal dose | Water absorption rate (ml/hr) | Total gut volume (ml) |
|-------------------|-----------------------------|----------------------|
| Glucose (100 mM)  | 781 1292*                   | 1036 1181 *          |
| Clonidine (0.3 mg) | 696 799*                    | 987 1830*            |
| Codeine (60 mg)   | 920 882                     | 1025 1921*           |
| Loperamide (18 mg)| 909 898                     | 985 1764*            |
| Somatostatin analog (100 µg) | 791 734 | 818 1377 |

*P < 0.05 vs control.
Current evidence suggests that opiates have an indirect effect on enterocytes. The effect of opiates can be blocked by the neurotoxin, tetradotoxin. Further, opiate receptors cannot be identified on rabbit ileal enterocytes. The intermediary agonist responsible for opiate effects on ion transport, however, has not been identified. Studies in the rabbit ileum suggest a nonadrenergic, noncholinergic mediator.

CENTRAL NERVOUS SYSTEM INVOLVEMENT IN THE ACTIONS OF OPIOIDS ON NET WATER AND ELECTROLYTE TRANSPORT ACROSS THE MAMMALIAN SMALL INTESTINE. D.R. Brown, F.L. Quinto, and M.A. Gillespie, Department of Veterinary Biology, University of Minnesota College Veterinary Medicine, 1988 Fitch Avenue, St. Paul, Minnesota 55108.

Opioids, such as morphine and codeine, have historically been used as potent and effective antidiarrheal agents. Although their activity has been attributed to their ability to alter intestinal motility, it is now clear that both opiate alkaloids and endogenous opioid peptides also decrease active anion secretion and enhance net fluid absorption through a local action in the intestinal mucosa. Moreover, recent evidence suggests that opiate antidiarrheal activity may be expressed through sites within the central nervous system (CNS) as well as at the level of the gut. For example, intestinal transit time of luminal contents is markedly increased by the CNS administration of opiate alkaloids.

We have examined the hypothesis that opioids decrease intestinal secretion by an action at CNS sites. Antisecretory activity was measured in rats either in isolated intestinal loops [cholera toxin (CT) experiments] or by single-pass perfusion techniques using [14C]PEG as a nonabsorbed marker of net water transport [prostaglandin E1 (PGE1) experiments]. The metabolically stable enkephalin analog, [D-Ala2, Met5]enkephalinamide (DAMA), given intracerebroventricularly at doses ≥3 μg, was found to decrease ongoing fluid secretion in the in situ rat jejunum induced by either intraluminal CT or intravenous PGE1. The antisecretory actions of DAMA occur within 30 min of its CNS injection and are manifested for >30 min. Pretreatment of rats with DAMA at intracerebroventricular doses <1 μg inhibits PGE1-induced CT- and water secretion with a similar time course. These antisecretory effects are inhibited by the opiate antagonist, naloxone. The peptide does not appear to act in other regions of the central nervous system (CNS) as well as at the level of the gut. For example, intestinal transit time of luminal contents is markedly increased by the CNS administration of opiate alkaloids.

Loperamide is one of the most frequently used and effective agents for the treatment of diarrheal disorders of diverse etiologies including chronic idiopathic diarrhea, irritable bowel syndrome, short-bowel syndrome, endocrine-associated diarrheas, and childhood diarrheal diseases. The compound has particularly wide appeal because of its relatively few side effects and safety. It appears to have both antimotility and antisecretory actions. In healthy volunteers loperamide predominantly alters motor function causing increased capacitance of the gut and delay in the passage of fluid through the intestine. Little if any effect for this drug was observed on basal or VIP-stimulated secretion. Others, however, have shown that loperamide inhibits the basal transport process and secretion stimulated by a variety of secretagogues including prostaglandins, cholera toxin, bisacodyl, castor oil, Ca ionophore A23187, and heat-labile and heat-stable E. coli toxins.

The mechanism of action of loperamide remains controversial. Several studies have shown it to have opiate-like effects on intestinal motility and secretion and specific binding to opiate receptors. A significant difference of this compound to opiates is the lack of analgesic or addictive properties common to opiates, a desirable feature attributed to its quaternary structure and hence poor permeability across the blood-brain barrier. Others have reported that it will inhibit prostaglandin release from guinea pig ileum, and this effect is not reversed by naloxone.

Loperamide does not appear to inhibit basal or stimulated anion or guanylate cyclase activity. However, loperamide has been shown to be a potent inhibitor of calmodulin, a calcium-dependent regulatory protein (a property not shared by other opiate agonists). When the ability of loperamide and other neuroleptics to bind calmodulin in vitro is compared to their antidiarrheal effects on castor-oil-induced secretion, a positive correlation can be demonstrated.

More recently, it has been shown that loperamide may selectively block receptor-activated calcium permeabilities in the plasma membrane. Specific binding of loperamide and related compounds to [3H]nitrendipine binding sites in rat brain membranes have been demonstrated. In isolated enterocytes, loperamide has been shown to specifically block stimulated increases in cytosolic calcium (measured by quin-2 fluorescence) by the gut peptides substance P (SP), neurotensin (NT), and bombesin (BB), but not by serotonin, a muscarinic agonist. These findings suggest that loperamide selectively blocks receptor-activated membrane permeability to calcium. However, exactly what role gut peptides such as substance P, neurotensin, and bombesin play in the homeostatic regulation or pathophysiological pro-

NON-ANTIMICROBIAL ANTIDIARRHEAL AGENTS

The antisecretory actions of DAMA occur within 30 min of its in situ injection and are manifested for >30 min. Pretreatment of rats with DAMA at intracerebroventricular doses <1 μg inhibits PGE1-induced CT- and water secretion with a similar time course. These antisecretory effects are inhibited by the opiate antagonist, naloxone. The peptide does not appear to act in other regions of the central nervous system (CNS) as well as at the level of the gut. For example, intestinal transit time of luminal contents is markedly increased by the CNS administration of opiate alkaloids.
cesses of intestinal electrolyte transport remains unclear. These findings may be more relevant to its action on smooth muscle.

In summary, the putative actions of loperamide on secretory and motility elements are numerous. Consequently, it has been difficult to sort out any predominant mechanism of action. It may be possible that loperamide’s effectiveness as an antidiarrheal agent rests on this broad spectrum of action. However, clinically, it is more likely that its predominant site of action is on intestinal motility, despite the existing in vitro data.

EFFECT OF Ca2+/CaM ANTAGONISTS ON SMALL INTESTINAL ELECTROLYTE TRANSPORT. M. Donowitz, H. Cheng, J. Wicks, and G.W.G. Sharp, Departments of Medicine and Physiology, Tufts University School of Medicine and New England Medical Center, Boston, Massachusetts 02111; and Department of Pharmacology, New York State Veterinary College and Cornell University, Ithaca, New York 14853.

Many classes of drugs are Ca2+/calmodulin antagonists. These drugs affect intestinal water and electrolyte transport, but because these drugs are hydrophobic, they exert effects related to their hydrophobic properties in addition to their Ca2+/calmodulin effects. To control for hydrophobic effects, synthesized compounds have been called naphthalene sulfonamides which are pairs of compounds differing only in the presence of a Cl atom and which have very similar hydrophobic properties (octanol-water partition coefficient), but have very different IC50 as Ca2+/calmodulin antagonists.

These compounds, W13 (Ca2+/calmodulin antagonist) and W12 (hydrophobic control) were studied on rabbit ileal active Na and Cl transport in the basolateral and after exposure to secretagogues acting through Ca2+ and camp. W13 but not W12 stimulated neutral active Na and Cl absorption, causing an equal increase in mucosal-to-serosal Na and Cl fluxes with the increase in Na being dependent on Cl ion in the bath and vice versa. This indicates that W13 stimulated the linked, neutral NaCl absorptive process. W12 also increased net Cl absorption, but not net Na absorption since it increased serosal-to-mucosal Na movement. The concentration dependence of W13 indicated a 50% effect at 42 μM, similar to other Ca2+/calmodulin dependent systems. W12 caused similar but smaller effects on transport, but with a 50% effect at 100–200 μM, also consistent with the ratio of W13/W12 effects reported for other Ca2+/calmodulin systems. Neither W13 nor W12 affected paracellular permeability based on dilution potentials, mannitol fluxes, and scanning EM of tight junctions. The effects of W13 and W12 were evaluated on changes in transport caused by Ca2+ ionophore A23187, serotonin, theophylline, and 8-Br-cAMP. Neither W13 nor W12 at concentrations up to 200 μM inhibited short-circuit current responses or changes in Na and Cl fluxes caused by any of these secretagogues.

We conclude that in rabbit ileum: (1) Ca2+/calmodulin inhibits the linked, neutral NaCl absorptive process under basal conditions; (2) previous reports of antisecretory effects of drugs with Ca2+/calmodulin antagonist properties likely were due to non-Ca2+/calmodulin antagonist properties, perhaps other hydrophobic effects of these drugs; and (3) the hydrophobic effects may be useful in selecting antisecretory drugs.

POTENTIAL TARGETS FOR PHENOTHIAZINES AND ISOQUINOLINE SULFONAMIDES IN INTESTINAL EPITHELIUM. Hugo R. de Jonge, Department of Biochemistry 1, Medical Faculty, Erasmus University, Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

The neuroleptics chlorpromazine (CPZ) and trifluoperazine (TFP) have been shown to antagonize chloratoxin- and heat-stable E. coli toxin (STα)-induced intestinal secretion in animals and man. Phenothiazine mimetics lacking the sedative action are potentially highly effective antidiarrheals. The antisecretory effect on the crypt cells however, requires rather high dose levels of CPZ and TFP which are inconsistent with a blockade of calmodulin (CaM) functions. Therefore, other potential targets involved in S-S coupling, ie, protein kinases, endogenous phosphorylation reactions, and polyphosphoinositide (PI) metabolism in the brush border membrane (BBM) were tested for their sensitivity to phenothiazine action. Similar experiments were carried out to test the selectivity of a new class of protein kinase antagonists, the isoquinoline sulfonamides. Their inhibitory effect is attributed to a direct interaction with the active center of the kinase and is competitive with respect to ATP. Protein kinase inhibition in the intact cell therefore requires much higher levels of these compounds than needed in PK assays in vitro. The outcome of both studies can be summarized as follows:

1. At concentrations below ~50 μM, both phenothiazines act as rather selective inhibitors of intestinal Ca2+/CaM kinase. Raising their levels above ~100 μM led to the additional inhibition of protein kinase C (PK-C). At concentrations above ~200 μM, both compounds started to inhibit the activity of PI-P kinase in the intestinal BBM, resulting in accumulation of PI-P and a decreased formation of PI-P2 and PA. Inhibition of cGMP and cAMP kinase activities could only be detected at millimolar levels of CPZ and TFP. These results suggest that their antisecretory action cannot be due to a blockade of cAMP- or cGMP-dependent phosphorylation but instead could be related to PK-C inhibition and/or a hydrophobic interaction with intestinal membranes as evidenced by the blockade of a crucial step in PI cycling.

2. When tested at low ATP levels (20 μM), the isoquinoline sulfonamides H7 and H8 acted as rather poor inhibitors of intestinal Ca2+/CaM kinase. Raising their levels above ~100 μM led to the additional inhibition of PK-C (IC50 = 13 and 30 μM), but highly potent antagonists of cAMP kinase (IC50 = 4.1 and 2.2 μM) and cGMP kinase (IC50 = 2.5 and 0.3 μM).

3. Using stripped rat proximal colon mounted in Ussing chambers as a model system to test the antisecretory action of H7 and H8 (following 5 min of preincubation), we obtained the following results: (a) H8 (70 μM) caused a selective inhibition (~80%) of the Ise response to 8-Br-cGMP and STα without altering the response to 8-Br-cAMP, carbocoll, or Bradykinin; dose-response curves for H8 inhibition of Ise were identical for 8-Br-cGMP and STα; (b) in the presence of 70 μM H8 and 8-Br-cGMP, subsequent addition of STα did not further increase Ise; therefore, additional effects of STα on other processes leading to secretion (eg, activation of the PI cycle) seem unlikely; (c) upon replacement of H8 by H7 (70 μM), an approximately eightfold less potent inhibitor of cGMP kinase, the Ise response to 8-Br-cGMP and STα was not significantly different from control values. It is concluded that H8 may selectively block cGMP-dependent phosphorylation and cGMP-mediated secretion in intact colonocytes without interfering with cAMP- or Ca2+-linked secretion. Moreover, STα seems to act exclusively through activation of cGMP kinase, at least in the colonic crypt cell.
ADRENERGIC AGONISTS AS ANTIDIARRHEAL AGENTS: A REVIEW OF PHYSIOLOGICAL AND CELLULAR MECHANISMS. Eugene Chang, Division of Gastroenterology, Department of Medicine, Columbia University, 630 West 168 Street, New York, New York 10032.

It has been well established that catecholamines have potent in vivo and in vitro antisecretory and proabsorptive effects on intestinal electrolyte transport. In rabbit ileum, for example, epinephrine (EPI) or norepinephrine (NE) enhance electrically neutral NaCl absorption and inhibit an apparent electrogenic HCO₃ secretion. Similarly, in rat colon, epinephrine has been shown to enhance NaCl absorption and inhibit anion secretion. These effects are predominantly mediated by postsynaptic alpha-2-specific adrenergic receptors which, by radiological binding studies, have been identified as enterocytes. Although other adrenergic agonists and dopamine have been reported to have similar actions to alpha-2-receptor activation, their relative contribution is unclear.

The mechanisms for alpha-2-receptor effects in gut transport have been partially elucidated. EPI decreases cholangio-toxin and prostaglandin E₂ augmented cAMP concentrations in rabbit ileal mucosa. This finding is consistent with the “classical” action of alpha-2 receptors described in other cell systems where receptor stimulation causes an activation of a GTP-binding regulatory protein (Ni or Gi) resulting in the inhibition of adenylate cyclase.

However, alpha-2 agonists have no effect on basal cyclic AMP levels despite large changes in basal transport rates. Recent speculation has been that stimulation of alpha-2 receptors may also interfere with the mobilization or metabolic consequences of intracellular free calcium (Ca). In support of this is the finding that EPI selectively inhibits the subsequent Ca-mediated secretory response of muscarinic agonists, substance P, and Ca ionophore A238187. No inhibition of the secretory response to 8-bromo-cyclic AMP is seen. When Ca-fluorescence techniques are used to measure cytosolic free Ca in isolated enterocytes, EPI had no effect on secretagogue-stimulated increases in Ca, suggesting that its effect may be instead the inhibition of a critical Ca-dependent regulatory step for electrolyte transport.

These agents are potentially useful for the treatment of diarrhea because of their wide spectrum of action, ie, effective against secretagogues that activate adenylate cyclase or increase intracellular free calcium. Clinical trials have been limited to a few cases of secretory and diarrheic diarrhea. In healthy volunteers, clonidine, an alpha-2 agonist appears to slow transit time and exclude by the blood-brain barrier, the clinical utility of these agents should increase.

The usefulness of currently available alpha-2 adrenergic agonists such as lidamine and clonidine for diarrheal disease is unfortunately limited by unpleasant and intolerable side effects. Most patients develop severe orthostatic hypotension, lethargy, and experience the withdrawal side effects of alpha-2 agonists. As newer compounds are developed that are more gut selective and excluded by the blood-brain barrier, the clinical utility of these agents should increase.

STRUCTURE-ACTIVITY RELATIONSHIPS OF α₂-ADRENERGIC COMPOUNDS ON ELECTROLYTE TRANSPORT ON THE RABBIT ILEUM AND RAT COLON: POSSIBLE DEVELOPMENT OF GUT SELECTIVE ANALOGS. K. Dharmasthaphorn, University Hospital, UCSD H-811-D, 225 Dickinson Street, San Diego, California 92103.

Clonidine, an α₂-adrenergic agonist, increases electrolyte absorption in the intestine and inhibits diarrhea. In an attempt to develop a gut-specific α₂-adrenergic compound for the treatment of diarrhea, we tested several imidazoline derivatives to determine which aspects of the molecule are gut-specific. The potency of each compound in the stimulation of electrolyte transport in the rabbit ileum and rat colon was determined using a modified Ussing chamber technique. These results were then compared with the ability of these drugs to lower blood pressure following intracisternal injection in spontaneously hypertensive rats, as well as with other α₂-adrenergic properties that have been defined in previous studies. Results indicate that all imidazoline derivatives interact with α₂-adrenergic receptors in the gut preparation but activate the ion transport processes to a variable extent, ie, all analogs tested are either agonist or antagonist for ion transport. Structure—activity relationships were derived; for the agonist property in the gut, the imidazoline derivative required (1) substitution (in order of potency) with halide > CH₃ or C₂H₅ > CH₂O or > OH at position 2 or 6 of the phenyl ring, or a simultaneous substitution at positions 3 and 4 with hydroxy groups, or certain other groups that independently enhance the agonist properties; and (2) the presence of a proper bridging unit between the phenyl and imidazoline rings. NH ≥ CH₂. In addition, it was found that certain compounds with a methoxy substitution of the phenyl ring displayed a dissociation between the intestinal ion transport potency and central hypotensive activities.

ANTIDIARRHEAL PROPERTY OF SOMATOSTATIN: POSSIBLE DEVELOPMENT OF GUT SELECTIVE SOMATOSTATIN ANALOGS. K. Dharmasthaphorn, University Hospital, UCSD H-811-D, 225 Dickinson Street, San Diego, California 92103.

The antisecretory function of somatostatin has been observed in the small and large intestine of a variety of animal models, including man. Our studies suggest that somatostatin blocks an intermediate step in the secretory processes beyond the step of cAMP production. The same antisecretory effect is observed in the rabbit and dog intestine. In man, somatostatin was also found to inhibit water secretion, particularly that caused by the overproduction of peptide hormones or neurotransmitters, while in the basal state it had little or no significant effect. The effectiveness of somatostatin has been demonstrated in patients suffering from severe watery diarrhea due to the carcinoid syndrome and VIPoma. A patient with severe diarrhea associated with colonic pseudoobstruction, and some patients with Crohn’s disease and short-bowel syndrome also responded well to somatostatin.

The fact that somatostatin has been shown to be quite effective in controlling diarrhea in patients with endocrine tumors and in certain hypersecretory states suggests the potential use of somatostatin as an antidiarrheal agent.

It may be possible to eliminate many of the adverse systemic side effects, provided that gut receptors for somatostatin are different from other receptors. In collaboration with Dr. Jean Rivier, Dr. Wylie Vale, and Dr. Marvin Brown, we have tested a series of somatostatin analogs for intestinal action. We found that substitution with L-alanine, or deletion of amino acids at Phe⁶, Phe⁷, Trp⁹, and Lys² of the somatostatin molecule, or deletion of Thr¹⁰, reduced the ion transport properties as well as the inhibition of growth hormone, insulin, and glucagon release to near zero. This indicates that Phe⁶, Phe⁷, Trp⁹, and Lys² are necessary for somatostatin receptor binding, while Thr¹⁰ serves...
as an essential spacer. More importantly, we have observed that substitution or deletion of Phe\textsuperscript{11} and Lys\textsuperscript{4} do not appear to be critical for biologic activities in the rabbit ileum and rat colon, as they are for some other endocrine effects. Alteration of either one of these amino acids may result in a gut-specific analog in the rat and rabbit. Whether the results can be extended to man still remains to be tested.

**CLINICAL EXPERIENCE OF SOMATOSTATIN ANALOG (COMPound 201-995, SANDOSTATIN\textsuperscript{a}) AS AN ANTIDIARRHEAL AGENT.** T.M. O'Dorisio and H.S. Meikhiyan, Division of Endocrinology, Department of Medicine, Ohio State University Hospital, Columbus, Ohio 43210.

Prior work has established that secretory diarrhea associated with metastatic carcinoid tumors and vasoactive intestinal peptide (VIP) -secreting tumors is improved by intravenous infusion of the potent native peptide/hormone inhibitor, somatostatin. The major drawback of somatostatin as an antidiarrheal agent has been the need for intravenous infusion and its short (2-4 min) plasma half-life. Further, the side effects of glucose intolerance, steatorrhea, and cholelithiasis are predictable from review of somatostatinoma case reports. A promising long-acting somatostatin analog (compound 201-995, Sandostatin\textsuperscript{a}) is presently available in the United States on a compassionate need basis for therapy in patients with symptomatic neuroendocrine tumors associated with refractory secretory diarrhea. This compound is an octapeptide (molecular weight, 1019.3) and shares a four amino acid homology with native somatostatin. Clinical studies have demonstrated the analog can be given subcutaneously, peaks within 30 min, and has a plasma half-life of 100-120 min with a duration of about 8 hr.

Sandostatin appears to be very effective in ameliorating symptoms of diarrhea and flushing in patients with progressive metastatic carcinoid tumors. In a recent study of malignant carcinoid syndrome, 19 of 25 patients (76%) had >50% reduction in secretory diarrhea with subcutaneous Sandostatin (dose range, 100 mg bid to 150 mg every 8 hr). In 10 of 12 patients (83%) with metastatic unresectable VIPoma/watery diarrhea syndrome, complete or partial resolution with reversal of hypokalemia and metabolic acidosis has been observed. Complete resolution of secretory diarrhea associated with diabetic gastroenteropathy has been reported in two of two patients studied with Sandostatin. Finally, in three of six patients with diarrhea, in whom no peptide excess state could be identified or bacterial or mechanical abnormality discerned, complete or partial resolution of diarrhea states (in this country) has been achieved with doses ranging between 50 and 200 mg twice or three times daily. Adverse effects thus far reported are minor, consisting of pain or burning at the site of injection, abdominal cramps, and transient loose stools. None of the reported side effects (in the United States) has been severe enough to necessitate stopping therapy. Development of antibodies to Sandostatin has not been observed either in patients treated for as long as 18 months. However, a phenomenon resembling tachyphylaxis, requiring adjustment of the dose, has been noted.

The somatostatin analog (compound 201-995, Sandostatin) appears to be a very promising and safe antidiarrheal agent in certain secretory diarrhea states associated with carcinoid syndrome, VIP-secreting tumors/watery diarrhea syndromes, and diabetic gastroenteropathy. Further, it may also be beneficial in certain secretory diarrhea states wherein the precise etiology cannot be determined.

**RABBIT ILEAL PROSTAGLANDINS: SITES OF PRODUCTION, PROFILES, AND STIMULI.** H.M. Berschneider, D.W. Powell, and L.D. Lawson, Division of Digestive Diseases and Nutrition and Core Center in Diarrheal Diseases, University of North Carolina, Chapel Hill, North Carolina 27514.

The short-circuit current (Isc) of rabbit ileal mucosa stripped of its muscle layers in solutions containing inhibitors of eicosanoid production are different from tissues stripped in normal Ringer solution and then mounted in Ussing chambers. When stripped in indomethacin (3 × 10\textsuperscript{-5} M), nordihydroguaiaretic acid (NDGA 10\textsuperscript{-4} M), or meperidine (10\textsuperscript{-4} M), (inhibitors of the cyclooxygenase, lipoxygenase, and phospholipase enzymes, respectively), the Isc was 50-100% lower than controls. Na and Cl fluxes measured in tissues stripped in the inhibitors showed stimulation of absorption as compared to controls, maximum stimulation occurring with meperidine which had a corresponding Ise of approximately zero. These findings suggest that stripping muscle layers from rabbit ileum releases eicosanoids which stimulate electrogenic ion secretion by the epithelial cells, thus accounting for the basal Ise in the Ussing chamber. The true basal Ise of normal rabbit ileum may be approximately zero, a condition accompanied by net Na and Cl absorptive rates of 3-5 μeq/hr/cm\textsuperscript{2}. Previous Ussing chamber studies of rabbit ileal transport have probably been accomplished in the setting of 'eicosanoid tone.'

The sites, profiles, and stimuli of eicosanoid production by rabbit ileum were studied in various components of the gut that were isolated with chemical and mechanical methods. Eicosanoids were measured by a combination of radioimmunoassay and HPLC. Epithelial cells, isolated as sheets of cells with a modification of the calcium chelation method of Weiser using EDTA (1.5 mM), maintained the ability to exclude trypan blue for up to 4 hr, were capable of transporting α-methylglucoside against a 15-fold gradient, and responded to PGE\textsubscript{2} and VIP with increases in cyclic AMP content. The remaining deep epithelialized ileum was studied separately or was further separated into the lamina propria, submucosa, and muscularis propria by a combination of blunt and sharp dissection. One hour was necessary for trauma-stimulated eicosanoid production to abate.

The prostaglandin profile from the ileal epithelial cell fraction (PGE\textsubscript{2} > PGF\textsubscript{2α} > PGE\textsubscript{2} = DHKPG > TXB\textsubscript{2}) was quite different from deep epithelialized ileum (PGE\textsubscript{2} = PG6KF\textsubscript{6} >> PGE\textsubscript{2} = DHKPG = TXB\textsubscript{2}), and the production of prostaglandins by the deep epithelialized ileum was 200 times the rate from the epithelial cells. Although the epithelial cells represented 67% of the protein content of ileum, it produced only 0.2% of PGE\textsubscript{2}. Conversely, the lamina propria and submucosa, which represented 12% of the protein in the gut wall, produced over 90% of the prostaglandins.

Bradykinin, A23187, arachidonic acid, and meullitin all stimulated PGE\textsubscript{2} synthesis by 200-400% in the intact ileum and deep epithelialized ileum, but bradykinin was without effect on the epithelial cell fraction or on the muscularis propria.

Thus, the major producer of eicosanoids in rabbit ileum is the lamina propria which is made up of inflammatory cells, fibroblasts, endothelial cells, and nerves. These results indicate that
the subepithelial tissues play an important regulatory role in intestinal water and electrolyte transport.

**ANTINFLAMMATORY AGENTS AND DIARRHEA. William F. Stenson, Washington University School of Medicine, 660 South Euclid, Box 8124, St. Louis, Missouri 63110.**

A number of important diarrheal diseases are characterized histologically by an intense inflammatory response. Infection of the colon with invasive bacteria including *Campylobacter* and *Shigella* results in an inflammatory response. Certain significant noninfectious diarrheal diseases are also marked by inflammation in the small bowel (sprue), colon (ulcerative colitis), or both (Crohn's disease). Diarrhea in these diseases results either from diminished salt and water absorption (ulcerative colitis) or a combination of decreased absorption and increased secretion (sprue). The pathogenesis of diarrhea in inflammatory states has not been well characterized but probably relates to loss or dysfunction of the enterocytes plus the actions of soluble mediators of inflammation on the enterocytes. Among the soluble mediators that have been proposed as playing significant roles in intestinal inflammation are the prostaglandins and products of the lipoxygenase pathway including leukotrienes B₄ and various mono hydroxy fatty acids (HETEs). Prostaglandins cause salt and water secretion in the small intestine and 5-HETE enhances chloride secretion in the rabbit colon in vitro. Other soluble mediators which may participate in intestinal inflammation include products of complement activation, platelet-activating factor, and kinins. Bradykinin induces salt and water secretion in the colon. In addition to the direct effects that some of the mediators have on intestinal epithelial secretion, almost all of these soluble mediators enhance vascular permeability to albumin and other macromolecules. Enhanced vascular permeability leads to tissue edema, a characteristic of inflamed intestinal mucosa. It is easy to imagine the presence of tissue edema impairing the absorption of salt and water from the intestinal lumen and contributing to diarrhea.

Elevated levels of prostaglandins and leukotrienes have been found in inflamed colonic mucosa in rat acetic acid colitis and human inflammatory bowel disease. Elevated levels of these mediators have been found in rectal dialysates of patients with ulcerative colitis. Both epithelial cells and inflammatory cells are capable of synthesizing prostaglandins, whereas leukotrienes appear to be produced by inflammatory cells, but not epithelial cells.

The role of soluble mediators in the amplification of the inflammatory response in diarrheal disease raises the possibility of treating these diseases by blocking the generation or action of these inflammatory mediators. This approach could include drugs that inhibit the synthesis of mediators and also receptor antagonists. It may be possible to target the delivery of these drugs to the gut by giving them as enemas or by administering them orally in forms that are poorly absorbed or unabsorbed. There is some indirect evidence that corticosteroids and sulphasalazine may have their beneficial effects in inflammatory bowel disease by inhibiting the synthesis of prostaglandins and leukotrienes. Sulphasalazine, at concentrations found in the colonic lumen, blocks both cyclooxygenase and lipoxygenase, whereas corticosteroids induce the synthesis of lipomodulin, a protein that inhibits phospholipase A₂ and, thus, blocks the release of arachidonic acid, the substrate for both the cyclooxygenase and lipoxygenase pathways. When patients with ulcerative colitis are treated with prednisolone, the levels of prostaglandin E₂ and leukotriene B₄ in their rectal dialysates decline. The availability of more selective inhibitors of arachidonate metabolism, particularly specific 5-lipoxygenase inhibitors, for targeted delivery to the inflamed intestine would give us a better insight into the role of arachidonate metabolites in the generation of inflammation in the intestine and may yield clinically useful drugs.

**NOVEL COMPOUNDS (BERBERINE AND NICOTINIC ACID). R. Bradley Sack, Department of International Health, Johns Hopkins University School of Medicine, 615 N. Wolfe Street, Baltimore, Maryland 21205.**

These two compounds share a number of common properties. Nicotinic acid (niacin) is one of the B complex of vitamins and is a normal component of food. Berberine is a natural plant alkaloid, which for several thousands of years has been used as a remedy for diarrheal diseases in India, China, and Burma. Both have recently been found to prevent and to reverse secretion in animal small intestine induced by chola toxin, and also by the heat-stable enterotoxin (ST) of *E. coli*, two toxins known to mediate their effects through different biochemical pathways. Both have also been given controlled clinical trials in humans with severe diarrheal disease (cholera), and both have given disappointingly less-than-anticipated therapeutic responses.

**Berberine.** Berberine, a bitter, yellow powder, is derived from the roots and bark of the plant *Berberis aristata*, a spiny, deciduous, evergreen shrub with yellow flowers (berberry bush) that grows widely in Asia. As one of a number of indigenous plant extracts being studied for its medicinal properties 30 years ago, it was found to markedly reduce the severity of *V. cholerae* infection in the infant rabbit model. Since then the drug has been studied extensively and found to have a number of antibacterial, antifungal, and antiparasitic properties. It is part of the regular pharmacopeia of India and China.

In several animal models (rats, mice, rabbits, and pigs), it has been found to both prevent and to reverse the secretory responses induced by cholera toxin and by both the heat-labile enterotoxin (LT) and ST of *E. coli*, when given either into the lumen of the secreting gut or given parenterally. It does not have an effect when given intraluminally into portions of the gut not involved in the stimulated secretory process, ie, isolated intestinal loops. The inhibitory effects are dose-related, and the effects at high doses are marked, with nearly complete inhibition of secretion. The mechanism of action of the drug is not completely defined, although it seems clearly to act through pathways which do not involve the cyclic nucleotides. In some models, it has been shown to increase basal water and sodium chloride absorption in the jejunum.

Because of its marked effect in animals, and because of its long history of use in humans, it has been studied in adults with severe diarrhea (cholera and noncholera) in Bangladesh and Burma in controlled trials. When given as a single 400-mg dose (approximately 10 mg/kg) to cholera patients, it resulted in a statistically significant decrease in stool volume of about 20% during the first 24 hr following administration. In noncholera patients, with lower stool outputs, the same dose resulted in a decrease in stool volume of about 40% in Bangladesh, but there were no differences in the Burmese study.

Berberine has not been tested on milder diarrheas, ie, those less than the severe category, and it has not been tested in

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children. The doses used in the clinical studies have been extrapolated from the animal data and the current known human use of the drug, and are somewhat arbitrary. There are no known adverse clinical effects of the drug (except hemolytic anemias have been reported in G-6-PD-deficient individuals) so that increased doses may have larger therapeutic effects.

Nicotinic Acid. Because nicotinic acid was known to prevent rises of cyclic AMP in isolated fat cells elicited by a variety of stimuli. Its effect on cholera toxin-mediated intestinal hypersecretion was studied. In rabbits it was found, at a dose of 100 mg/kg, to both prevent and reverse the intestinal secretion and the increase in cyclic AMP effected by cholera toxin. Additional studies in pigs, at the same dose, gave the same antisecreatory results, not only with cholera toxin, but also within the ST toxin of E. coli. In the pig studies, however, the effect was found to be independent of any changes in cyclic AMP or cyclic GMP. Also in the pig studies, the effect was only seen with nicotinic acid at acid pH; no effect was seen at neutral pH. No effects on baseline water and sodium chloride were seen.

Nicotinic acid was then studied, in a controlled way, in adults with cholera in Bangladesh, at a dose of either 1 or 2 g given in divided doses over a 24-hr period. At the lower dose, no significant reduction in stool volume was found, but at the high dose a significant reduction of about 30% was found, as compared to concurrent controls. No significant side effects were seen from the drug.

Suggestions for New NIH Funding Initiatives in the Area of Diarrheal Diseases

PATHOPHYSIOLOGY OF INVASIVE INFECTIOUS DISEASES

Purpose

As a result of the May 8–9, 1986, conference, we recommend a new program announcement for the study of infectious diarrhea. The Division of Digestive Disease and Nutrition (DDDN) of the National Institute of Diabetes, and Digestive and Kidney Diseases (NIDDK) and the Microbiology and Infectious Disease Program (MIDP) of the National Institute of Allergy and Infectious Disease (NIAID) has recently announced a need for research on enterotoxin intestinal mucosal receptors and microbiol adherence (NIH Guide for Grants and Contracts, Vol. 15, May 23, 1986, page 17). We suggest that they now seek submission in other areas of infectious diarrheas and encourage submission of scientifically meritorious grant applications to study the pathophysiology of invasive infectious diarrhea of viral, bacterial, and parasitic origin. Research in this area might include:

1. Pathophysiologic studies of diarrheogenic enteric viruses. These might include studies of viral receptors; viral cell-to-cell invasion mechanisms; viral effects on enterocyte DNA and on DNA expression; virally induced inflammatory responses; and viral effects on plasma membrane phospholipid metabolism.

2. Mechanisms by which nonviral microorganisms invade the intestinal epithelium and mucosa, including studies of bacterial products involved, host defense mechanisms, and the role of inflammatory mediators in the physiologic response to infection.

Background

Diarrheal diseases remain one of the major causes of mortality worldwide and are the leading cause of infant mortality, with estimates of 5–10 million deaths per year occurring in Africa, Asia, and Latin America, and the claim that 13% of children less than 5 years old in Brazil die from diarrheal diseases (R.L. Guerrant, in Microbial Toxins and Diarrheal Diseases, CIBA Foundation Symposium, 112, 1985). In the United States, diarrhea is one of the leading causes of morbidity as judged by loss of time from work, although few patients die. The causes for acute diarrheal diseases are largely infectious, with at least 20% of the cases being due to viruses, especially rotaviruses, and approximately 10% due to parasitic diseases, including Entameba histolytica and Giardia lamblia. In addition, recently recognized infectious causes of diarrhea such as Cryptosporidia cause up to 5% of acute diarrheal diseases in some developing countries, while other recently recognized bacterial causes such as Campylobacter cause 10% of acute diarrhea.

Emphasis on diarrheal diseases research has been on attachment factors and mechanisms by which several bacterial products produce intestinal secretion, with most extensive study of mechanisms of action by cholera toxin and heat-labile and heat-stable E. coli enterotoxins. Understanding second messenger functions in the intestine and other cells...
has largely been based on studies of several enterotoxins, using them as activators. This is particularly true for cholera toxin which stimulates adenylate cyclase activity by acting on a guanine nucleotide binding protein while Bordetella pertussis toxin acts to inhibit another guanine nucleotide binding protein and either releases inhibition of adenylate cyclase or affects cellular Ca\(^{2+}\) handling. A recent program announcement by NIDDK and NIAID seeks additional research on these enterotoxins and adherent factors which lead to colonization of the bowel at specific sites by microorganisms, including parasites. In contrast, there is little known concerning the pathophysiology of viral and invasive bacterial and parasitic diarrheal diseases. The mechanisms by which microorganisms invade the intestinal cells and damage normal intestinal cell function remain largely unknown. Consequently, a program announcement for infectious diarrheal diseases is suggested emphasizing these areas in which the pathophysiology is poorly understood, including:

1. Pathophysiologic studies of diarrheagenic enteric viruses, especially the ways viruses interact with epithelial cells, the nature of viral receptors, and the role of inflammatory intermediates in diarrhea and how products of enterocyte plasma membrane phospholipid metabolism are affected by the viruses.

2. Mechanisms by which invasive bacteria invade the intestinal epithelium, including the effects of bacterial products, host defense mechanisms, pathways of invasion, and the role of inflammation in the response to infection.

3. Understanding the pathophysiology of parasitic diarrheal diseases including site specificity, production of enterotoxins, attachment, and invasion factors, and the role of inflammatory intermediates and interactions with host defense mechanisms.

**ANTISECRETORY DRUGS AND ORAL NUTRIENT-ELECTROLYTE SOLUTIONS IN SECRETORY DIARRHEAS**

**Purpose**

As a result of the May 8-9, 1986, conference, we recommend that the Division of Digestive Diseases and Nutrition (DDDN) of the National Institute of Diabetes, and Digestive and Kidney Diseases (NIDDK) and the Microbiology and Infectious Diseases Program (MIDP) of the National Institute of Allergy and Infectious Disease (NIAID) encourage submission of scientifically meritorious grant applications for the development of human or animal models to test antisecretory drugs and oral nutrient-containing electrolyte solutions for the treatment of secretory diarrheas.

In the past 10 years the use of oral replacement solutions in the treatment of secretory diarrheas, particularly those of infectious etiology, has represented a major breakthrough in halting the mortality and morbidity of those diseases in developing nations. The present program announcement is prompted by two developments.

1. Recent evidence suggests that the use of polypeptides and glucose polymers in oral replacement solution may be more efficacious than the standard glucose-electrolyte solution in replenishing water and electrolyte losses in patients with severe diarrhea. Use of such carbohydrate- and protein-enriched solutions might also diminish diarrheal volume. Development and testing in humans of optimal oral nutrient-electrolyte replacement solutions remains a desirable research goal.

2. Use of antisecretory compounds could limit diarrheal output and diminish the duration of diarrhea, thus decreasing morbidity and mortality. Although World Health Organization-sponsored investigation is proceeding in humans with infectious diarrheas, the development of suitable animal models in which to test concepts and formulations prior to their application to man would be of great benefit. Accordingly, a program announcement is suggested seeking applications for the development of such animal models which would be both simple to use and effective in the assessment of specific antidiarrheal drugs.

**Background**

The efficacy of oral replacement solutions in the treatment of secretory diarrheas has been repeatedly confirmed. In fact, use of oral replacement solutions is one of the more impressive applications of basic science knowledge to treatment of diseases in man. The ability of glucose to promote water and electrolyte absorption in the face of secretory diarrheas remains the single most important therapeutic advance in the last century in the treatment of these diseases. Nonetheless, glucose- or amino acid-supplemented oral electrolyte solutions have not been found to reduce diarrhea, although when given in sufficient quantity, they can replace diarrheal...
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losses. Preliminary evidence from human studies suggests that the use of polymeric forms of carbohydrates and proteins ("rice ORT") improves water and electrolyte absorption beyond that promoted by glucose alone. It is likely that a large osmotic penalty is not paid by the addition of polymeric sugars or amino acids to oral replacement solutions. The working hypothesis is that as glucose and amino acids are liberated by digestive tract hydrolases, they are quickly absorbed. With rice absorption, water and electrolytes that are largely derived from fluid secreted into the gut lumen by the diarrheal process are also absorbed. Currently, one of these "super" oral replacement solutions ("rice ORS") is being tested in man. A saving in time, effort, and cost, and perhaps an increased safety might be gained by pretesting such "super" oral replacement solutions in suitable human or animal models. Animal models of infectious diarrhea, particularly rotavirus and toxigenic E. coli, which are the leading causes of diarrheal diseases in developing nations, might allow more rapid advances in this field. Animal models presently employed include in vivo perfusion techniques and static isolated loops as well as in vitro gut-sack and Ussing chamber techniques. None of these models sufficiently mimics normal whole animal physiology to readily allow extrapolation of the results obtained in such systems to the intact human. Furthermore, infant physiology is quite different from the adult, further complicating the interpretation of results. What is critically needed are in vivo whole-animal or human models of diarrhea which will allow assessment of efficacy of these oral replacement solutions in both dehydrated and normally hydrated animals.

Studies in the last decade have uncovered a host of potentially useful antitoxic secretory agents. When studied in in vitro and in vivo animal systems, a variety of agents, including opiates, alpha-2-adrenergic agonists, somatostatin, neuropeptide Y, anti-inflammatory agents, and phenothiazines showed impressive antitoxic or proabsorptive properties. However, when applied to humans with diarrheal illnesses, only a relatively few compounds (eg, chlorpromazine, berberine, nicotinic acid) have shown any efficacy and, even then, are of only borderline clinical usefulness. This suggests that the current animal models for assessment of the antitoxic and proabsorptive effects of drugs are inadequate in their application to humans, especially to infants and children. Accordingly, infant animal models of secretory diarrhea would be extremely useful for evaluating of antitoxic compounds. Appropriate animal models would also allow testing of oral or parenteral drugs alone or in combination in various age groups with infectious enteritides. Testing could also be accomplished in both the hydrated and dehydrated states, the latter more closely approximating natural disease in humans. Such models would allow identification of efficacious agents and refinement of drug dosage schedules or combinations of drugs that might be useful in the treatment of diarrheas. These models should allow assessment of water and electrolyte balance as well as concrete assessments of morbidity and mortality.

Examples of potentially useful models are triple-lumen tube, water marker, perfusion studies in humans (Schiller et al. Studies of the mechanism of the antidiarrheal effects of codeine. J Clin Invest 70:999, 1982) and animal models (eg, piglets) where water markers can be continuously fed to unanesthetized, infected neonates or infants (Lecce JG, Balsbaugh RJ, Clare DA, King MW. Rotavirus and hemorrhagic entero-pathogenic Escherichia coli in porcine diarrhea of pigs. J Clin Microbiol 16:715, 1982).

INTESTINAL CELL CULTURE

Purpose

As a result of the May 8-9, 1986, conference, we recommend that the Division of Digestive Diseases and Nutrition of the National Institute of Diabetes and Digestive and Kidney Diseases seeks contracts to develop intestinal epithelial cell cultures. Research on the expression and regulation of various intestinal epithelial cell absorptive and secretory functions would be greatly facilitated by the development of immortalized cell lines derived from normal small intestine and colon of vertebrates. Cancer cell lines with differentiated subsets cloned for specific epithelial functions would also be useful. Such cell culture systems would provide a basis for understanding the cellular mechanisms underlying a number of diarrheal diseases. Diarrheal disease is the largest single disease-related cause of mortality and morbidity in the world at large. In the United States, it is one of the two leading causes of admissions to pediatric emergency rooms and pediatric inpatient services. Among American adults, it is less prevalent but still a major public health concern. The large majority of severe diarrheas in
Cooperative agreements or contracts are encouraged to develop and characterize immortalized intestinal epithelial cell cultures that will provide models for the study of absorptive and secretory processes and for the study of microbial interactions with these processes. Applications should be related to, but not necessarily limited to, studies in one or more of the following areas: (1) immortalization of differentiated, normal intestinal epithelial cells with viral DNA; (2) development of techniques for growing cells into confluent monolayers suitable for transepithelial transport studies; (3) studies of matrices (ie, collagens, fibronectin, etc) useful for cell growth and differentiation; (4) coculture of intestinal epithelial cells with various mesenchymal cells that may influence growth and differentiation of the former; (5) hormonal requirements for cell growth and differentiation; (6) study of factors leading to or inhibiting dedifferentiation of cultured cells; (7) development of cell culture systems derived from different segments of the normal intestinal tract; (8) characterization of the transport properties of cultured cells; (9) cloning of specific subsets of epithelial cells derived from both normal and cancer cells lines; and (10) development of cDNA libraries of cloned cell lines.

Awardees of Cooperative Agreements or Contracts would be required to submit new long-lasting or immortalized intestinal cell lines to the NIH cell culture bank so that they can be made generally available for research.

Background

At present only one cell line derived from normal intestine is generally available (IEC-6 and IEC-18, essentially identical lines derived from fetal rat small intestine). These are cryptoid cells which have not been successfully differentiated into villus cells, except when inoculated into rat embryos. Monoclonal antibodies to various surface and internal cell proteins have been generated. These have proven useful for tracing the expression of these proteins in other intestinal epithelial cells, both normal and malignant. Three colon cancer cell lines are available, one of which (CaCo) has been found to adopt some of the functions and structural features of the small intestine following critical changes in the culture medium. The principal means for transforming these colon cancer cells into small intestine-type cells is omission of glucose from the culture medium. The cells transformed from wild-type culture have been cloned to yield different types of small intestinal cells, for example, goblet cells and brush border enriched absorptive cells. Another colon cancer cell line (T-84) is secretory in nature and has been grown to confluence for transepithelial transport studies. It is a useful model for studies of active chloride secretion.

Immortalization of primary cell cultures has occurred on occasion spontaneously but can now be more systematically induced through the use of viruses and viral DNA hybrids in which the replication unit of the transforming virus DNA has been deleted. The origin-defective simian virus 40 (SV40) mutant 6-1 has been useful in transforming human cells. However, the low efficiency of transformation achieved by DNA transfection has been a major drawback. More recently, this efficiency has been increased by use of recombinant adenovirus-SV40 virions (Van Doren K, Glugman Y. Efficient transformation of human fibroblasts by adenovirus-simian virus 40 recombinants. Mol Cell Biol 4:1653, 1984). Because of the high efficiency of transformation, the origin-defective chimeric virus is a convenient system for establishing SV40-transformed cell lines from any human cell type susceptible to infection by adenovirus 5. This most likely includes intestinal cells. The C-mic oncogene has been used to immortalize certain kidney cell lines and may also prove useful for intestinal cells.

Cryptoid cell lines are more readily propagated and perpetuated than are more differentiated (absorptive) cell lines. Techniques for inducing differentiation of cryptoid cells into absorptive cells need to be developed. In the case of malignant colon cells (CaCo), the simple expedient of galactose substitution for glucose in the culture medium has proven successful. The use of growth factors, hormonal and other types, has thus far been little explored for intestinal cells.

Cooperative agreements are encouraged for the development and characterization of immortalized cell cultures derived from various segments of the intestinal tract. Studies are encouraged in the following areas: (1) attempts at immortalization using viral DNA; (2) development of techniques for growing cells into confluent monolayers suitable for transepithelial transport studies; (3) studies of matrices useful for growing cells (ie, collagens, fibro-
nectin, etc) to examine the relation between specific matrices and cell growth and differentiation; (4) coculture of intestinal epithelial cells with various mesenchymal cells to examine their developmental interactions; (5) hormonal requirements for cell growth and differentiation; (6) cloning of specific subsets of epithelial cells derived from both normal and cancer cell lines; (7) development of cDNA libraries of cloned cells; and (8) study of factors leading to cell transformation and dedifferentiation.

REGULATION OF INTESTINAL WATER AND ELECTROLYTE TRANSPORT BY ENTERIC NERVOUS, ENDOCRINE, AND IMMUNE SYSTEMS

Purpose
As a result of the conference of May 8–9, 1986, we recommend that the Division of Digestive Diseases and Nutrition (DDDN) of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) encourage the submission of scientifically meritorious grant applications for studies of the regulation of intestinal ion and water transport. Investigator-initiated grants, program project submissions, and training grant applications are encouraged in the following or related areas:

1. Regulation of the cellular mechanisms of enterocyte ion transport by the enteric nervous system and mucosal endocrine cells including any of the following: regulation by the autonomic nervous system including the enteric nervous system (ENS); regulation by the central nervous system (CNS); and regulation of enterocyte function or modulation of the ENS by intestinal endocrine cells through classic hormonal actions or through paracrine effects.

2. Regulation of enterocyte or ENS function by elements of the immune system including intraepithelial lymphocytes, macrophages, T cells, B cells, and mast cells, as well as mesenchymal cells in the lamina propria such as fibrocytes, endothelial cells, and smooth muscle elements.

3. Role of ENS in modulating changes in enterocyte function brought about by luminal or systemic influences; eg, luminal nutrients, toxins or circulating hormones.

Background
In the past two decades an impressive body of knowledge has emerged from the study of intestinal ion and water transport, particularly in defining the ion transport mechanisms at both the apical and basolateral membranes of the intestinal cell and how intracellular messengers such as cyclic AMP, cyclic GMP, and calcium regulate these ion carriers and channels. In no way have scientists achieved a complete understanding of the intracellular and membrane events which lead to intestinal water and electrolyte absorption and secretion and, indeed, additional research is necessary in this area. However, there is even less understanding of the neural, humoral, and paracrine control of epithelial function. Only recently has it become clear that the enteric nervous system is a separate and somewhat autonomous section of the nervous system with a number of neurons approximating that of the spinal cord. The ENS may innervate enteroendocrine cells which regulate enterocyte function through classic endocrine or paracrine mechanisms. Furthermore, elements of the ENS, either nerves or endocrine cells, are important in the regulation of the ENS itself. Thus, enterocyte or ENS function may be modulated by neural projections from the CNS. Areas of research that might be addressed include:

1. Definition of neurotransmitter, neuromodulator, paracrine, and endocrine agents that take part in intestinal enterocyte regulation, including whether these elements affect the enterocyte directly or whether they modulate enteric nervous system function that subsequently alters enterocyte function.

2. A specific definition of mucosal endocrine cells including the identification of their effector substances and whether the discharge of these substances is regulated by intraluminal or neurohumoral events.

3. Mapping of extrinsic and intrinsic enteric nerves and how local spinal or ganglia reflexes regulate the ENS.

A potentially important, but even less well understood, controlling mechanism for intestinal water and electrolyte transport is the immune system and other mesenchymal elements of the lamina propria. Eicosanoids produced by resident blood cells (intraepithelial lymphocytes, T cells, B cells, macrophages, and polymorphonuclear leukocytes) may be important stimulants of intestinal secretion. Similarly, effector substances from mast cells, leukocytes, and macrophages, such as serotonin, adenosine or its analogs, kinin-generating factors, super-
oxide, and oxy-radicals, might be important regulators of intestinal water and electrolyte transport, epithelial cell damage, or epithelial repair. Other potential elements in the lamina propria which might produce mediators include vascular endothelium, fibrocytes, and smooth muscle cells. Their roles in modulating intestinal electrolyte transport need further definition as well. While these elements may not be important in all diarrheal diseases, they seem most likely to be important in the pathophysiology of inflammatory bowel disease, invasive viral and bacterial diarrheas, parasitic disease, and probably in food allergy.

Studies of immune regulation of enterocyte function might include: (1) a cataloguing of the specific elements in the lamina propria which respond to stimuli with secretion of effector substances; (2) an understanding of the neurohumoral regulatory control of effector release by laminal proprial elements; (3) a definition of which effector substances are released from inflammatory cells and how they may affect the enterocyte (this would include study of eicosanoids, adenosine compounds, histamines, serotonin, and other antacoids); (4) in depth study of mast cell biology in the gut including the sources of mast cell, neurohumoral control of mast cell function, and identification of mast cell products and their release; and (5) study of how intestinal inflammation regulates intestinal enterocyte and epithelial function (such studies might seek an understanding of how lymphokines and other agents regulate epithelial function).

**DRUG THERAPY OF CHRONIC DISABLING DIARRHEA**

**Purpose**

As a result of the conference of May 8–9, 1986, we recommend that the Division of Digestive Diseases and Nutrition (DDDN) of the National Institute of Diabetes, and Digestive and Kidney Diseases (NIDDK) seek applications for Cooperative Agreements or Contracts for multiinstitutional, cooperative studies of the therapy of chronic disabling diarrheas. Multicenter trials of treatment are sought for debilitating chronic diarrheas such as short-bowel syndrome, refractory steatorrheas of any type, AIDS-related diarrhea, secretory diarrheas, diabetic diarrhea, and radiation enteritis. Such cooperative agreements could include funds for support of the principal investigator, a biostatician, an epidemiologist, and a nurse-practitioner to plan the study or to direct the investigation once it has begun. Funds could be requested for data management and computer analysis. The proposal should include treatment protocols and mechanisms to allow individual pharmaceutical firms to share in the cost of the individual studies of their drugs or agents. The cooperative agreement/contract should include definition of the diarrheal diseases to be studied, how the patients are to be evaluated and categorized, and how efficacy of treatment is to be determined. It should include a listing of participating study groups or institutions and evidence of cooperation from at least one pharmaceutical firm whose agent is to be tested.

**Background**

In developing nations, acute diarrhea is a leading cause of morbidity and mortality, particularly among infants and children. In developed nations, diarrheal diseases are primarily an economic burden. In humans they account for considerable lost time from work and moderate expenditure for health care evaluation and treatment. If one includes inflammatory bowel disease, infectious diarrhea, and functional bowel disease, it has been estimated that in 1980 $2 billion yearly were expended in direct health care costs, not including economic loss in the work place. There are groups of individuals whose disabling diarrhea causes considerable morbidity and cost. These are patients with previous intestinal resections leaving them with short-bowel syndrome, with idiopathic refractory malabsorptive disease, with secretory diarrheas usually from endocrine tumors, with radiation enteritis, with the idiopathic diarrhea of diabetes, and those with AIDS. No one medical center has sufficient numbers of these patients to effectively study the pathophysiology of their diseases or to assess drug efficacy in their treatment. There are a number of potentially useful antisecretory compounds or drugs which have not been studied in any organized fashion in these disease states. Accordingly, the present contract/cooperative agreement would seek funds to organize a multicenter effort to prospectively study the treatment of these or other disabling diarrheas with antisecretory or proabsorptive compounds.

The essential elements of phase one of the study would include funds for a principal investigator to head the study and support for a biostatistician and an epidemiologist as well as funds for data management.

The second phase of the agreement/contract...
would be to actually initiate studies of patients with specific antidiarrheal agents. This phase of investigation would require that the planning group and a number of institutions take part in a multicenter trial. The cooperating institutions should define specifically which diseases they wish to include in the study. They should include an operative definition of the diseases or states to be investigated; plans for evaluation of the pathophysiology of these diseases; agreements with at least one pharmaceutical firm to defray the costs of study of at least one putative therapeutic agent in the treatment of these diseases; concrete plans that will allow the unambiguous evaluation of drug efficacy; and data management storage and computer capabilities for analyzing the data that result from this multicenter trial. Funds for nurse-practitioners might be requested; ideally, the cost of such assistants would be paid in part by grants from pharmaceutical companies.