

Pathogenesis of Rotavirus-Induced Diarrhea

Preliminary Studies in Miniature Swine Piglet

DAVID Y. GRAHAM, MD, JEFFREY W. SACKMAN, and MARY K. ESTES, PhD

The pathogenesis of diarrhea caused by rotavirus infection was studied in miniature swine piglets. The animals were inoculated orally with 2×10^7 plaque-forming units of porcine rotavirus (OSU strain). During the height of diarrhea, intestinal function was investigated by in vivo perfusion of a 30-cm segment of proximal jejunum and a 30-cm segment of distal ileum. Absorption of Na^+ and water decreased and 3-O-methylglucose transport was markedly reduced, $P < 0.01$ compared to control animals. Mucosal lactase and sucrase levels were depressed in both the jejunum and ileum, $P < 0.001$. Na^+, K^+ -ATPase activity was significantly depressed only in the ileum, $P < 0.001$. These changes were associated with a marked reduction in villous height, suggesting that the diarrhea could be an osmotic diarrhea due to nutrient (carbohydrate) malabsorption. Fresh stool samples were obtained and analyzed immediately for Na^+ , K^+ , osmolarity, glucose, and lactose; the osmotic gap was also determined. Stool osmolarity continually increased from 248 ± 20 mosm/liter prior to inoculation to 348 ± 20 mosm/liter at 75 ± 1 hr postinoculation ($P < 0.005$); the majority of the fecal osmotic gap could be accounted for by the amount of lactose present in the stools. Stool sodium increased from 34 ± 6 mM prior to inoculation to a maximum of 65 ± 4 mM at 53 ± 1 hr postinoculation, $P < 0.001$. There was no significant change in potassium concentration. The present investigation suggests that rotavirus-induced diarrhea is due to virus destruction of enterocytes lining the intestinal villi, thus reducing the mucosal surface area and important digestive enzymes. This destruction leads to an osmotic diarrhea due to nutrient (primarily carbohydrate) malabsorption. A possible contributing role of unopposed secretion from the crypt cannot be excluded from this study.

Rotavirus-induced gastroenteritis is a worldwide problem. Acute diarrhea is the most important

cause of illness, malnutrition, and death among children in developing countries. The best available data estimate that annually 100 million diarrheal episodes affect children below 5 years of age, resulting in 5 million deaths (1, 2). Rotaviruses are the most common cause of acute nonbacterial gastroenteritis requiring hospitalization in infancy and early childhood.

Our knowledge of the pathophysiology of rotavirus diarrhea is limited and is based upon morphological studies in experimental animals (3-10) and biopsies of intestinal mucosa from children with gastroenteritis (11). These studies have shown that the virus destroys the enterocytes lining the villi of the small intestine, leading to progressive shortening of

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From the Digestive Disease Section, Veterans Administration Medical Center, and Baylor College of Medicine, Houston, Texas.

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Address for reprint requests: Dr. David Y. Graham, Room 612, Bldg 1A, VA Medical Center, 2002 Holcombe Blvd., Houston, Texas 77211.

the villi, increases in crypt depth, and reduction of villous mucosal enzymes including sucrase, lactase, and Na^+, K^+ -ATPase. Although there have been few studies of intestinal function in rotavirus gastroenteritis, a number of detailed studies have evaluated gastroenteritis caused by transmissible gastroenteritis virus (TGE) of swine, a completely unrelated virus (a coronavirus) (12–17). These studies on TGE merit comment as this has been the most extensively studied viral diarrhea. TGE is an extremely virulent, and frequently lethal, infection of piglets associated with essentially complete destruction of the villous epithelium within 12 hr after inoculation (18, 19). Reduced mucosal Na^+, K^+ -ATPase activity and defective glucose-stimulated Na^+ transport have been demonstrated. TGE-induced diarrhea has been reported in the absence of mucosal damage (13, 15), although this has not been confirmed, and most studies have shown that the functional disturbances occur only after enterocyte destruction and shedding of virus into the lumen. The clinical severity of the disease is influenced by the age of the host.

More recent studies have demonstrated diminished glucose transport (20) *in vivo*, during the period when TGE-induced diarrhea was most severe. This observation is consistent with previous experiments with TGE that demonstrated that the diarrhea could be made to start or stop simply by feeding or withholding milk (19). Although it is not clear whether the data from the TGE model is directly applicable to rotaviral-induced disease, an *in vitro* Ussing chamber study showed similar alterations of glucose-stimulated Na^+ transport (21), suggesting that the two diseases have similar pathogenesis.

We report studies on the pathogenesis of rotavirus-induced diarrhea in mini-swine piglets. An integrated analysis of rotavirus infection *in vivo* was performed with the goals of (1) identifying a susceptible animal, (2) producing a reproducible disease, and (3) evaluating the histological and functional abnormalities responsible for the diarrhea. The mini-swine piglet was chosen as the animal model because of similarities between human and porcine digestive physiology and because its small size makes it a convenient laboratory animal. Additional attractive features of the mini-swine model include the fact that mini-swine are monogastric, secretory IgA is the major intestinal immunoglobulin, and the OSU strain of porcine rotavirus is easily cultivatable in the laboratory.

MATERIALS AND METHODS

Animals and Virus. Miniature swine piglets were an inbred cross between the Pitman-Moore and Sinclair strains (University of Texas System Cancer Center, Science Park Veterinary Division, Bastrop, Texas). Piglets were transferred to the vivarium at 3–5 days of age, housed in rabbit cages, and fed a milk-based formula (Similac®, Columbus, Ohio) three to four times daily throughout the study. Two days after arrival, piglets were transferred to cages in a separate building and inoculated orally with 2×10^7 plaque-forming units (22) of porcine rotavirus (OSU strain, obtained by needle aspirate of cecal contents of a gnotobiotic piglet, infected, and kindly supplied by Dr. E. Bohl) in 1.0 ml using a feeding needle (Popper & Sons, New Hyde Park, New York). Sodium bicarbonate (100 μmol , 1.0 ml, pH 7.4) was administered first, immediately followed by the rotavirus inoculum. Animals were observed frequently and signs of disease were recorded.

***In Vivo* Perfusion Studies.** Piglets were fasted for 8–12 hr prior to beginning perfusion experiments. Two or three days after inoculation, piglets were anesthetized with 20 mg/kg Ketamine, intramuscularly, followed by 25 mg/kg pentobarbital, intraperitoneally. The abdomen was opened through a midline incision and two 30-cm intestinal segments (a jejunal segment beginning 5–10 cm distal to the ligament of Treitz and an ileal segment extending proximally beginning 10 cm proximal to the ileocecal junction) were isolated and their ends cannulated. After cleansing the cannulated bowel with 30 ml warm perfusate solution, a single-pass perfusion study was performed using a multichannel peristaltic pump (Model P-3, Pharmacia, Sweden). The infusion rate was determined before and after each perfusion study.

Two perfusion solutions were employed. The first contained 145.9 mM Na^+ , 4.73 mM K^+ , 132.3 mM Cl^- , 1.17 mM Mg^{2+} , 0.84 mM Ca^{2+} , 1.17 mM SO_4^{2-} , and 20 mM HCO_3^- (osmolarity = 310 mosm/liter). The second solution was similar except 135.9 mM Na^+ , 122.3 mM Cl^- , and 34 mM [^{14}C]-3-*O*-methyl-*D*-glucose (MGLU) (Amersham Radionuclides, Arlington Heights, Illinois) were present (osmolarity = 321 mosm/liter). Six 20-min effusate samples were collected into preweighed, covered containers. The volume recovered was determined by weighing the samples to the nearest 0.1 mg (Mettler Instruments, Hightstown, New Jersey). Steady state was assumed if the volume collected varied <2% between collections. Water transport was calculated by subtracting the volume infused from the volume recovered using the final 60–80 min of perfusion. Aliquots of those collections from the second solution (containing MGLU) were analyzed for radioactivity by counting in a toluene base fluor (Scintiverse, Fisher Scientific, Pittsburgh, Pennsylvania) using a Packard 3801 liquid scintillation counter. Sodium and MGLU transport were calculated by subtracting the amount recovered (volume times concentration) from the amount infused.

Specimen Collection and Analysis. At the end of the perfusion experiments, the piglet was killed by an intracardiac administration of concentrated KCl. The small bowel was quickly removed, placed on ice, and the

perfused segments removed. The length of each segment was measured; the wet weight was determined to the nearest 0.1 mg, and the dry weight was determined after drying to constant weight under an infrared heat lamp for 24 hr. Segments of bowel (4–6 cm) immediately distal to the jejunal perfusion site, immediately proximal to the ileal perfusion site, and in the mid-small intestine were removed for enzyme determinations and histology. Mucosal homogenates were prepared by opening the bowel along the mesenteric border and scraping the mucosa using a glass slide. The mucosa was homogenized (Sovrall Omni-Mixer) in 20–30 ml ice-cold 150 mM NaCl, pH 7.2, and the samples frozen (-22°C).

Disaccharidase activity was determined within 2 weeks of the perfusion experiments according to the method of Dahlqvist (23) with modifications. The sample (0.2 ml) was added to 0.2 ml 100 mM substrate (lactose or sucrose) at pH 5.6 or 6.0 for lactase or sucrase, respectively. Aliquots were analyzed for glucose during the next 60 min using a Beckman glucose analyzer. One unit of enzyme activity was defined as $1\ \mu\text{mol}$ glucose liberated per minute per gram protein. Protein was determined according to the method of Lowry et al (24). $\text{Na}^{+},\text{K}^{+}$ -ATPase was determined according to the method of Hokin et al (25).

Histological specimens were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. Stained sections were coded and examined (blindly) by one of us (D.Y.G.) using a calibrated micrometer eyepiece to determine crypt depth and villous height. Well-oriented sections were selected for measurement, and at least five measurements were done from each portion of the bowel.

Analysis of Stools. Fresh stool samples were obtained from rotavirus-inoculated and control piglets throughout the experiments. A clean stainless-steel tray was placed under a piglets and the piglet was continuously observed. When a stool was passed without urine it was immediately collected and centrifuged at $12,500\ \text{g}$ for 30 min (Microfuge 12B, Beckman Instruments, Palo Alto, California) (within 10 min of excretion) to obtain stool water. Stool water then was analyzed immediately. Formed stools were homogenized with an equal volume of distilled water prior to analysis. The sodium and potassium concentrations were determined by flame photometry (Instrument Labs, Lexington, Massachusetts), osmolality by vapor pressure depression (Wescor Instruments, Logan, Utah), and glucose by glucose oxidase (glucose analyzer, Beckman Instruments, Palo Alto, California). Lactase was determined by hydrolyzing 0.2 ml stool water with 400 units lactase (Sigma Chemicals, St. Louis, Missouri) in 0.8 ml of 0.1 M maleate buffer, pH 5.6, for 24–30 hr at 37°C and determining the resulting concentration of glucose. A standard 100 mM lactose solution was included in each lactase assay. The assay was shown to have reached an endpoint within 20 hr, and quantitative recovery assays provided 93–104% recovery. Interassay variations of 10–12% and intraassay variations of <9% were observed.

Stool osmotic gap was calculated from the formula: osmotic gap = stool osmolality $- 2(\text{Na}^{+} + \text{K}^{+})$.

Statistics. All data are presented as the mean \pm stan-

dard error. Student's two-tailed t test was used to determine significant differences.

RESULTS

Clinical Signs of Infection in Piglets

Within 18 hr postinoculation (PI), all rotavirus-inoculated piglets exhibited profuse diarrhea with loss of appetite, frequently with vomiting. Diarrhea progressed, became severe approximately 36 hr PI, and continued through the maximum period tested, 78 hr. Perfusion studies were performed during the height of diarrhea.

Analysis of Intestinal Function and Structure

***In Vivo* Transport.** To evaluate the effect of rotavirus infection on intestinal function, perfusion experiments were performed 48–78 hr PI. Uninoculated litter-mates were studied at similar times. The perfusion rates were $2.203 \pm 0.004\ \text{ml/min}$ for jejunal perfusions and $2.341 \pm 0.005\ \text{ml/min}$ for ileal perfusions. Water and sodium transport data for perfusates without 3-*O*-methylglucose are presented in Figure 1. Water transport was significantly depressed 48–72 hr PI in both the jejunum ($P < 0.05$) and ileum ($P < 0.05$). Sodium transport significantly decreased in the jejunum ($P < 0.05$) but not in the ileum. Water and glucose transport data for 3-*O*-methylglucose-containing perfusates are presented in Figure 2. Water transport was significantly depressed in the jejunum ($P < 0.02$), but not in the ileum. 3-*O*-methylglucose transport was depressed following inoculation in the jejunum ($P < 0.001$) and ileum ($P < 0.01$). Sodium transport was reduced in the infected intestine compared to control animals (226 ± 213 vs $-254 \pm 94\ \mu\text{mol/hr/g}$ dry weight in the jejunum and 563 ± 305 vs $51 \pm 87\ \mu\text{mol/hr/g}$ dry weight in the ileum for the controls and infected animals, respectively). These differences did not achieve statistical significance; however, sodium absorption was linearly correlated to glucose absorption in both the jejunum ($r = 0.67$, $P < 0.01$) and the ileum ($r = 0.77$, $P < 0.01$).

Mucosal Enzymes. Mucosal lactase and sucrase levels were depressed in both the jejunum ($P < 0.01$) and ileum ($P < 0.01$) following infection (Figure 3). In addition, lactase and sucrase levels were depressed in the mid-intestine (0.5 ± 0.1 and 0.4 ± 0.1 units/g protein, respectively; compared to controls of 6.7 ± 2.5 and 6.3 ± 0.4 , $P < 0.05$). $\text{Na}^{+},\text{K}^{+}$ -ATPase remained unchanged in the jejunum and was significantly depressed compared to

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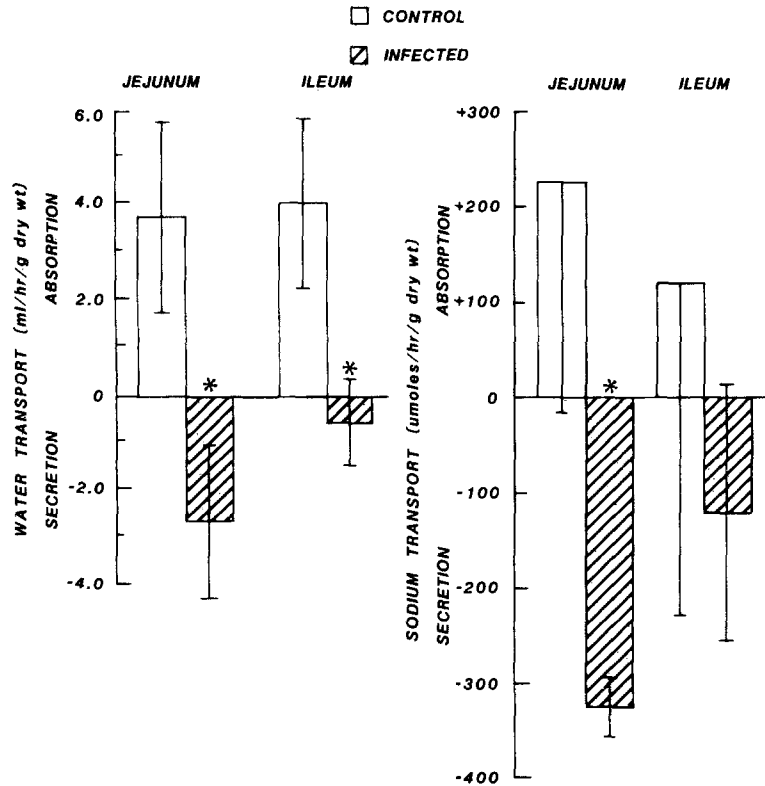


Fig 1. Net transport of water and sodium obtained when jejunal and ileal intestinal segments of piglets were perfused with a balanced electrolyte solution. Significant differences are indicated by an *. There were eight control and eight infected animals perfused.

that of control animals in both the mid-intestine (0.3 ± 0.1 compared to 0.9 ± 0.1 units/mg protein, $P < 0.001$) and ileum (3.5 ± 0.4 compared to 7.8 ± 0.6 units/mg protein, $P < 0.001$).

Histology. Villous height and crypt depth were unaffected in the duodenum (Table 1). There was considerable villus atrophy in the proximal jejunum, mid-intestine, and ileum. This impression was confirmed by measuring the villus height and crypt depth: villous height (μm) in the proximal jejunum, mid-intestine, and ileum from control piglets was significantly greater than in infected piglets. Crypt depth was increased in the jejunum and mid-intestine after rotavirus infection, but remained unchanged in the ileum.

Stool Analysis

To evaluate whether the changes in intestinal function and structure were associated with nutrient (carbohydrate) loss resulting in an osmotic diarrhea, stools were studied for electrolyte and

sugar concentration. Figure 4A illustrates the time course of changes in stool sodium, potassium, and osmolarity after rotavirus inoculation. Stool sodium increased from 34 ± 6 mM prior to inoculation to a maximum of 65 ± 4 mM at 53 ± 1 hr PI, $P = 0.001$. There was no significant change in potassium concentrations during the same period (58 mM prior to inoculation to a maximum of 66 mM at 30 ± 4 hr PI). Stool osmolarity increased continually from 248 ± 20 mosm/liter prior to inoculation to 348 ± 20 mosm/liter at 75 ± 1 hr PI, $P = 0.005$. Fecal osmotic gap prior to inoculation was uniformly <20 mosm/liter (13 ± 3 , Figure 4B). The majority of the fecal osmotic gap observed postinfection could be accounted for by the amount of lactose present in the stools (Figure 4B). By 24 hr PI, the mean osmotic gap was significantly elevated, 38 ± 8 mosm/liter ($P < 0.01$). The maximum stool osmotic gap was observed at 75 ± 1 hr PI (95 ± 13 mosm/liter). The maximum fecal concentration of lactose was 77 ± 8 mM (75 ± 1 hr PI), whereas the fecal glucose

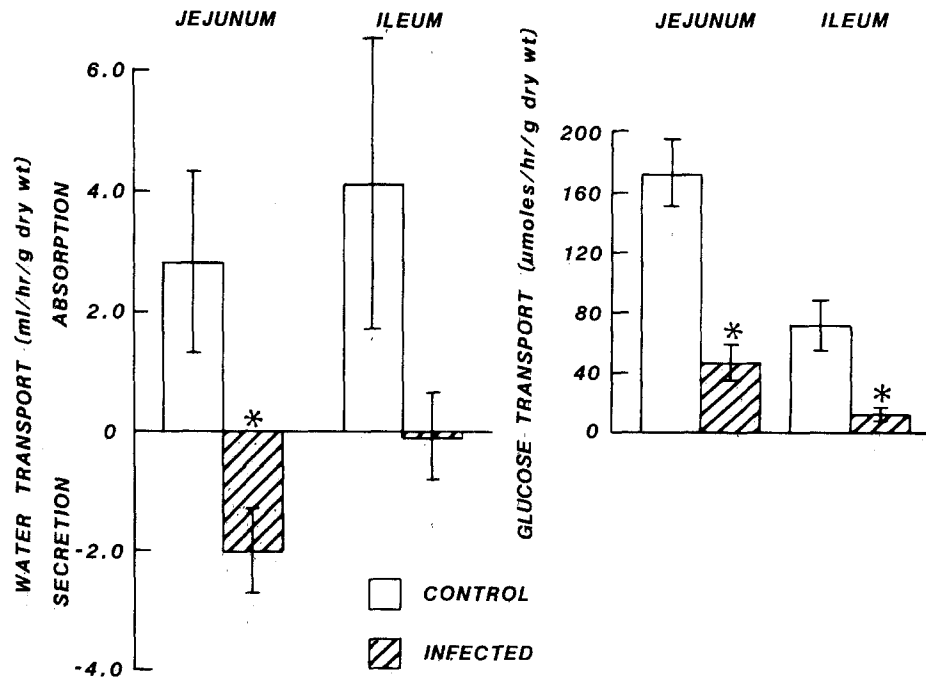


Fig 2. Net transport of water and mucosal to serosal transport of 3-O-methylglucose obtained when jejunal and ileal intestinal segments were perfused with a balanced electrolyte solution containing 34 mM glucose (as 3-O-methylglucose) are shown. Significant differences are indicated by an *. There were six control and eight infected animals perfused.

concentration never exceeded 10 mM. Neither lactose nor glucose appeared in the stools of control piglets throughout the course of observation.

DISCUSSION

The present investigation along with previous morphologic studies suggests a relatively straightforward mechanism for the diarrhea in rotavirus-induced gastroenteritis: (1) the virus invades the enterocytes lining the intestinal villi, (2) the infection is lytic and causes the destruction of the enterocytes, resulting in a reduction of mucosal surface area and important digestive enzymes (thus the diarrhea follows the infection and appears when progeny virus is seen in the stools), (3) an osmotic diarrhea results from nutrient (primary carbohydrate) malabsorption, and (4) the mucosa is initially repopulated with immature cuboidal epithelial cells (with reduced absorptive function) followed by the regeneration of a normal villous architecture.

Net secretion would result from reduced absorption, increased secretion, or both. This study cannot distinguish between these possibilities. If crypt secretion contributes to rotavirus-induced diarrhea,

the mechanism is unlikely to be mediated by adenylate cyclase as normal theophylline stimulation of chloride secretion occurs, and similar cyclic AMP levels are present in isolated enterocytes from rotavirus-infected and control piglets (21). In the other well-studied viral enteritis, TGE, diarrhea in infected piglets can be started and stopped by giving or withholding milk (19) suggesting that normal, yet unopposed, crypt secretion may exert only a minor contribution to viral-induced diarrhea associated with destruction of small intestinal enterocytes.

Normally the colon functions to absorb water and electrolytes that enter from the small bowel, thus reducing diarrhea. In carbohydrate-induced diarrhea, the diarrhea may be reduced or prevented by bacterial hydrolysis of malabsorbed carbohydrate to short-chain fatty acids that are largely absorbed (salvaged) by the colonic mucosa (26). This is an important adaptive phenomenon which was apparently lacking during the acute phase of rotaviral diarrhea in piglets, as the osmotic gap could be accounted for by mono- and disaccharides. This is not surprising as colonic adaptation was not immediately present upon administration of disaccharides to children with congenital disaccharidase

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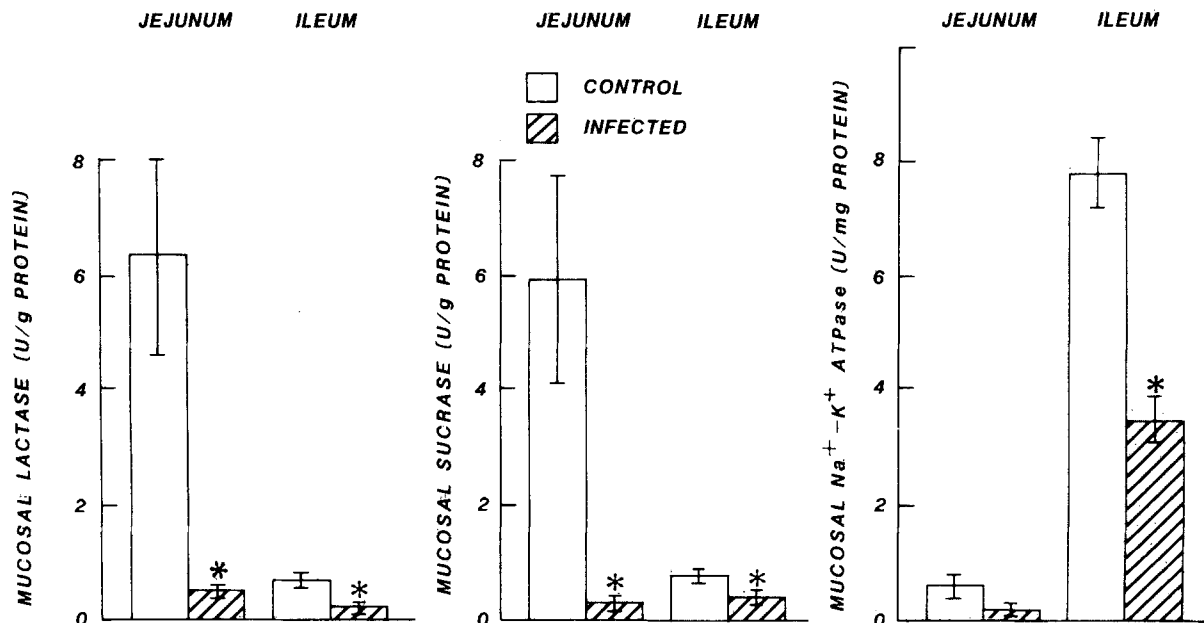


Fig 3. Mucosal enzyme levels following rotavirus infection (N = 10-12) jejunal and ileal lactase, sucrase, and Na⁺,K⁺-ATPase levels in mucosal homogenates obtained 48-72 hr postinoculation with rotavirus or media.

deficiency. In those children, continuous administration of disaccharides was required before adaptation occurred, and adaptation was manifested by a reduction in the amount of malabsorbed lactose, associated with increasing amounts of lactate in the stools, and reduction in the severity of diarrhea (27, 28). Thus, nutrient salvage by the colon is not immediate but requires the presence of carbohydrate for a definite time. It is currently unknown whether the colon has a role in viral gastroenteritis, and this deserves further study.

Infants and small children with rotavirus gastroenteritis usually come to the attention of physicians because of severe dehydrating gastroenteritis. The initial clinical description of rotavirus gastroenteritis in children reported that excess fecal sugar was not detected and assumed that the diarrhea is not caused by malabsorption (29). Carbohydrate malabsorption has subsequently been reported in a number of clinical studies including those evaluating rehydration solutions in children with rotaviral diarrhea (30-41), and it is now considered the rule. In studies comparing glucose- and sucrose-containing rehydration solutions (38), a large osmotic gap was present (86 mosm/liter) but not commented on by the authors. We have examined the fecal water from one of our own children with rotavirus-induced diarrhea and the osmotic gas was 290 mosm/

liter (unpublished observation). Malabsorption of xylose, fat, and nitrogen have also been reported during the acute phase of rotaviral diarrhea, and recovery of absorptive function has been slow (42, 43).

We have demonstrated that in piglets, as in infants, carbohydrate absorption (and presumably other nutrients) is markedly impaired during rotavirus enteritis and that a large amount of ingested sugar is lost in the stool. These findings may have implications on the composition of solutions (feedings) to be used for the periods of rehydration and recovery from rotaviral gastroenteritis. In cholera and enterotoxigenic *E. coli*-induced diarrheas, the

TABLE 1. COMPARISON OF SMALL INTESTINE MUCOSAL STRUCTURE IN CONTROL AND INFECTED PIGLETS*

	Duodenum	Upper jejunum	Mid-jejunum	Distal ileum
Villus height (μm)				
Control	425 ± 50	395 ± 55	500 ± 105	480 ± 50
Infected	315 ± 40	165 ± 35	150 ± 45	335 ± 50
P	NS	<0.01	<0.001	<0.05
Crypt depth (μm)				
Control	170 ± 15	150 ± 15	150 ± 15	130 ± 10
Infected	195 ± 20	220 ± 20	205 ± 15	135 ± 20
P	NS	<0.01	<0.01	NS

*N = 12 control and 9 infected animals.

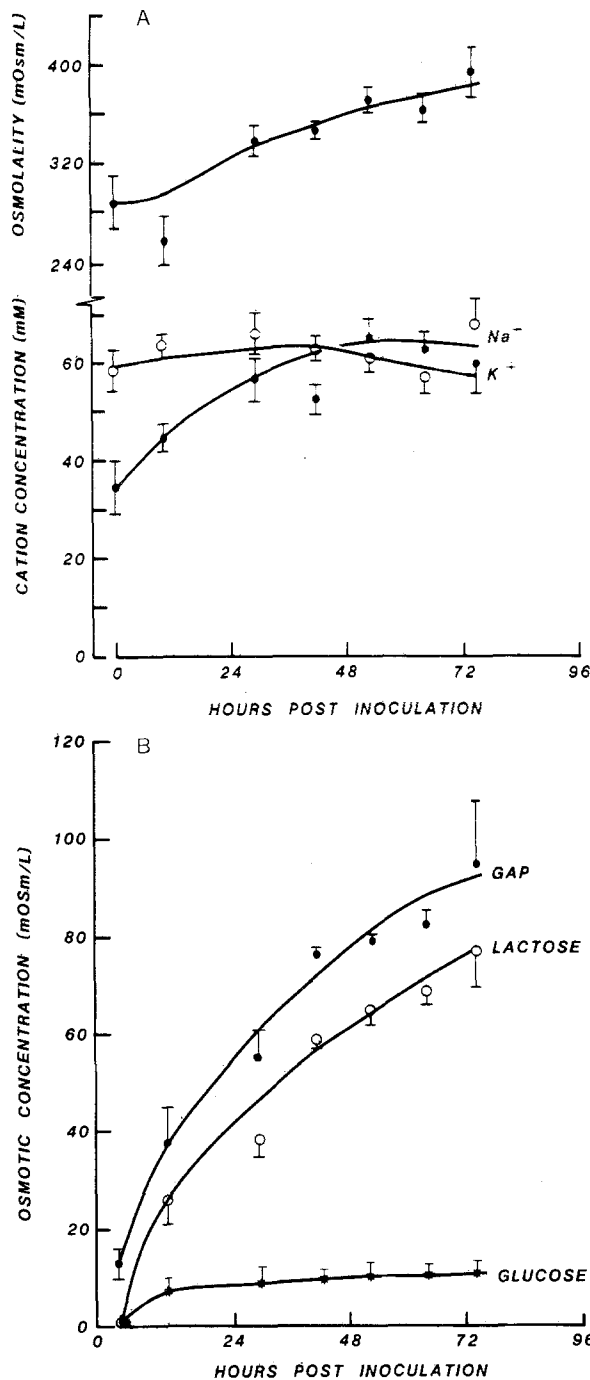


Fig 4. Fecal electrolyte composition after rotavirus infection of piglets. Figure 4A illustrates the time course of changes in stool sodium, potassium, and osmolality after rotavirus inoculation. Figure 4B illustrates that the majority of the fecal osmotic gap could be accounted for by the lactose present in the stools.

ability to absorb glucose is intact, and this mechanism can be exploited with glucose-electrolyte solutions (44). Since viral gastroenteritis has a mark-

edly different pathogenesis, possibly a different therapy should be considered (40). A solution containing glucose in excess of that amount able to be absorbed, such as the WHO rehydration solution designed primarily for the treatment of cholera patients, may not be the best choice for rotavirus-induced gastroenteritis. The WHO solution has been shown to be effective when administered to hospitalized children, but the failure rate has been in the range of 10% (39, 45). A nutrient-dense solution containing a mixture of absorbable nutrients, each employing different absorptive pathways, might be preferable to the current WHO solution but would require intensive testing before adoption. A solution containing a mixture of nutrients has been used in secretory diarrhea, ie, a glucose-glycine mixture has been shown to be more effective than a glucose solution in the treatment of cholera (46) and was also shown to be effective in the treatment of rotavirus-induced diarrhea in piglets (47).

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