EFFECTS OF INTESTINAL MICRO-ORGANISMS ON FLUID AND ELECTROLYTE TRANSPORT IN THE JEJUNUM OF THE RAT

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TOXINS produced by *Escherichia coli* and *Vibrio cholerae* are well known to affect intestinal fluid and electrolyte transport (Pierce, Greenough and Carpenter, 1971; Pierce and Wallace, 1972; Finkelstein, 1976). Similar effects have been described with *Clostridium perfringens* (Duncan and Strong, 1969), staphylococcal enterotoxin B (Sussman, Ryan and Shields, 1970) and *Shigella flexneri* and *Shigella sonnei* (Keusch and Jacewicz, 1977). Several other Gram-negative enteric organisms have also been shown to be enterotoxigenic including certain strains of *Klebsiella* spp. and *Enterobacter* spp. (Klipstein *et al.*, 1973) as well as of *Citrobacter* spp., *Proteus* spp., *Aeromonas* spp., *Serratia* spp. and *Pseudomonas* spp. (Wadström *et al.*, 1976). However, studies of enterotoxin production in organisms other than those of Gram-negative species have been limited.

Reports from South East Asia, Africa and the Caribbean (Mata *et al.*, 1972; Gracey *et al.*, 1973; Heyworth and Brown, 1975; Rowland and McCollum, 1977) have shown the upper intestinal secretions of malnourished children to be contaminated with excessive numbers of a variety of micro-organisms. Many of these are not usually considered to be enteropathogenic, but we have already shown that jejunal sugar absorption is impaired by several of these organisms (Gracey *et al.*, 1975; Burke, Houghton and Gracey, 1977). In view of this, and because transport of fluid and electrolytes in the upper intestine is known to be influenced by *Vibrio cholerae* and cholera toxin (McGonagle *et al.*, 1969; Banwell *et al.*, 1970) it seemed appropriate to investigate whether similar effects might be caused by a wider spectrum of intestinal organisms. The present study was concerned with the effect of such organisms on the jejunal transport of water and electrolytes in live rats.

MATERIALS AND METHODS

Strains. Except for the enterotoxigenic strain B7A of *Escherichia coli*, which was isolated from an adult, all micro-organisms used were isolated from the upper intestinal contents of malnourished Indonesian children (Gracey *et al.*, 1975). The isolates comprised individual strains of the following species: *Staphylococcus epidermidis*, *Streptococcus faecalis*, *Salmonella paratyphi* B, *Shigella sonnei*, *Klebsiella pneumoniae*, *Candida albicans*, *Candida tropicalis*; in addition two *Escherichia coli* isolates were tested, one belonging to serotype 055 and one non-enteropathogenic strain.

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Preparation of culture filtrates. Bacteria and yeasts that had been stored at -20°C were grown under aerobic conditions as pure cultures in 100-ml volumes of a modified phosphate-buffered peptone medium containing 1% Bacto-Peptone (Difco Laboratories, Michigan, Detroit, USA) in 250-ml Erlenmeyer flasks. After incubation at 37°C in a shaking waterbath at 50 oscillations per min. for 18 h, cultures were checked for purity and total cell counts were made by microscopy. For growth of *Candida* spp., the pH of the medium was lowered to 6.0. Cell-free preparations were made by centrifuging the cultures at 3000 r.p.m. for 30 min. and filtering the supernate through a Millipore type HA filter (Millipore Corp., Bedford, Massachusetts, USA). For all control experiments, uninoculated medium was treated identically.

Perfusion fluid. The phosphate buffer (pH 7.25) contained KH_2PO_4 0.5988 g, Na_2HPO_4 7.0423 g and NaH_2PO_4 1.04232 g per litre. Immediately before the experiments, sodium chloride 1.753 g per litre and mannitol 9 g per litre were added to simulate physiological and isotonic conditions. Osmolality of the test solutions was 290 (standard error $[\text{SE}] \pm 5$) mosM.

Phenol red $5 \mu\text{g}$ per ml (British Drug Houses, London) was added to the cell-free filtrate as a non-absorbable marker for measurements of water transport. Recovery of phenol red was 99% ($\text{SE} \pm 1.58$).

Absorption experiments. Adult male rats (Wistar inbred albino) weighing 200–250 g were obtained from a long-established colony in the University of Western Australia. They were fasted overnight, but allowed water *ad libitum* before the experiment.

Rats were anaesthetised by the intraperitoneal injection of urethane (ethyl carbamate) (1 g per kg body weight), and surgery was carried out under a heating lamp to maintain the body temperature.

Two cannulae were introduced through the gut wall, one at each end of an intact segment of jejunum approximately 20 cm in length, and tied in position with black silk. The gut segment was washed with 3 ml of the buffer solution described above, and then drained by gently blowing air through it.

The segment was replaced in the abdominal cavity and perfused with the undiluted culture filtrate at 2 ml per h. After equilibration for 30 min., perfusate was collected over a period of 60 min. At the end of the perfusion, the test segment was drained and washed with 5 ml of buffer, and its length was measured under the tension of a standard weight.

A second series of perfusion experiments was designed to investigate the relationship between net water and electrolyte flux and time of exposure to the cell-free supernate. The jejunal segment was prepared as described above and perfused at a rate of 10 ml per h. A 10-min. period was allowed for equilibration and samples were then collected after a further 15, 30 and 60 min. After 60 min. the segment was drained, washed, and measured as before.

Biochemical assays. Sodium and potassium were determined in initial and final solutions by flame photometry, and chloride by coulometric titration. Phenol red was determined spectrophotometrically at 560 nm; samples and washings were centrifuged and 0.5-ml volumes of fluid were made alkaline with 1 ml of 1N NaOH. In the experiments in which samples were taken at 15, 30 and 60 min., the 15- and 30-min. values were corrected for the amount of phenol red obtained in the washing at the end of the 60-min. period.

Expression of results. Net water flux is expressed as change in volume per cm of intestine per h and for sodium, potassium and chloride in μmoles per cm of intestine per h. For statistical calculations Student's *t* test was used, after taking $\log_{10}$ of the observed values, as variance was proportional to the size of the observations. A level of $P < 0.05$ was considered significant.

RESULTS

Net water flux. With the exception of the *Streptococcus faecalis* isolate, the non-enteropathogenic strain of *Escherichia coli* and the *Salmonella paratyphi* B isolate, all the organisms studied were associated with a decrease in net movement of water out of the jejunal lumen. As shown in table I, net water

TABLE I
Effect of cell-free culture filtrates on net flux of water after perfusion*
of rat jejunum in vivo for 1 h

Organism	Number of experiments	Mean net water flux (ml per cm per h) from lumen (SE in parenthesis)	Significance (P) of difference from controls
<i>Gram-positive cocci</i>			
<i>Staphylococcus epidermidis</i>	12	0.007 (0.003)	<0.0005
<i>Streptococcus faecalis</i>	12	0.049 (0.001)	NS
<i>Enterobacteriaceae</i>			
Non-pathogenic <i>E. coli</i>	6	0.051 (0.002)	NS
<i>E. coli</i> 055	9	-0.047 (0.003)†	<0.005
<i>E. coli</i> B7A	12	0.023 (0.001)	<0.0025
<i>Salmonella paratyphi B</i>	12	0.042 (0.001)	NS
<i>Shigella sonnei</i>	12	0.020 (0.001)	<0.005
<i>Klebsiella pneumoniae</i>	11	0.001 (0.002)	<0.0025
<i>Candida species</i>			
<i>Candida albicans</i>	11	0.022 (0.002)	<0.0025
<i>Candida tropicalis</i>	11	0.019 (0.001)	<0.0005
Controls	10	0.041 (0.001)	

* Perfusion flow rate = 2 ml per h.

† Negative value represents secretion into the intestinal lumen.

NS = Not significant.

flux was affected by filtrates of the isolates of *Staphylococcus epidermidis* (identified by lack of DNase and coagulase), *Escherichia coli* 055, *Escherichia coli* B7A, *Shigella sonnei*, *Klebsiella pneumoniae*, *Candida albicans* and *Candida tropicalis*. However, only *Escherichia coli* 055 produced net secretion of water into the intestinal lumen during the 1-h period of perfusion.

This pattern of net water movement was consistent after perfusion for 15, 30 and 60 min., but there was a decrease in net water flux with time for all organisms except the non-enteropathogenic *Escherichia coli* and the *Streptococcus faecalis* and *Salmonella paratyphi B* isolates. After 15 min., differences from control values were significant for all other organisms, and they increased with time. These results are shown in table II.

Net sodium movement. Table III shows the net jejunal sodium flux after perfusion for 60 min. Changes in net sodium flux paralleled the effects of net water movement for each organism studied. The values for sodium movement out of the lumen were significantly less than the control values ($P < 0.005$) for the isolates of *Staphylococcus epidermidis*, *Escherichia coli* 055, *Escherichia coli* B7A, a *Klebsiella* sp., *Candida albicans* and *Candida tropicalis*. Although the *Shigella sonnei* isolate caused a decrease in net sodium flux out of the lumen, the difference from the control values was not statistically significant. Secretion of sodium into the intestinal lumen was found with the *Escherichia coli* 055 and *Klebsiella pneumoniae* isolates.

TABLE II

Effect of cell-free culture filtrates on net water flux after perfusion of rat jejunum in vivo for 15, 30 and 60 min.*

Organism	Net water flux (ml per cm intestine per h) from lumen (SE in parenthesis) at					
	15 min.		30 min.		60 min.	
<i>Gram-positive cocci</i>						
<i>Staphylococcus epidermidis</i>	0.020	(0.001)	0.018	(0.001)	0.016	(0.003)
<i>Streptococcus faecalis</i>	0.062	(0.003)	0.056	(0.002)	0.053	(0.002)
<i>Enterobacteriaceae</i>						
Non-pathogenic <i>E. coli</i>	0.058	(0.003)	0.060	(0.002)	0.058	(0.004)
<i>E. coli</i> 055	0.022	(0.002)	0.015	(0.001)	0.033	(0.002)
<i>E. coli</i> B7A	0.034	(0.002)	0.025	(0.002)	0.013	(0.001)
<i>Salmonella paratyphi B</i>	0.063	(0.004)	0.048	(0.003)	0.049	(0.001)
<i>Shigella sonnei</i>	0.022	(0.001)	0.012	(0.002)	0.005	(0.001)
<i>Klebsiella pneumoniae</i>	0.018	(0.001)	0.008	(0.001)	0.001	(0.004)
<i>Candida species</i>						
<i>Candida albicans</i>	0.039	(0.003)	0.031	(0.001)	0.029	(0.002)
<i>Candida tropicalis</i>	0.041	(0.003)	0.029	(0.002)	0.022	(0.001)
Controls	0.055	(0.003)	0.055	(0.001)	0.051	(0.001)

* Perfusion flow rate = 10 ml per h.

Figures in bold type are significantly different ($P < 0.01$) from control values.

Net potassium movement. Net potassium flux followed the same pattern as sodium flux, in that decreased movement out of the jejunal lumen occurred with the *Candida albicans* and *Candida tropicalis* isolates. The decreased potassium flux found with the *Shigella sonnei* isolate was not significantly different from the control values. Secretion of potassium into the intestinal lumen was seen with the isolates of *Escherichia coli* 055, *Escherichia coli* B7A, *Klebsiella pneumoniae* and *Candida tropicalis*.

DISCUSSION

Decreased net movement out of the intestinal lumen or actual secretion of water, sodium or potassium into the intestinal lumen was found in intestinal perfusion experiments in live rats with culture filtrates of single isolates of *Staphylococcus epidermidis*, *Escherichia coli* 055, *Escherichia coli* B7A, *Shigella sonnei*, a *Klebsiella* sp., *Candida albicans* and *Candida tropicalis*. The isolates of *Streptococcus faecalis*, non-enteropathogenic *Escherichia coli* and *Salmonella paratyphi B* did not have this effect.

Enterotoxins that affect intestinal movement of water and electrolytes are well known to be produced by enteropathogenic *Escherichia coli* (Guerrant *et al.*, 1973) such as the isolates (055 and B7A) that we examined (Du Pont *et al.*, 1971). *Shigella sonnei* (Keusch and Jacewicz, 1977) and *Klebsiella* spp.

TABLE III

Effect of cell-free culture filtrates on net intestinal flux of sodium and potassium after perfusion of rat jejunum in vivo for 1 h

Organism	Mean net ion flux (μ .moles per cm intestine per h) from lumen (SE in parenthesis)			
	Sodium		Potassium	
<i>Gram-positive cocci</i>				
<i>Staphylococcus epidermidis</i>	2.41	(0.21)	0.01	(0.008)
<i>Streptococcus faecalis</i>	7.27	(0.13)	0.20	(0.004)
<i>Enterobacteriaceae</i>				
Non pathogenic <i>E. coli</i>	6.45	(0.14)	0.18	(0.004)
<i>E. coli</i> 055	-5.23	(0.13)	-0.33	(0.006)
<i>E. coli</i> B7A	-2.73	(0.12)	-0.06	(0.006)
<i>Salmonella paratyphi B</i>	5.44	(0.16)	0.12	(0.009)
<i>Shigella sonnei</i>	5.89	(0.22)	0.11	(0.008)
<i>Klebsiella pneumoniae</i>	-0.34	(0.30)	-0.13	(0.002)
<i>Candida species</i>				
<i>Candida albicans</i>	3.12	(0.16)	0.05	(0.009)
<i>Candida tropicalis</i>	0.41	(0.12)	-0.13	(0.01)
Controls	6.16	(0.11)	0.29	(0.08)

Figures in bold type are significantly ($P < 0.05$) less than the control values.

(Klipstein *et al.*, 1973) have also been shown to produce an enterotoxin. These organisms might therefore have been expected to affect net water flux in our experiments. Secretion of fluid was seen only with *Escherichia coli* 055 during the period of perfusion, but isolates of *Escherichia coli* B7A, *Shigella sonnei* and *Klebsiella* spp. were associated with a decrease in net water movement out of the lumen. Failure to demonstrate net secretion of fluid is probably related to the time necessary for the toxins to act, and this has been variously estimated with different experimental methods. With cholera toxin, the time lag has been variously reported to be 4 h (Leitch, Iwert and Burrows, 1966), 30 min. (Swallow, Code and Freter, 1968) and 15 min. (Carpenter and Greenough, 1968). Field (1971) has shown that cyclic AMP causes an increase in intestinal short-circuit current within a few minutes, but the effect of cholera enterotoxin, while apparently related, is delayed. Perfusion of jejunal segments in the rabbit demonstrated an effect on net fluid movement 10 min. after exposure to cholera toxin (McGonagle *et al.*, 1969) and the difference from control values increased with time. This is similar to the results of our perfusion studies. However, McGonagle *et al.* (1969) reported secretion of fluid into the lumen during this period; in our experiments there was a reduction in the net flux out of the lumen with all organisms tested except for (a) the isolates of *Streptococcus faecalis*, *Salmonella paratyphi B* and a non-enteropathogenic *Escherichia coli*, which had no effect on net water movement, and (b) the *Escherichia coli* 055 isolate, which produced secretion of fluid. This difference between our findings

and those of McGonagle *et al.* (1969) is probably due to a difference in technique. McGonagle *et al.* (1969) used dilution of phenol red in the fluid collected from the jejunal segment as the measure of net fluid movement, but did not comment on their recovery of the marker. With the same method of measurement, all specimens collected from the jejunal segment in our experiments with the organisms affecting net water flux showed dilution of phenol red; they would thus be considered to have shown secretion of fluid into the intestinal lumen. By washing out the segment at the end of the perfusion study and appropriately correcting the phenol red value found in the perfusion fluid, the apparent dilution of perfusate proved to represent a reduction in water movement out of the lumen. If this step is omitted, our results show the same pattern as those of McGonagle *et al.* (1969). Enterotoxigenic strains of *Escherichia coli* (Pierce and Wallace, 1972), *Shigella* spp. (Keusch and Jacewicz, 1977) and *Klebsiella* spp. (Klipstein *et al.*, 1973) have been described, and our results are consistent with these reports. However, a *Staphylococcus epidermidis* isolate and two species of *Candida* also affected net fluid and electrolyte flux in a similar way. The effects of cell-free filtrates of these organisms on water and electrolyte movement may be related to the production of enterotoxins or to the elaboration of other metabolic by-products. This is perhaps particularly surprising with *Staphylococcus epidermidis*, but the strain used was identified on the basis of lack of DNase and coagulase, and could not have been confused with *Staphylococcus aureus*, which is known to produce enterotoxin (Sussman, Ryan and Shields, 1970).

We have previously found that cell-free supernates from cultures of several organisms isolated from the upper intestinal tract of malnourished children decreased absorption of monosaccharides (Burke, Houghton and Gracey, 1977). This effect on sugar transport is not always associated with an effect on net water movement. For example sugar absorption was impaired by the *Streptococcus faecalis* isolate, but water flux was not affected, while the *Escherichia coli* 055 isolate had a striking effect on water movement, but did not inhibit sugar absorption. However, the *Candida albicans* and *Candida tropicalis* isolates affected both sugar and water transport. These observations suggest that more than one metabolite or "toxin" is involved in producing these effects on intestinal transport.

It is not known whether such factors are produced *in vivo* but the finding that organisms isolated from the upper intestine of malnourished children may affect sugar and water transport in the small bowel is potentially important in understanding the pathogenesis of diarrhoea—a major factor in the ill health of malnourished children. Our results indicate that overgrowth of organisms in the small intestine should be considered as possibly contributing to diarrhoea, even when they are not recognised enteric pathogens.

SUMMARY

Culture filtrates of micro-organisms isolated from the upper intestinal secretions of malnourished children and grown in pure culture were shown to

impair the intestinal absorption of water and electrolytes in live rats. Decreased net movement out of the intestinal lumen, or actual secretion of water, sodium or potassium into the intestinal lumen, was found with culture filtrates of single isolates of *Staphylococcus epidermidis*, *Escherichia coli* 055, *Escherichia coli* B7A, *Shigella sonnei*, *Klebsiella pneumoniae*, *Candida albicans* and *Candida tropicalis*. These organisms have been found to contaminate upper intestinal secretions in malnourished children and it is suggested that the effects observed in these experiments might be relevant to the production of the diarrhoea that is a dominant clinical feature of childhood malnutrition.

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