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Escherichia coli Heat Stable (STa) Enterotoxin and the Upper Small Intestine: Lack of Evidence in Vivo for Net Fluid Secretion

M.L. Lucas, M.M.M. Thom, J.M. Bradley, N.F. O'Reilly, T.J. McIlvenny, Y.B. Nelson
Laboratory of Gastrointestinal Physiology, Division of Neuroscience & Biomedical Systems, Institute of Biological & Medical Science, Glasgow University, Glasgow, G12 8QQ, Scotland, Great Britain

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Abstract. Heat stable (STa) enterotoxin from *E. coli* reduced fluid absorption in vivo in the perfused jejunum of the anaesthetized rat in Krebs-phosphate buffer containing lactate and glucose (nutrient buffer), in glucose saline and in glucose free saline. Bicarbonate ion enhanced fluid absorption of 98 ± 7 (6) μl/cm/h was very significantly ($P < 0.0001$) reduced by STa to 19 ± 4 (6) μl/cm/h, but net secretion was not found. When impermeant MES substituted for bicarbonate ion, net fluid absorption of 29 ± 3 (6) μl/cm/h was less ($P < 0.01$) than the values for phosphate buffer and bicarbonate buffer. With STa in MES buffer, fluid absorption of 3 ± 2 (6) μl/cm/h was less than ($P < 0.001$) that in the absence of STa and not significantly different from zero net fluid absorption. *E. coli* STa did not cause net fluid secretion in vivo under any of the above circumstances. Neither bumetanide nor NPPB when co-perfused with STa restored the rate of fluid absorption. In experiments with zero sodium ion-containing perfusates, STa further reduced fluid absorption modestly by 20 μl/cm/h. Perfusion of ethyl-isopropyl-amiloride (EIPA) with STa in zero sodium ion buffers prevented the small increment in fluid entry into the lumen caused by STa, indicating that the STa effect was attributable to residual sodium ion and fluid uptake that zero sodium-ion perfusates did not eradicate. These experiments, using a technique that directly measures mass transport of fluid into and out of the in vivo proximal jejunum, do not support the concept that *E. coli* STa acts by stimulating a secretory response.

Key words: *E. coli* — Secretion — Intestine — Heat-stable STa — Enterotoxin — Jejunum — Fluid absorption

Correspondence to: M.L. Lucas; email: M.Lucas@bio.gla.ac.uk

Introduction

Many strains of *E. coli* produce heat-stable (STa) enterotoxin that causes diarrhoeal disease (Nataro & Kaper, 1998) by preventing sodium chloride and fluid absorption. However, a generalized theory of secretory diarrhoea envisages that absorption losses are exacerbated by simultaneous fluid movement into the lumen, driven by epithelial cell chloride-ion secretion (Field et al., 1978). Increased chloride-ion and fluid secretion, perhaps from crypt enterocytes, is a widely accepted paradigm to explain derangement of fluid uptake, to which STa is assumed to conform. Fluid secretion is therefore almost always an assumed outcome of enterotoxin action whenever reduced net fluid absorption is detected. However, all categories of evidence for fluid secretion can be challenged, as studies using techniques that unequivocally measure mass transfer of fluid into the lumen have not put the matter beyond doubt. When fluid absorption was measured in vivo by recovery of perfusate volume, although inhibited absorption was detected, secretion was never found after exposure to STa, contradicting the published literature including previous reports from this laboratory (McEwan & Lucas, 1990) using indicator dilution. This lack of secretion in vivo contradicts the secretory view of STa action in particular but more importantly perhaps also the epithelial cell chloride secretory model in general. The present paper reports on experiments based on the present, assumed state of knowledge of the secretory process, undertaken with a view to testing more rigorously the secretion hypothesis. These experiments found no evidence for a secretory component to the action of STa enterotoxin on the small intestine. The lack of fluid secretion in vivo in circumstances where it might reasonably be expected to occur means that the current model of bacterial

enterotoxins catastrophically enhancing normally occurring epithelial cell chloride secretion may require revision.

Materials and Methods

In Vivo Perfusion Procedures

Absorption of fluid in vivo from perfused jejunal loops was measured using a recirculation procedure (Schanker et al., 1958). Adult Sprague-Dawley female rats (200–250 g) were anaesthetized by an initial intraperitoneal sodium pentobarbitone (Sagatal) at a dose of 70 mg/kg body weight, followed by periodic doses to maintain abolition of the hind limb flexor withdrawal reflex. Body temperature was kept at 37°C by a heating table controlled by a rectal thermistor. After trachaeotomy to maintain a patent airway, a midline incision was made in the abdominal wall along the *linea alba* and the pylorus was ligated. The duodenum was sectioned and approximately 5 ml of warm isotonic saline were introduced through a cannula (length 3 cm, i.d. 1 mm, o.d. 3 mm) to allow identification of the first loop of proximal jejunum immediately distal to the ligament of Treitz. A second ligature was tied here 10 cm distal to the first, an incision was made and an entry cannula of similar dimensions was tied into the proximal jejunum. A wider exit cannula (length 3 cm, i.d. 3 mm, o.d. 5 mm) was then inserted about 25 cm distal to the entry cannula and a further ligature tied off the end of the distal intestine to prevent blood loss through the intramural blood vessels. The entry and exit cannulae were drilled-out solid, polystyrene rodding, grooved so that the suture thread gripped adequately to prevent leakage. This was preferred to tying the loop ends on to polyethylene tubing. The isolated loop was flushed carefully with warm saline to remove debris and finally with air to expel residual fluid. The isolated loops were perfused at 1.8 ml/min by means of a peristaltic pump (Crouzet 82344, UK) from an initial volume of 25 ml in a reservoir maintained at 37°C. At the end of each experiment, perfusate was pumped into the reservoir and all tubing flushed with air. The abdomen was opened, the jejunal location of the loop was confirmed and it was flushed out with air using a 20-ml syringe. Residual fluid in the loop was collected in the fluid reservoir under gravity drainage and the final recovered volume was measured to the nearest 0.1 ml. The length and wet weight of the loop were also measured, allowing fluid absorption to be calculated as μl per cm length of intestine per hour and per gram wet weight. The excised loop was dried to constant weight in an oven at 100°C, allowing results also to be expressed per milligram dry weight. At the end of the experiment, the animals were humanely killed by anaesthetic overdose. In enterotoxin experiments, the perfusate contained STa at a concentration of 80 ng/ml.

Assay Procedures

Glucose was estimated by the glucose oxidase enzyme method (Raabo & Terkildsen, 1960). Lactate was estimated using the lactic acid dehydrogenase assay (Hohorst, 1957), protein in the solution by the Coomassie blue technique (Bradford, 1976), phosphate by ammonium molybdate reaction (Fiske & Subbarow, 1925). Bicarbonate ion was measured enzymatically by phosphoenolpyruvate decarboxylase assay (Forrester et al., 1976). Sodium and potassium ions were assayed by flame photometry with a Corning 410C photometer. Chloride ion was measured photometrically at 460 nm by the reduction of mercuric thiocyanate method (Hamilton, 1966). Osmolarity was measured using a Wescor vapor pressure osmometer.

Estimation of Fluid Movement

Fluid absorption was measured by the volume recovery method (Fedorak & Allen, 1989) from the difference between the initial and the final recovered volume. The intention was to use sodium ferrocyanide as an inert marker but subsequent investigations showed a systematic error between estimates from the recovered volume method and the inert marker method, consistent with in vivo absorption of ferrocyanide. In addition, a readily detectable one ml loss of fluid from the reservoir represents a 4% reduction in fluid volume. Indicator methods have difficulty matching this degree of precision given an assay variability that is increased by perfusion through the intestine, as additional protein precipitation procedures compound the variability. Assessment of volume to the nearest 0.1 ml represents a 0.4% accuracy, which indicator methods cannot equal. It is the case that recovered volume allows only one assessment of fluid absorption per loop experiment, while marker methods ostensibly allow serial measurements. However, on the grounds of precision, which was important in experiments to decide whether secretion had occurred, the fluid recovery method was selected as the most reliable way of estimating fluid transport.

Composition of the Perfusates

Various perfusates were circulated through the proximal jejunal loops. Initially, as indicated, some of these contained ferrocyanide as an inert marker. This was discontinued but is recorded here for completeness of description of the perfusates. In the first series of experiments (*see* Table 1), a nutrient buffer was used, which consisted of Krebs-phosphate buffer additionally containing 10 mM glucose and 15 mM sodium lactate but with the sodium ion concentration adjusted to that of unmodified Krebs phosphate buffer. Glucose saline was a solution containing 100 mM glucose and 100 mM sodium chloride. Both buffers contained 5 mM potassium ferrocyanide as the unabsorbable marker for estimation of the fluid volume. Saline (Table 1) was a simple solution of 150 mmoles/l sodium chloride without ferrocyanide. The 100 mM sodium bicarbonate perfusate (*see* Table 2) contained 100 mmoles/l of bicarbonate ion and 50 mmoles/l of chloride anion. The MES buffer had 100 mM MES (morpholinium ethanesulphonic acid) replacing the bicarbonate ion of the previous buffer. This solution was perfused through the lumen without gassing with the 95:5% (v/v) O_2:CO_2 gas mixture and both perfusates contained ferrocyanide as inert marker, although this later was discontinued and no subsequent perfusates contained ferrocyanide. Later bicarbonate perfusates (*see* Table 3) were either the 100 mM sodium bicarbonate/50 mM sodium chloride mixture or 150 mM sodium bicarbonate. Where ethyl-isopropyl amiloride or bumetanide was used, this was dissolved first in DMSO vehicle and a similar amount of vehicle added to control experiments. Sodium ion substitution was achieved (Table 5) by substituting 150 mM choline chloride or by 300 mM mannitol for sodium chloride in the perfusates.

Source of Chemicals

E. coli STa and most other chemicals, including assay reagents, were purchased from Sigma Chemical (Poole, UK). Sagatal (sodium pentobarbitone) was obtained from Rhone Merieux Ltd (Harrow, UK). Coomassie blue was obtained from Perbio, Pierce, Illinois.

Statistical Analysis of Data

Results are given as the mean and standard error, with the number of experiments, equal to the number of animals, in parenthesis.

Table 1. The effect of *E. coli* STa enterotoxin (80 ng/ml) on in vivo fluid and electrolyte absorption from 150 mM saline, glucose-saline and lactate-phosphate buffer perfusates recirculated through the proximal jejunum of the anaesthetized rat

	Fluid Transport (µl/cm/h)	Sodium Transport (µmol/cm/h)	Chloride Transport (µmol/cm/h)	Bicarbonate Transport (µmol/cm/h)	Potassium Transport (µmol/cm/h)	Osmolyte Transport (µosmol/cm/h)
Glucose saline	124.6 ± 11.8 (6)	14.0 ± 1.4 (6)	16.9 ± 1.0 (6)	−1.0 ± 0.5 (6)	−0.74 ± 0.06 (6)	36.0 ± 3.5 (6)
with STa	90.9 ± 15.5 (6)*	8.5 ± 1.1 (6)***	9.9 ± 1.8 (6)**	−0.6 ± 0.4 (6)	−0.76 ± 0.08 (6)	27.7 ± 4.4 (5)
Lactate buffer	52.8 ± 6.1 (6)	8.1 ± 1.1 (6)	10.8 ± 0.3 (6)	−1.9 ± 0.6 (6)	0.09 ± 0.02 (6)	20.6 ± 1.6 (6)
with STa	21.8 ± 5.0 (6)***	4.4 ± 1.0 (6)*	2.7 ± 1.7 (6)***	−1.2 ± 0.9 (6)	−0.04 ± 0.03 (6)	4.0 ± 2.4 (6)$^{=}$

Results are expressed as the mean and the standard error of the mean with the number of experiments (equal to the number of animals) in parentheses. Statistical significance was calculated by Student's *t*-test. *: $P < 0.05$; $P < 0.02$; ***: $P < 0.01$; $^{=}$: $P < 0.001$.

Table 2. The effect of *E. coli* STa enterotoxin (80 ng/ml) on in vivo fluid and electrolyte absorption from bicarbonate buffer (100 mM) and from morpholinoethanesulfonate (100 mM) buffer perfusate recirculated through the proximal jejunum of the anaesthetized rat

	Fluid Transport (µl/cm/h)	Sodium Transport (µmol/cm/h)	Chloride Transport (µmol/cm/h)	Bicarbonate Transport (µmol/cm/h)	Potassium Transport (µmol/cm/h)	Osmolyte Transport (µosmol/cm/h)
Bicarbonate (100 mM) perfusate	98.1 ± 7.0 (6)	14.5 ± 1.1 (6)	−3.6 ± 0.3 (6)	16.7 ± 0.7 (6)	0.15 ± 0.01 (6)	30.7 ± 1.6 (6)
with STa	18.8 ± 4.0 (6)$^{=}$	3.3 ± 1.4 (6)$^{=}$	−6.3 ± 1.0 (6)	5.9 ± 1.4 (6)$^{=}$	−0.01 ± 0.02 (6)	−0.3 ± 0.6 (6)$^{=}$
MES (100 mM) perfusate	28.6 ± 3.2 (6)	3.9 ± 0.5 (6)	1.5 ± 1.0 (6)	−1.0 ± 0.5 (6)	0.08 ± 0.01 (6)	10.0 ± 1.0 (6)
with STa	3.2 ± 2.2 (6)$^{=}$	−0.8 ± 0.8 (6)**	−0.3 ± 1.3 (6)	−0.6 ± 0.4 (6)	0.01 ± 0.02 (6)	−0.9 ± 3.3 (6)*

Results are expressed as the mean and the standard error of the mean with the number of experiments (equal to the number of animals) in parentheses. Statistical significance was calculated by Student's *t*-test. *: $P < 0.05$; **: $P < 0.02$; ***: $P < 0.01$; $^{=}$: $P = 0.0001$.

Implementation of all statistical analysis was done using BMDP (1981). Multiple comparisons of means were done by analysis of variance (P1V) within BMDP, with statistical significance calculated by Student's *t*-test for separate means, after Bonferroni type correction for significance limits. Variables were also standardized by dry and by wet weight of tissue, but are not presented here since no significant difference to the conclusions that were drawn arose from these alternative standardizations.

Results

Preliminary Experiments

Experiments to confirm secretion after STa perfusion were intended to precede screening of likely anti-secretory compounds. Using the method of perfusate recovery, severely reduced net fluid absorption was found, but not net fluid secretion in perfused small intestinal loops, a finding at variance with the majority of the published literature. The inability of *E. coli* STa to cause net fluid secretion was not confined to one particular set of circumstances because investigations were also carried out in the ileum to compare with the jejunum, in younger and in older rats, in Sprague-Dawley versus Wistar strains, in male and female rats separately, at the lower STa dose of 40 ng/ml and at the higher dose of 80 ng/ml, with preparation of the enterotoxin in saline versus distilled water to prevent any denaturation of the peptide, with extracts of native toxin from P16-infected scouring pig at doses between 10–100 mouse units/ml rather than the Sigma toxin, STa from Japanese sources and finally with differing lot numbers of Sigma STa enterotoxin. Experimental protocols were also varied where each animal provided a control and an experimental loop, then later each loop acted as its own control where normal values were first established and then STa was added later in the protocol. In all experiments, not reported here because they were identically negative, no net secretion was found, though fluid absorption was consistently reduced.

The perfused in-vivo loops transport D-glucose from the lumen from an initial concentration of 5 mM that falls linearly over the three hour period, indicating that active transport persists throughout the experimental period. The addition of 10^{-5} M phlorizin to the perfusate stopped glucose absorption. Haematoxylin and eosin histological sections of the lumen after perfusion show no structural abnormality. Loops prepared in this laboratory are unlikely to differ from those prepared elsewhere. They have normal intestinal function and are not under undue intraluminal pressure that might counteract any prevailing secretion, since a manometer at the exit cannula showed outflow pressure to be less than 1 cm

Table 3. The effect of 100 µM luminal bumetanide and 10 µM 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) on the action of *E. coli* STa (80 ng/mL) on in vivo electrolyte, osmolyte and fluid absorption in the rat proximal jejunum

	Fluid transport (µl/cm/h)	Sodium transport (µmoles/cm/h)	Chloride transport (µmoles/cm/h)	Bicarbonate transport (µmoles/cm/h)	Potassium transport (µmoles/cm/h)	Osmolyte transport (µosmoles/cm/h)
Bicarbonate perfusate (100 mM)	99.2 ± 9.2 (9)	16.5 ± 1.6 (9)	−2.9 ± 0.8 (9)	22.1 ± 1.2 (9)	−0.14 ± 0.03 (10)	24.6 ± 2.9 (9)
Bicarbonate perfusate (100 mM) with STa	13.3 ± 4.3 (9)$^{=}$	3.4 ± 1.2 (7)$^{=}$	−0.1 ± 2.5 (7)	9.7 ± 1.8 (7)$^{=}$	−0.15 ± 0.03 (7)	1.2 ± 5.4 (7)$^{=}$
Bicarbonate perfusate (100 mM) with STa and 100 µM bumetanide	0.6 ± 7.0 (6)$^{=}$	1.4 ± 1.4 (6)	−0.7 ± 1.5 (6)	9.4 ± 3.0 (6)***	−0.36 ± 0.11 (6)	−0.8 ± 3.1(6)$^{=}$
Bicarbonate perfusate (150 mM) with STa	22.3 ± 4.2 (6)	4.0 ± 3.5 (6)	−10.5 ± 0.7 (6)	6.3 ± 2.1 (6)	−0.40 ± 0.03 (6)	10.1 ± 6.1 (6)
Bicarbonate buffer (150 mM) with STa and 10 µM NPPB	34.1 ± 8.0 (6)	9.2 ± 3.6 (6)	−10.5 ± 0.8 (6)	8.8 ± 2.4 (6)	−0.37 ± 0.06 (6)	17.9 ± 6.4 (6)

Results are expressed as the mean and the standard error of the mean with the number of experiments (equal to the number of animals) in parentheses. Statistical significance was calculated by *t*-test.
***: $P < 0.01$; $^{=}$: $P = 0.0001$.

H_2O. The failure to detect secretion is unlikely to be because of poor tissue viability or because of unusual perfusion circumstances that would vitiate any attempt to detect secretion. Finally, in two experiments, hypertonic mannitol solutions (150 mM saline additionally containing 300 mM mannitol) were perfused through two loops, resulting in 5–6 ml of fluid entering the perfusate reservoir, representing about 40 µl/cm/h of osmotically induced fluid entry. Hence the perfused loop preparation and recovered perfusate technique can detect fluid secretion, if it is there to be found.

Perfusion of the Proximal Jejunum with Various Nutrient Perfusates

The effect of STa on fluid absorption was investigated by comparing the net fluid absorption of a control group of animals (with perfused loops without STa), with an experimental group with perfused loops with STa. Initial experiments (Table 1 and Fig. 1) were carried out with a saline perfusate (150 mM NaCl) without additional electrolytes or nutrients that might enhance absorption or otherwise make secretion difficult to detect. In the absence of STa, fluid absorption was 59.7 ± 6.8 (10) µl/cm/h, which was significantly higher than the 25.2 ± 5.6 (10) µl/cm/h ($P < 0.02$) in the presence of STa. Sodium ion uptake with STa perfusion was also less at 5.8 ± 1.3 (10) µmol/cm/h ($P < 0.05$) compared with 9.7 ± 1.2 (10) µmol/cm/h in the controls. Bicarbonate and potassium ion entry into the lumen was identical in both cases. Osmolyte transport was at −2.8 ± 4.0 (10) µosmol/cm/h not significantly different from zero from STa perfusates, but highly significantly less than the 12.4 ± 2.8 (10) µosmol/cm/h in STa-free saline perfusates. There were no differences in the rate of secretion of soluble protein, which could be regarded as a proxy for mucus secretion and similarly none for the appearance of phosphate anion in the perfusate (*see* Table 6). These variables were measured for some of the other perfusates, but this was discontinued as there were no differences that might account for, or add to the difference between STa and control loops. There was therefore evidence for the anticipated reduction in electrolyte and fluid absorption with STa, but none for the net secretion of fluid.

Similar results were obtained with a lactate-phosphate buffer containing glucose, included on the assumption that secretion might require nutrients, as other groups had used Tyrode's solution as a perfusate. At first, the effect of STa on fluid absorption from phosphate buffer, including 15 mmol/1 of lactate and 10 mmol/1 of glucose, was investigated. On perfusion of this nutrient-containing buffer (Fig. 1), jejunal fluid absorption was approximately

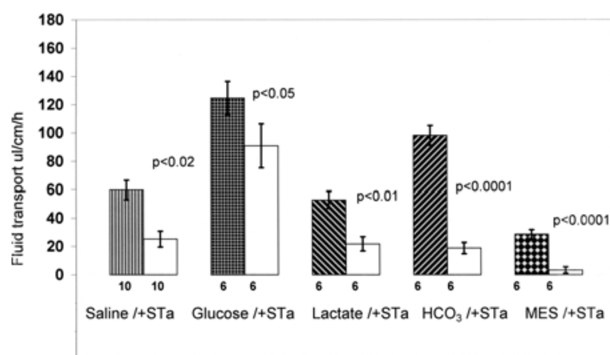

Fig. 1. The effect of 80 ng/ml of *E. coli* STa enterotoxin on net fluid absorption from the proximal jejunum of the anaesthetized rat perfused with saline, glucose saline, lactate saline, bicarbonate saline and morphinolinium-ethanesulfonate saline. Results are expressed as the mean and standard error of the mean with the number of experiments (equal to the number of animals) at the foot of the column. Clear columns are perfusates containing STa. Statistical significance refers to the significance of the difference between perfusate alone and perfusate with STa. Positive values mean net absorption.

52.8 ± 6.1 (6) µl/h/cm. With STa in the perfusate, fluid absorption was significantly less ($P < 0.005$) at 21.8 ± 5.0 (6) µl/h/cm. The rate of fluid absorption in the presence of STa enterotoxin was still significantly ($P < 0.01$) greater than zero net fluid absorption. As the rates of glucose and lactate absorption were unaffected by STa, changes in net fluid absorption were not a consequence of differences in substrate absorption and perhaps of unabsorbed nutrient in the lumen. Sodium ion uptake was reduced ($P < 0.02$) from 8.1 ± 1.1 (6) to 4.4 + 1.0 (6) µmoles/cm/h, as was chloride ion uptake. Bicarbonate and potassium entry into the lumen did not differ between control and experimental loops. Potassium ion leakage was also smaller in this series than in later experiments because the buffer contained potassium ferrocyanide as a non-absorbable marker and hence the gradient for diffusion into the lumen was not so steep as in other experiments where potassium ion was absent from the luminal perfusates. Total osmolyte transport at 4.0 ± 2.4 (6) µosmol/cm/h was very much less ($P < 0.0001$) after STa exposure than the 20.6 ± 1.6 (6) µosmol/cm/h associated with the control loops. There was therefore no evidence of secretion in this buffer after the jejunum was exposed to STa.

The effect of higher concentrations of glucose was also investigated by using Krebs-phosphate buffer containing 100 mM glucose, but with sodium chloride concentration reduced to maintain osmolarity, since intraluminal D-glucose was reported to initiate sodium ion secretion (See & Bass, 1993). With high luminal glucose concentrations, fluid absorption was enhanced to values above those from the previous nutrient buffer solution of 52.8 ± 6.1 (6) µl/h/cm to 124 ± 11.8 (6) µl/cm/h. Fluid absorption was

reduced only to 90.9 ± 16 (6) µl/cm/h in the presence of STa. Glucose absorption was insignificantly reduced from 29 ± 2 (6) to 22 ± 4 (5) µmoles/cm/h in the presence of STa, this reduction being accountable by glucose entrained in the absorbed fluid. The significant reduction in fluid absorption with STa exposure was associated with significant reductions in sodium ion and chloride ion uptake, as it was with the lactate perfusate. Since glucose was a very significant fraction of the total osmolarity and transport was unaffected by STa, the uptake of osmolyte was less with STa, but this did not reach significance with this number of experiments. Secretion was therefore not found in either of the nutrient-containing buffers and for that reason, simpler electrolyte perfusates were investigated.

Since electrolytes, such as bicarbonate anion, stimulate fluid absorption, STa might effect secretion only with bicarbonate anion present at relatively high concentration in the jejunum. High jejunal bicarbonate concentrations are normally present because of pancreatic secretion. Bicarbonate is also known to be absorbed there. Experiments (Table 2 and Fig. 1) investigated whether STa could inhibit the enhanced fluid absorption caused by luminal bicarbonate ion and might require bicarbonate anion to effect net fluid secretion. When 100 mM bicarbonate ion substituted for chloride ion, the resulting net fluid absorption of about 98.1 ± 6.8 (6) µl/h/cm was approximately twice ($P < 0.001$) the value of 52.8 ± 6.1 (6) µl/h/cm detected in the phosphate nutrient buffer (Fig. 1). Fluid absorption was 18.8 ± 3.4 (6) µl/h/cm in the presence of STa, a reduction of about 80% compared with control values ($P < 0.0001$). The fluid absorption in the presence of STa was identical in the high bicarbonate and the phosphate nutrient buffer, both values approximating about 20 µl/h/cm. Sodium ion uptake fell significantly ($P < 0.0001$) from 14.5 ± 1.1(6) µmoles/cm/h to 3.3 ± 1.4 (6) µmoles/cm/h, bicarbonate anion uptake likewise fell ($P < 0.0001$) from 16.7 ± 0.7 (6) µmoles/cm/h to 5.9 ± 1.4 (6) µmoles/cm/h and osmolyte uptake fell ($P < 0.0001$) from 30.7 ±1.6 (6) µosmoles/cm/h to −0.3 ± 0.6 (6) µosmoles/cm/h. Although STa profoundly reduced electrolyte and fluid absorption there was no evidence of net fluid secretion in this buffer.

Given that bicarbonate stimulated fluid absorption and is a very permeable anion, the experiment was repeated with the very poorly absorbed 2-N-morpholino-ethanesulfonate (MES) anion substituting for bicarbonate. When the perfusate contained MES at a concentration of 100 mM to replace bicarbonate ion, fluid absorption was 28.6 ± 3.2 (6) µl/h/cm (Table 2), which was below ($P < 0.01$) that in phosphate nutrient buffer and very significantly ($P < 0.001$) below the value in bicarbonate buffer. Nevertheless, some fluid absorption persisted in MES buffer, as net fluid absorption differed significantly ($P < 0.001$) from zero fluid uptake. With STa in the MES-buffered perfusate (Fig. 1), mean fluid absorption was approximately 3.0 ± 2.2 (6) µl/h/cm, lower ($P < 0.001$) than in the absence of STa and no longer significantly different from zero fluid absorption. Sodium ion uptake persisted in the MES buffer at 3.9 ± 0.5 (6) µmoles/cm/h, which was significantly different ($P < 0.02$) from −0.8 ± 0.8 (6) µmoles/cm/h for MES buffer and STa. Similarly, osmolyte uptake of 10.0 ± 1.0 (6) µmoles/cm/h was less than in bicarbonate buffer but was still higher than the zero mean of −0.9 ± 3.3 (6) µmoles/cm/h for MES and STa. Even at this low level of fluid uptake in the controls, fluid, sodium ion and osmolyte absorption were reduced to nil, but net fluid secretion did not arise with STa perfusion.

Luminal Perfusion with Compounds that Affect Chloride Ion Transport

The effect of the chloride channel blocker, NPPB (5-nitro-2-(3-phenylpropylamino)-benzoate) and the NKCC1 inhibitor bumetanide on STa action was tested. The assumption was that the chloride transport-inhibiting compounds would restore fluid uptake that had been inhibited by STa. When 100 mM bicarbonate buffer was perfused (Table 3 and Fig. 2) through proximal jejunum, fluid transport was 99.2 ± 10.4 (9) µl/cm/h and was significantly higher than the 13.0 ± 4.3 (9) µmoles/cm/h when STa was added to the perfusate. Sodium ion transport ($P < 0.0001$), bicarbonate ($P < 0.01$) and total osmolyte transport ($P < 0.02$) were inhibited. Chloride ion and potassium entry in the luminal perfusate were not inhibited by STa. However, the reduced fluid absorption of 13.0 ± 4.3 (9) µmoles/cm/h after STa exposure was not restored by 100 µM luminal bumetanide but may have been further reduced. The reduced fluid absorption in the presence of STa was still significantly non-zero, but it was no longer significantly different from zero in the presence of bumetanide. Paralleling the changes in fluid absorption, sodium ion, bicarbonate anion and osmolyte uptake were significantly reduced with STa perfusates and remained so in the presence of bumetanide. Chloride and potassium ion entry did not increase with STa perfusion and were not altered by bumetanide. The results with the NKCC1 inhibitor bumetanide were also paralleled by co-perfusion with 10 µM NPPB, a compound also known to inhibit increase in short-circuit current after STa exposure. In the higher 150 mM sodium bicarbonate buffer, fluid transport in the presence of STa was 22.3 ± 4.2 (6) µmoles/cm/h and was not significantly different at 34.1 ± 8.0 (6) µmoles/cm/h when NPPB was co-perfused. There were no differences in sodium ion, bicarbonate anion and osmolyte transport between STa alone and STa

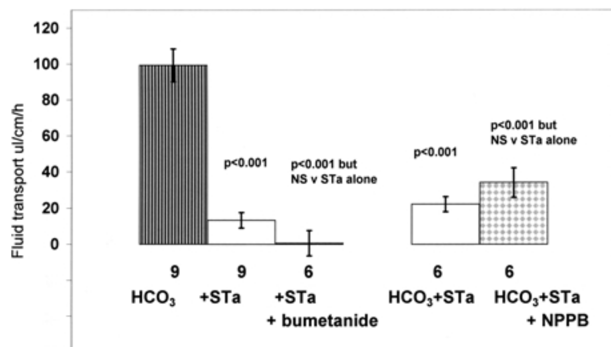

Fig. 2. The effect of 10 µM 5-nitro−2-(3-phenylpropylamine)-benzoate (NPPB) and of 100 µM bumetanide on fluid absorption in vivo from the rat proximal jejunum inhibited by perfusion with 80 ng/ml of *E. coli* STa enterotoxin in bicarbonate perfusate. Results are expressed as the mean and standard error of the mean with the number of experiments (equal to the number of animals) at the foot of the column. Statistical significance refers to the effect of STa on absorption from bicarbonate perfusate and where stated, the lack of significance (NS) between STa alone and STa with the stated drug. Positive values mean net absorption.

with NPPB co-perfusion, and potassium ion and chloride ion movement into the lumen were also not altered by NPPB.

Effect of STa Enterotoxin in the Absence of Fluid Absorption

An attempt was made to minimize fluid uptake prior to perfusion with STa on the grounds that with minimal fluid uptake, STa should cause discernible net fluid secretion. For this reason, experiments were done (Table 4 and Fig. 3) with zero sodium ion perfusates with and without STa co-perfusion. With 150 mM choline chloride perfusate, there was fluid entry into the lumen of −12.1 ± 3.3 (9) µmoles/cm/h, significantly ($P < 0.001$) less than the 59.7 ± 6.8 (10) µmoles/cm/h when 150 mM sodium chloride was perfused, as was expected and also significantly ($P < 0.02$) different from zero net fluid movement, indicating a small amount of leakage of fluid into the lumen. When STa was in the perfusate, fluid entry into the lumen was significantly higher ($P < 0.0001$) at −29.2 ± 4.2 (8) µmoles/cm/h. When mannitol was substituted for choline chloride, there was a similar modest but statistically significant amount of fluid entry into the lumen of −11.7 ± 5.5 (7) µmoles/cm/h that was also enhanced to −30.1 ± 7.1 (6) µmoles/cm/h when STa was co-perfused. Along with the fluid entry into the lumen, osmolyte and sodium ion also entered rather than left the lumen, sodium ion entry being significantly higher ($P < 0.02$) after STa perfusion. With choline chloride as perfusate, chloride transport was not significantly different from nil and in mannitol perfusate, chloride ion entered the lumen at statistically

Table 4. The effect of luminal perfusion of ethyl-isopropyl-amiloride (EIPA) on in vivo electrolyte, osmolyte and fluid absorption from 150 mM sodium bicarbonate perfusate in the rat proximal jejunum

	Fluid transport (µl/cm/h)	Sodium transport (µmoles/cm/h)	Chloride transport (µmoles/cm/h)	Bicarbonate transport (µmoles/cm/h)	Potassium transport (µmoles/cm/h)	Osmolyte transport (µosmoles/cm/h)
Bicarbonate perfusate control	81.6 ± 12.3 (8)	11.1 ± 2.6 (8)	−14.7 ± 1.0 (8)	20.1 ± 3.4 (8)	−0.29 ± 0.05 (8)	22.7 ± 3.7 (8)
Bicarbonate perfusate with 10 µM EIPA	85.9 ± 12.4 (6)	10.8 ± 2.6 (6)	−13.6 ± 1.6 (6)	22.3 ± 1.6 (6)	−0.24 ± 0.06 (7)	22.8 ± 4.0 (6)
Bicarbonate perfusate with 100 µM EIPA	86.8 ± 17.7 (6)	10.9 ± 3.3 (6)	−11.6 ± 1.1 (6)	21.1 ± 2.6 (6)	−0.20 ± 0.02 (6)	22.4 ± 5.9 (6)

Results are expressed as the mean and the standard error of the mean with the number of experiments (equal to the number of animals) in parentheses.

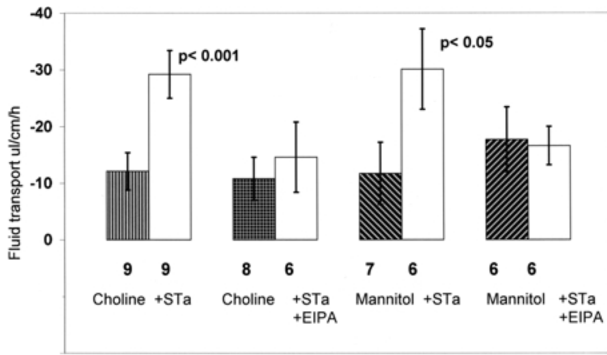

Fig. 3. The effect of 80 ng/ml of *E. coli* STa enterotoxin on fluid absorption in vivo from the rat proximal jejunum from zero [Na^+] perfusates with or without 100 μM ethyl-isopropyl-amiloride (EIPA). Results are expressed as the mean and standard error of the mean with the number of experiments (equal to the number of animals) at the foot of each column. Statistical significance refers to the difference of fluid transport from perfusate with and without STa. Negative values mean the appearance of fluid in the lumen.

significant rates. To answer the question whether the relatively small amount of fluid entry into the lumen caused by STa was enhanced by secretion or not, the experiments were repeated with perfusates additionally containing EIPA (ethyl-iso-propyl-amiloride). Initially, experiments were undertaken using EIPA in 150 mM sodium bicarbonate perfusate to see whether fluid absorption could be inhibited without recourse to zero sodium ion perfusates (Table 5). In the presence of sodium ion, neither 10 nor 100 μM EIPA had any effect on fluid or electrolyte movement and for this reason recourse was made to sodium ion-free buffers. The lack of an effect of EIPA at high sodium ion concentrations also ruled out investigating the effect of EIPA and STa in a normal sodium ion environment. When 100 μM EIPA was perfused in both the choline chloride and the mannitol perfusate, fluid transport was unchanged compared with no EIPA perfusion, i.e., rates of electrolyte and fluid movement into the lumen were similar to controls. With perfusate containing EIPA and STa, there also was no increment in fluid entry since with mannitol perfusate, fluid entry was −17.7 ± 5.7 (6) μmoles/cm/h with EIPA, but −16.6 ± 3.4 (6) μmoles/cm/h with EIPA and STa. With a choline chloride perfusate, fluid entry was −10.8 ± 3.8 (8) μmoles/cm/h in the presence of EIPA, which did not differ from −14.6 ± 6.2 (6) μmoles/cm/h in the presence of both EIPA and STa. Similarly, there were no EIPA-dependent changes in sodium or chloride ion movement depending on the buffer or changes in osmolyte, when it was co-perfused with STa.

Discussion

Diarrhoeal disease of bacterial etiology frequently causes severe dehydration, particularly in cholera

Table 5. The effect of luminal perfusion in vivo with *E. coli* STa (80 ng/μl) on electrolyte, osmolyte and fluid absorption from the proximal jejunum of the anaesthetized rat from initially sodium ion-free perfusates with and without 100 μM ethyl-iso-propyl-amiloride (EIPA)

	Fluid transport (μl/cm/h)	Sodium transport (μmole/cm/h)	Chloride transport (μmole/cm/h)	Bicarbonate transport (μmole/cm/h)	Potassium transport (μmole/cm/h)	Osmolyte transport (μosmoles/cm/h)
Choline	−12.1 ± 3.3 (9)	−10.4 ± 1.1 (9)	4.1 ± 2.9 (9)	−2.4 ± 0.2 (9)	−0.11 ± 0.04 (9)	−10.6 ± 3.2 (9)
Choline + STa	−29.2 ± 4.2 (8)***	−11.1 ± 1.1 (8)	3.5 ± 1.9 (8)	−2.7 ± 0.4 (8)	−0.18 ± 0.07 (8)	−14.6 ± 3.8 (8)
Choline/EIPA	−10.8 ± 3.8 (8)	−9.6 ± 0.8 (8)	5.5 ± 3.6 (8)	−1.6 ± 0.2 (8)	−0.24 ± 0.03 (8)	−6.0 ± 4.2 (8)
Choline/EIPA + STa	−14.6 ± 6.2 (6)	−9.8 ± 0.5 (6)	2.3 ± 1.8 (6)	−2.0 ± 0.3 (6)	−0.24 ± 0.02 (6)	−3.8 ± 4.3 (6)
Mannitol	−11.7 ± 5.5 (7)	−10.2 ± 0.8 (6)	−12.8 ± 0.6 (7)	−2.9 ± 0.1 (7)	−0.05 ± 0.01 (6)	−17.1 ± 2.0 (7)
Mannitol + STa	−30.1 ± 7.1 (6)*	−14.5 ± 1.1 (6)**	−18.0 ± 4.7 (6)	−3.3 ± 0.7 (6)	−0.09 ± 0.04 (6)	−20.9 ± 5.1 (6)
Mannitol/EIPA	−17.7 ± 5.7 (6)	−15.8 ± 2.4 (6)	−15.5 ± 4.4 (6)	−1.9 ± 0.4 (6)	−0.17 ± 0.07 (6)	−29.2 ± 7.9 (6)
Mannitol/EIPA + STa	−16.6 ± 3.4 (6)	−12.2 ± 1.5 (6)	−11.1 ± 2.1 (6)	−1.9 ± 0.4 (6)	−0.09 ± 0.02 (6)	−22.3 ± 3.7 (6)

Results are expressed as the mean and the standard error of the mean with the number of experiments (equal to the number of animals) in parenthesis. Statistical significance was calculated by *t*-test. * = $P < 0.05$; ** = $P < 0.02$; *** = $P < 0.01$.

Table 6. The effect of luminal perfusion in vivo with *E. coli* STa (80 ng/μl) on phosphate anion and protein appearance in the proximal jejunum of the anaesthetized rat

	Phosphate transport (μmoles/cm/h)	Protein appearance (ng/cm/h)
Saline	−0.29 ± 0.09 (6)	−0.19 ± 0.04 (6)
Saline + STa	−0.17 ± 0.05 (7)	−0.14 ± 0.03 (7)
HCO_3^-	−0.21 ± 0.06 (12)	−0.17 ± 0.6 (12)
HCO_3^- + STa	−0.26 ± 0.08 (12)	−0.19 ± 0.6 (12)
Lactate perfusate	−0.42 ± 0.10 (6)	−0.19 ± 0.06 (6)
Lactate perfusate + STa	−0.23 ± 0.14 (6)	−0.09 ± 0.04 (6)
MES	−0.17 ± 0.03 (6)	−0.14 ± 0.03 (6)
MES + STa	−0.31 ± 0.07 (6)	−0.11 ± 0.02 (6)

Results are expressed as the mean and the standard error of the mean with the number of experiments (equal to the number of animals) in parentheses.

where catastrophic fluid loss occurs. Early physiological investigations identified the epithelium as the site of the derangement, since it remained intact in the presence of *Vibrio cholerae*. Epithelial integrity implied the existence of a cellular secretion process, the driving force for which was later attributed (Field, 1971) to secretion from the epithelial cell of chloride ion, which, accompanied by sodium ion, causes osmotic movement of water into the lumen. Enhanced chloride ion secretion is the general model (Barrett, 2001; Field, 2003) for enterotoxin action, including STa. This heat-stable enterotoxin is reported to cause net chloride ion secretion in vitro (Field et al., 1978; Guandalini et al., 1982; Vaandrager et al., 2000), fluid secretion in vivo in the ligated loop (Hamilton et al., 1978; Tantisira, Jodal & Lundgren, 1990; Cohen et al., 1992; Young & Levin, 1992; Beubler, Badhri & Schirgi-Degen, 1992; Rolfe & Levin, 1994; Ieda et al., 1999), in the in vivo perfused loop (Klipstein & Engert, 1978; Eklund, Jodal & Lundgren, 1985; Rolston & Mathan, 1992; Volant et al., 1997; Turvill et al., 1999; Mourad & Nassar, 2000) and in the suckling mouse assay (Guerrant et al., 1980; Schulz et al., 1997). STa also increases short-circuit current in vitro (Cuthbert et al., 1994; Kuhn et al., 1994; Rolfe & Levin, 1994; Vaandrager et al., 2000; Guba et al., 1996) and in T84 cells (Huott et al., 1988; Barrett, 1991; Forte et al., 1993). When STa-induced fluid secretion was investigated in vivo using fluid recovery, it was not found, contradicting many previous reports, including one (McEwan & Lucas, 1990) from this laboratory. Experimental protocols were then varied extensively but under no circumstances was it detected. The question arose, does STa actually cause secretion or is its mode of action restricted to inhibition of fluid absorption? In many studies, assumptions in the methodology or the lack of mass transport measurements make the claim of secretion difficult to substantiate. Before considering the data showing a lack of secretion, it is important to examine why other methods purporting to show secretion are unsatisfactory.

A common method of assessing fluid transport, which does not measure the mass transport of fluid as such, is by monitoring indicator dilution. With polyethylene glycol 4000, sufficiently non-trivial fractions of the indicator are absorbed (Miller & Schedl, 1970; Sladen & Harries, 1972; Winne & Goerig, 1982; Pappenheimer & Reiss, 1987; Fasano et al., 1997) in vivo to introduce systematic error. With losses of just 2% in short loop experiments, e.g., a 10 ml solution perfused for 20 minutes through a 10 cm loop, secretion would falsely be estimated to be about 60 μl/cm/h, slightly higher than average cited values. This extent of indicator loss is within the typical 95–105% recovery band for acceptance of this type of experiment. Since results with incomplete recovery of marker are discarded (Volant et al., 1997; Mourad & Nassar, 2000), the attempt to be *rigorous* paradoxically obscures the fact that indicator methods are intrinsically unreliable. Experiments of a short duration close to the half-time of indicator diffusion into the unstirred layer are also problematical. Diffusion of marker into the unstirred layer reduces its luminal concentration. The combination of real absorptive and apparent diffusional loss of indicator normally only underestimates fluid absorption but in the challenged, non-absorbing intestine, it would misleadingly indicate secretion.

Evidence for fluid secretion is indirect when the increase in short-circuit current in vitro is the measured variable (Barrett & Keely, 2000; Forte, et al., 1993; Rolfe & Levin, 1994; Cuthbert, et al., 1994; Kuhn et al., 1994, Guba et al., 1996). The increase in current after STa exposure accords with chloride secretion, but is also compatible with increased electrogenic uptake of cation. Chloride conductance increases in the apical membrane occur in T84 cells after *E. coli* STa exposure (Chao et al., 1994), facilitating movement in either direction as the current-voltage relation shows. Although enhanced chloride ion *exit* from pre-loaded cells is found after STa exposure, so is *entry* into T84 (Huott et al., 1988) and colonic cells (Goldstein et al., 1994). An increased uptake rather than secretion of chloride ion is found with *Salmonella* virulence factor in human embryonic 293 cells (Feng, Wente & Majerus, 2000) and may be a general feature of distressed cells responding to enterotoxin challenge. This ambiguity in interpretation also extends to isotopic flux measurements that are regarded as definitive evidence that an enhanced chloride secretory flux causes the hypothesized secretion. Most reports of secretion after secretagogue action show an enhanced flux towards the lumen according to the convention (Field, Fromm & McColl, 1971), in rabbit ileum after exposure to cholera toxin (Sheerin & Field, 1977),

and to STa enterotoxin in rabbit ileum and weanling pig (Hamilton, Roe & Nielsen, 1977; Field et al., 1978; Guandalini et al., 1982; Vandraager et al., 2000). In these studies, Jsm flux is equated with secretion and Jms flux to absorption, with both assumed to be independent of one another. These assumptions derive from an incompletely formulated mathematical description of isotope distribution between compartments (Solomon, 1964). An appropriately formulated model (Lucas, 2005) shows that a Jsm flux is determined both by absorptive and secretory processes. This is an important objection to the flux evidence for the epithelial cell chloride secretory model, since an enterotoxin-mediated reduction in the absorptive *uptake* of chloride ion is detectable both as a reduction in the Jms flux and as an increase in the Jsm flux. Given this reciprocity property, enhanced chloride ion flux towards the lumen after enterotoxin exposure fails as a category of evidence for enhanced secretion.

This leaves studies that attempt to measure fluid movement directly, excluding those which do not show statistically significant secretion (Charney & Dansky, 1990; Rolfe & Levin, 1994; Mourad & Nassar, 2000) or which have a flawed calculation protocol (Cohen et al., 1992) that subtracts the heavier weight of an STa-treated loop from the lighter weight of a control loop. Many techniques fail to control for change in sub-epithelial volume. Screening techniques measuring gut to body weight ratio will misattribute increases in interstitial volume post toxin to fluid secretion (Guerrant et al., 1980; Schulz et al., 1997; Flagella et al., 1999). Intestinal vasodilatation with filtration of fluid into the sub-epithelial space may indeed be an aspect of enterotoxin action but is not the anticipated outcome of the chloride secretion model. Loop weight to loop length is also problematical as sub-epithelial volume can alter and be restored after pharmacological intervention (Thiagarajah et al., 2004) with no certainty that fluid secretion took place and was interrupted by such intervention. This is particularly true of those chloride channel blockers that additionally cause vasodilatation or at least relaxation of smooth muscle (Cruickshank, 2003). Similarly, in vivo experiments that weigh a denervated but blood perfused loop together with perfusate (Eklund et al., 1985) will measure change in submucosal fluid volume as well as luminal fluid movement. The weighed loop is particularly susceptible to volume artefact since vasomotor activity does change sub-epithelial volume. In addition, since sympathetic denervation itself causes secretion (Moreau, 1868; Teitelbaum et al., 1993), inhibition of normal absorption by STa would enhance the rate of denervation secretion, without actually participating in any secretory process. Consequently, evidence for STa-induced fluid secretion in this preparation is also equivocal.

A final group of experiments with short incubation times in vivo and short loop lengths have a serious source of error in the form of a flushing-solution artefact. In the absence of absorption, a necessarily incompletely flushed 10 cm loop in a prone animal provides about 100 μl of solution, only recoverable by gravity drainage at the end of the experiment (Sladen & Harries, 1972) and may account for reported secretion of −10 to −15 μl/cm of fluid after STa action (Farack et al., 2000). Short incubations are very susceptible to this artifact, which may explain differences in the assessment of secretion within the same laboratory (Table 9: Young & Levin, 1990 v. Table 1: Nzegwu & Levin, 1994), where also secretion in the jejunum from the starved animal became absorptive after one hour of perfusion, consistent with too brief a time to allow clearance of the injected fluid load plus the unknown residual flushing solution. Short duration saline control experiments without enterotoxin also show secretion (Beubler et al., 1992; Ieda et al., 1999), while longer perfusion times (Sund, 1975) show net fluid absorption. It is unlikely that secretion is the natural state of the proximal jejunum, as empty loops in vivo do not accumulate fluid. A likelier explanation is that short duration installation in short ligated loops leads to the correct conclusion of cessation of absorption but also to the false one that STa causes secretion.

The experiments here used volume recovery to assess the likelihood of secretion because all other procedures have methodological difficulties inherent in them that make detection of secretion problematical. What are the methodological features that might also lead to misinterpretation in the perfused loop experiments presented here? Incomplete washout of flushing solution will occur in these experiments as in others but is unlikely to be misleading because of the longer duration of the experiments. Over three hours, 50 μl/h/cm secretion should cause about four mls of fluid to accumulate in the reservoir and would be readily detected but in fact was not. The failure to show secretion after STa cannot be ascribed to a specific failure here because hyperosmotic mannitol will induce net fluid entry into the loops. Fluid absorption can change by about 80 μl/h/cm after STa exposure. If secretion contributes substantially to this, net secretion might occur in phosphate and certainly in MES perfusate but was not found. For the secretory hypothesis to remain plausible under these circumstances, secretion must always be a fixed but lesser fraction of the rate of absorption, must be small and depend on the anion in the lumen, none of which is a predicted aspect of the chloride secretion model.

Evidence for the model is that the STa-dependent increase in short-circuit current in vitro is reversed by chloride-transport inhibitors such as bumetanide or 5-nitro-2-(3-phenylpropylamino)-benzoate (Huott

et al., 1988; Nobles, Diener & Rummel, 1991; Forte et al., 1993; Kuhn et al., 1994). If short-circuit current reflects chloride ion-dependent fluid secretion, then bumetanide or NPPB should reverse any inhibition of net fluid absorption caused by STa, but it did not do so. This indicates that the changes in short-circuit current and associated pharmacological properties do not match the actions of STa on fluid absorption. While bumetanide reduces the increase in short-circuit current and is an NKCC1 inhibitor, against prediction, NKCC1 knockout mice still respond to STa (Flagella et al., 1999). The inability of chloride-channel blockers to restore fluid absorption implies no involvement of chloride ion in the action of STa. This lack was to some extent also confirmed by an absence of excess chloride ion in the luminal solutions as has been claimed (Young & Levin, 1990) for bethanechol-stimulated secretion using chloridimetry. In the presence of STa, in vivo bicarbonate ion uptake was inhibited but chloride ion movement into the lumen was reduced, not enhanced. Anions were detected by enzymatic and chemical methods that showed no mutual interference. In contrast, the electrical chloridimetric method cannot distinguish bicarbonate from chloride anodic deposition. The absence of increased perfusate chloride ion concentration in our experiments further argues against a chloride ion secretory process for STa in the small intestine, if this is a property of the chloride secretion model, as was advocated for bethanechol stimulated secretion.

An alternative way of demonstrating lack of a secretory process was to suppress absorption to create optimal conditions for detecting secretion. If all sodium ion-dependent fluid absorption were inhibited, then the addition of STa to sodium ion-free perfusates should allow secretion to be detected. Removal of sodium ion from the perfusate reduced net absorption from about 60 μl/cm/h to approximately −15 μl/cm/h, as others have found (Humphreys & Earley, 1971; Hubel, 1973). There was therefore minor fluid movement into the lumen rather than the zero net movement that might be anticipated. It is known that sodium ion-free conditions do not persist in the upper small intestine, since sodium ion enters the lumen significantly in vivo, sufficient to cause the luminal sodium ion concentration to rise (Hindle & Code, 1962). The movement of sodium ion down its concentration gradient accounts fully for the small amount of fluid entry into the lumen by transiently providing an osmotic driving force. There is also intestinal hyperaemia when sodium ion is replaced by choline (Lee, 1977) and the likely increased capillary pressure may squeeze interstitial fluid past a non-absorbing epithelium. This entry of sodium ion into the lumen was independent of the existence of a chloride ion gradient since minor fluid entry also occurred in choline perfusates where no chloride ion gradient was present. Net sodium ion entry into the lumen with almost exactly the same change in net osmolarity in sodium ion-free buffers has been observed before in vivo (Humphreys & Earley, 1971) and can account for the small amount of net fluid entry that might otherwise be attributed to secretion.

When STa was added, net fluid entry into the lumen increased by about 15 μl/cm/h in both choline chloride and mannitol perfusates. If this did represent secretion, then the secretory component is minor, since STa alters fluid transport from sodium bicarbonate perfusates by about 70 μl/cm/h. However, this small increment is unlikely to be secretion as envisaged by the secretory hypothesis. As the mucosal surface does not become completely sodium ion-free in vivo but has a higher concentration than the lumen, likely because of interstitial fluid acting as a source of sodium ion (Lucas & Cannon, 1983), sodium ion is likely to diffuse into the lumen and participate in NHE exchange. Residual sodium ion and associated fluid uptake is likely to be small but still inhibitable by STa. For this reason, the NHE:3 inhibitor ethyl-isopropyl-amiloride (Orlowski, 1993) was added to the perfusates. With EIPA and physiological concentrations of sodium ion, fluid uptake was unaffected, consistent with the known properties for EIPA to function best at low sodium ion concentrations. Consequently, the desirable experiment of first adding EIPA and then perfusing with STa in a high sodium ion perfusate was not possible. In low-sodium ion perfusates with EIPA present, sodium ion absorption might be expected to be completely inhibited. Any increase in fluid entry into the lumen after STa exposure would be attributable to some form of enhanced secretory fluid flux, as envisaged by the chloride secretion hypothesis. When loops were perfused with sodium ion-substituted perfusates containing 100 μM EIPA, STa had no additional effect on fluid movement. This makes it highly unlikely that *E. coli* STa acts, even partially, by enhancing a secretory component to net fluid transport in the upper small intestine. A noteworthy aspect of EIPA effects was that it did not precisely match the action of STa as it should for an NHE:3 inhibitor, as fluid absorption was -10.8 ± 3.8 (8) μl/cm/h with EIPA in choline buffer but was significantly different ($P < 0.05$) from the value of -29.2 ± 4.2 (8) with STa alone. EIPA may therefore have additional amiloride-like effects but it was beyond the accuracy of the in vivo loop methodology to resolve this point.

Diarrhoeal disease is a widely occurring, often fatal disease and it is appropriate to argue the case against epithelial cell chloride ion secretion within the limits of the inferences that can be drawn. The present experiments do not contradict the fact that fluid losses occur in diarrhoeal diseases or claim that secretion does not occur under any circumstance. However, it seems unlikely that *E. coli* STa does

cause fluid secretion in the jejunum in vivo as envisaged, since it was not found despite intensive investigation. The body of evidence for secretion can be grouped into relatively few categories of evidence that show that while intestinal tissue is *affected* by STa enterotoxin, they do not incontrovertibly demonstrate secretion. Observations provided by these methods, if correctly interpreted, do not, therefore, necessarily conflict with the present in vivo findings that STa reduces fluid absorption but does not act by causing increased fluid secretion. Many of the techniques used to demonstrate apparent secretion with other toxins give similar results with STa enterotoxin. STa will apparently cause increases in short-circuit current, apical cell membrane conductance and alterations in Ussing-chamber chloride ion fluxes, but these are unlikely to be phenomena associated with fluid secretion if this does not occur in vivo in this tissue, with this enterotoxin. Other enterotoxins may cause secretion that is blockable by interrupting the chloride channels in the apical cell membrane, but it is highly unlikely that STa does act in this way. Since the same criteria are used to demonstrate notional secretion with other enterotoxins as were used with STa, this is to some extent a challenge to chloride ion-dependent secretion as a general model for increased fluid entry into the lumen. It is outside the scope of this paper to review the likely causes of the heavy fluid losses in cholera and related diseases but evidence that is only *consistent* with epithelial cell chloride and fluid secretion should be assessed against the earlier hypothesis of fluid filtration, which was discounted rather than disproven, as an explanation for diarrhoeal disease.

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References

Barrett, K.E. 1991. Immune-related intestinal chloride secretion III. Acute and chronic effects of human mast cell mediators on chloride secretion by a human colonic epithelial cell line. *J. Immunology* **147:**959–964

Barrett, K.E., Keely, S.J. 2000 Chloride secretion by the intestinal epithelium: Molecular basis and regulatory aspects. *Annu. Rev. Physiol.* **62:**535–572

Beubler, E., Badhri, P., Schirgi-Degen, A. 1992. 5-HT receptor antagonists and heat-stable *Escherichia coli* enterotoxin-induced effects in the rat. *Eur. J. Pharmacol.* **219:**445–450

BMDP. 1981. Biomedical Programmes manual. UCLA Press, *University of California*.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**247–254

Chao, A.C., de Sauvage, F.J., Dong, Y.-J., Wagner, J.A., Goeddel, D.V., Gardner, P. 1994. Activation of intestinal CFTR Cl^- channel by heat-stable enterotoxin and guanylin via cAMP dependent protein. *EMBO J.* **13:**1065–1072

Charney, A.N., Dansky, H.M. 1990. Additive effects of ileal secretagogues in the rat. *Gastroenterology*. **98:**881–887

Cohen, M.B., Nogueira, J., Laney, Jr., D.W., Conti, T.R. 1992. The jejunal secretory response to *Escherichia coli* heat-stable enterotoxin is prolonged in malnourished rats. *Pediatric Res.* **31:**228–233

Cruickshank, S.F., Baxter, L.M., Drummond, R.M. 2003. The Cl^- channel blocker niflumic acid releases Ca^{2+} from an intracellular store in rat pulmonary artery smooth muscle cells. *Brit. J. Pharmacol.* **140:**1442–1450

Cuthbert, A.W., Hickman, M.E., MacVinish, L.J., Evans, M.J., Colledge, W.H., Ratcliffe, R., Seale, P.W., Humphrey, P.P.A. 1994. Chloride secretion in response to guanylin in colonic epithelia from normal and transgenic cystic fibrosis mice. *Brit. J. Pharmacol.* **112:**31–36

Eklund, S., Jodal, M., Lundgren, O. 1985. The enteric nervous system participates in the secretory response to the heat stable enterotoxins of *Escherichia coli* in rats and cats. *Neuroscience* **14:**673–681

Farack, U.M., Asher, A., Elsenhans, B., Schüette-Lückenga, B., Gerzer, R. 2000. Effect of loperamide on mucosal guanylyl cyclase activity in rat jejunum following *Escherichia coli* heat-stable toxin-induced fluid accumulation. *Pharmacol. Toxicol.* **86:**78–82

Fasano, A., Uzzau, S., Fiore, C., Margaretten, K. 1997. The enterotoxic effect of zona occludens toxin on rabbit small intestine involves the paracellular pathway. *Gastroenterology*. **112:**839–846

Fedorak, R.N., Allen, S.L. 1989. Effect of somatostatin analog (SMS 201–995) on *in vivo* intestinal fluid transport in rats. A limited systemic effect. *Dig. Dis. Sci.* **34:**567–572

Feng, Y., Wente, S.R., Majerus, P.W. 2001. Overexpression of the inositol phosphatase SopB in human 293 cells stimulates cellular chloride influx and inhibits nuclear mRNA export. *Proc. Natl. Acad. Sci. USA* **98:**875–879

Field, M. 1971. Intestinal secretion: effect of cyclic AMP and its role in cholera. *New Engl. J. Med.* **284:**1137–1143

Field, M., Fromm, D., McColl, I. 1971 Ion transport in rabbit ileal mucosa. I. Na and Cl fluxes and short-circuit current. Amer. J. Physiol. 220:1388–1396

Field, M., Graf, L.H. Jr., Laird, W.J., Smith, P.L. 1978. Heat-stable enterotoxin of *Escherichia coli*: In vitro effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. *Proc. Natl. Acad. Sci. USA* **75:**2800–2804

Fiske, C.H., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:**375–400

Flagella, M., Clarke, L.L., Miller, M.L., Erway, L.C., Gianella, R.A., Andringa, A., Gawenis, L.R., Kramer, J., Duffy, J.J., Doetschman, T., Lorenz, J.N., Yamoah, E.N., Cardell, E.L., Shull, G.E. 1999. Mice lacking the basolateral Na-K-2Cl cotransporter have impaired epithelial chloride secretion and are profoundly deaf. *J. Biol. Chem.* **274:**26946–26955

Forrester, R.L., Wataji, L.J., Silverman, D.A., Pierre, K.J. 1976. Enzymatic method for the determination of CO_2 in serum. *Clin. Chem.* **22:**243–245

Forte, L.R., Eber, S.L., Turner, J.T., Freeman, R.H., Fok, K.F., Currie, M.G. 1993. Guanylin stimulation of Cl^- secretion in human intestinal T84 cells via cyclic guanosine monophosphate. *J. Clin. Invest.* **91:**2423–2428

Goldstein, J.L., Sahi, J., Bhuva, M., Layden, T.J., Rao, M.C 1994. *Escherichia coli* heat-stable enterotoxin-mediated colonic Cl^- secretion is absent in cystic fibrosis. *Gastroenterology*. **107:**950–956

Guandalini, S., Rao, M.C., Smith, P.L., Field, M. 1982. cGMP modulation of ileal ion transport: in vitro effects of *Escherichia coli* heat-stable enterotoxin. *Am. J. Physiol.* **243:** G36–G41

Guba, M., Kuhn, M., Forssmann, W.G., Classen, M., Gregor, M., Seidler, U. 1996. Guanylin strongly stimulates rat duodenal HCO_3^- secretion: proposed mechanism and comparison with other secretagogues. *Gastroenterology* **111:**1558–1568

Guerrant, R.L., Hughes, J.M., Chang, B., Robertson, D.C., Murad, F. 1980. Activation of intestinal guanylate cyclase by heat stable enterotoxin of *Escherichia coli*: studies of tissue specificity, potential receptors, and intermediates. *J. Infect. Diseases* **142:**220–228

Hamilton, D.L., Johnson, M.R., Roe, W.E., Nielsen, N.O. 1978. Effects of intraluminal glucose on intestinal secretion induced by heat stable and heat labile *Escherichia coli* enterotoxin, cholera toxin and theophylline. *Canad J. Comp. Med.* **42:**89–96

Hamilton, D.L., Roe, W.E., Nielsen, N.O. 1977. Effects of heat-stable and heat labile *Escherichia coli* enterotoxins, cholera toxin and theophylline on unidirectional sodium and chloride fluxes in the proximal and distal jejunum of weanling swine. *Can. J. Comp. Med.* **41:**306–317

Hamilton, R.S. 1966. A direct photometric method for chloride in biological fluids employing mercuric thiocyanate and perchloric acid. *Clin. Chem.* **12:**1–8

Hindle, W., Code, C.F. 1962. Some differences between duodenal and ileal sorption. *Amer. J. Physiol.* **203:**215–220

Hohorst, H.J. 1957. Enzymatic determination of L (+) lactic acid. *Biochem. Z.* **328:**509–521

Hubel, K.A. 1973. Effect of luminal sodium concentration on bicarbonate absorption in rat jejunum. *J. Clin. Invest.* **52:**3172–3179

Humphreys, M.H., Earley, L.E. 1971. The mechanism of decreased intestinal sodium and water absorption after acute volume expansion in the rat. *J. Clin. Invest.* **50:**2355–2367

Huott, P.A., Liu, W., McRoberts, J.A., Giannella, R.A., Dharmsathaphorn, K. 1988. Mechanism of action of *Escherichia coli* heat stable enterotoxin in a human colonic cell line. *J. Clin. Invest.* **82:**514–523

Ieda, H., Naruse, S., Kitigawa, M., Ishiguro, H., Hayakawa, T. 1999. Effects of guanylin and uroguanylin on rat jejunal fluid and electrolyte transport: comparison with heat-stable enterotoxin. *Regulatory Peptides.* **79:**165–171

Jodal, M., Lundgren, O., Naftalin, R.J. 1987. Changes in vivo in rat ileal fluid uptake and mucosal volume induced by changes in the tonicity of the luminal perfusion fluid. *J. Physiol.* **391:**P29

Klipstein, F.A., Engert, R.F. 1978. Reversal of jejunal water secretion by glucose in rats exposed to coliform enterotoxins. *Gastroenterology.* **75:**255–262

Krebs, H.A., Henseleit, K. 1932. Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyler's Z. Physiol. Chemie* **210:**33–66

Kuhn, M., Adermann, K., Jähne, J., Forssmann, W.G., Rechkemmer, G. 1994. Segmental differences in the effects of guanylin and *Escherichia coli* heat-stable enterotoxin on Cl^- secretion in human gut. *J. Physiol.* **479:**433–440

Lee, J.S. 1977. Epithelial cell extrusion during fluid transport in canine small intestine. *Amer. J. Physiol.* **232:**E408–E414

Lucas, M.L. 2005. Amendments to the theory underlying Ussing chamber data of chloride ion secretion after bacterial enterotoxin exposure. *J. Theor. Biol.* **234:**21–37

Lucas, M.L., Cannon, M.J. 1983. Measurement of sodium ion concentration in the unstirred layer of rat small intestine by polymer Na^+-sensitive electrodes. *Biochim. Biophys. Acta* **730:**41–48

McEwan, G.T.A., Lucas, M.L. 1990. The effect of *E. coli* STa enterotoxin on the absorption of weakly dissociable drugs from rat proximal jejunum in vivo. *Brit. J. Pharmacol.* **101:**937–943

Miller, D.L., Schedl, H.P. 1970. Total recovery studies of nonabsorbable indicators in the rat small intestine. *Gastroenterology* **58:**40–46

Moreau, A. 1868. Ueber die Folgen der Durchschneidung der Darmnerven. *Zentralbl. Med. Wissenschaft* **14:**209–211

Mourad, F.H., Nassar, C.F. 2000. Effect of vasoactive intestinal polypeptide (VIP) antagonism on rat jejunal fluid and electrolyte secretion induced by cholera and *Escherichia coli* enterotoxin. *Gut* **47:**382–386

Nataro, J.P., Kaper, J.B. 1998. Diarrheagenic *Escherichia coli. Clin. Microbiol. Rev.* **11:**142–201

Nobles, M., Diener, M., Rummel, W. 1991. Segment-specific effects of the heat-stable enterotoxin of *E. coli* on electrolyte transport in the rat colon. *Eur. J. Pharmacol.* **202:**201–211

Nzegwu, H.C., Levin, R.J. 1994. Fluid hypersecretion induced by enterotoxin STa in nutritionally deprived rats: jejunal and ileal dynamics in vivo. *Exp. Physiol.* **79:**547–560

Orlowski, J. 1993. Heterologous expression and functional properties of amiloride high affinity (NHE-1) and low affinity (NHE-3) isoforms of the rat Na/H exchanger. *J. Biol. Chem.* **268:**16369–16377

Pappenheimer, J.R., Reiss, K.Z. 1987. Contribution of solvent drag through intracellular junctions to absorption of nutrients by the small intestine of the rat. *J. Membrane Biology* **100:**123–136

Raabo, E., Terkildsen, T.C. 1960. On the enzymatic determination of blood glucose. *Scand. J. Clin. Lab. Invest.* **12:**402–409

Rolfe, V., Levin, R.J. 1994. Enterotoxin *Escherichia coli* STa activates a nitric oxide-dependent myenteric plexus secretory reflex in the rat ileum. *J. Physiol.* **475:**531–537

Rolston, D.D.K., Mathan, V.I. 1992. Effect of base precursors on water and electrolyte transport during oral hydration solution perfusion in secreting rat intestine. *Dig. Dis. Sci.* **37:**47–52

Schanker, L.S., Tocco, D.J., Brodie, B.B., Hogben, C.A.M. 1958. Absorption of drugs from the rat small intestine. *J. Pharmaceut. Exp. Therapeutics* **123:**81–88

Schulz, S., Lopez, M.J., Kuhn, M., Garbers, D.L. 1997. Disruption of the guanyl cyclase-C gene leads to a paradoxical phenotype of viable but heat stable enterotoxin-resistant mice. *J. Clin. Invest.* **100:**1590–1595

See, N.A., Bass, P. 1993. Glucose-induced ion secretion in rat jejunum-a mucosal reflex that requires integration by the myenteric plexus. *J. Auton. Nerv. System* **42:**33–40

Sheerin, H.E., Field, M. 1977. Ileal mucosal cyclic AMP and Cl secretion: serosal vs. mucosal addition of cholera toxin. *Amer. J. Physiol.* **232:**E210–E215

Solomon, A.K. 1964. Validity of tracer measurements of fluxes in kidney tubules and other three compartment systems. *In:* Snell, F.M., Noell, WK., (eds) Transcellular membrane potentials and ionic fluxes. Chapter IV. Gordon & Breach, New York, London

Sladen, G.E., Harries, J.T. 1972. Studies on the effects of unconjugated dihydroxy bile salts on rat small intestinal function in vivo. *Biochim. Biophys. Acta* **288:**443–456

Sund, R.B. 1975. The effect of dodecylsulphate upon net sodium and water transport from tied jejunal loops in anaesthetized rats. *Acta Pharmacol. Toxicol.* **37:**282–296

Tantisira, M.H., Jodal, M., Lundgren, O. 1990. Further studies of the changes in alkaline secretion, transepithelial potential difference and net fluid transport induced by the heat-stable enterotoxin of *Escherichia coli* (STa) in the rat jejunum in vivo. *Acta Physiol. Scand.* **140:**557–565

Teitelbaum, D.H., Sonnino, R.E., Dunaway, D.J., Stellin, G., Harmel, R.P. 1993. Rat jejunal absorptive function after intestinal transplantation. Effects of extrinsic denervation. Digest. Dis. Sci. **38:**1099–1104

Thiagarajah, J.R., Broadbent, E., Hsieh, E., Verkman, A.S. 2004. Prevention of toxin-induced intestinal ion and fluid secretion by a small-molecule CFTR inhibitor. *Gastroenterology*. **126:**511–519

Turvill, J.L., Kasapidis, P., Farthing, M.J.G. 1999. The sigma ligand, igmesine, inhibits cholera toxin and *Escherichia coli* enterotoxin induced jejunal secretion in the rat. *Gut* **45:**564–569

Vaandrager, A.B., Bot, A.G.M., Ruth, P., Pfeifer, A., Hofmann, F., De Jonge, H.R. 2000. Differential role of cyclic GMP-dependent protein kinase II in ion transport in murine small intestine and colon. *Gastroenterology*. **118:**108–114

Volant, K., Grishina, O., Descroix-Vagne, M., Pansu, D. 1997. Guanylin-, heat-stable enterotoxin of *Escherichia coli*- and vasoactive intestinal polypeptide-induced water and ion secretion in the rat intestine in vivo. *Eur. J. Pharmacol.* **328:**217–227

Winne, D., Görig, H. 1982. Appearance of ^{14}C-polyethylene glycol 4000 in intestinal venous blood: influence of osmolarity and laxatives, effect on net water flux determination. *Naunyn-Schmiedeberg's Arch. Pharmakol.* **321:**149–156

Young, A., Levin, R.J. 1990. Diarrhoea of famine and malnutrition: investigations using a rat model. 1 Jejunal hypersecretion induced by starvation. *Gut* **31:**43–53

Young, A., Levin, R.J. 1992. Intestinal hypersecretion of the refed starved rat: a model for alimentary diarrhoea. *Gut* **33:**1050–1056