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Comparison of the Effects of *E. coli* STa with *E. coli* LT, *Clostridium difficile* Toxin A and Osmotic Burdens on Small Intestinal Fluid Transport: Additional Proof that STa is Not a Secretory Enterotoxin

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Using a recirculation procedure to perfuse anaesthetised rat jejunum, *E. coli* STa enterotoxin can be shown to inhibit net fluid absorption profoundly, while not causing net fluid secretion, provided fluid measurement is by mass or volume. This observation contradicts many reports of STa causing secretion, implying that the recovered volume technique in the anaesthetised animal over a period of some hours cannot detect secretion because of conjectured or unspecified flaws. Experiments are presented here confirming the viability of the perfusion protocol used in this laboratory but also demonstrate that if secretion were to be occurring, the recovered volume protocol would detect it. It will only return a negative finding, if secretion does not occur. To this end, the effect of two secretory toxins on intestinal fluid movement in a closed loop preparation

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were studied to demonstrate that the anaesthetic, intestinal preparation or perfusion duration did not hinder the demonstration of net secretion when the intestine was exposed to *E. coli* LT and C. *difficile toxin* A.. It is evident that STa itself only reduces net absorption but can appear to be secretory if driving forces such as luminal osmotic pressure or capillary hydrostatic pressure through vasodilatation are introduced, as was likely to have occurred with pithing and theophylline. The recognition that STa is a non-secretory enterotoxin necessarily falsifies several alternative methods that claim to demonstrate secretion. Since STa is not secretory many other substances identified by these methods need also not be secretory and alternative explanations must be found to explain their action. The importance of recognising that action on the small intestine cannot be attributed to a secretory mechanism within the enterocyte adds further weight to the concept that where net secretion does occur, the likely mechanism for it is a combination of increased vasodilatation together with increased hydraulic conductivity.

Keywords: Intestine; secretion; diarrhoeal disease; E. coli STa; enterotoxin; enterocyte.

1. INTRODUCTION

Almost a half century ago, the means by which large volumes of fluid entered the small intestine in cholera was proposed [1,2] to be secretory processes residing within the villous enterocyte (epithelial) cells. This view revived a neglected hypothesis from an earlier century [3a, b], namely that the epithelial layer caused secretion, since it was often intact in severe cholera, yet fluid secretion persisted. The attribution, at that time, of secretion solely to the enterocytes was a critical *non-sequitur* since the possibility of it originating from the sub-epithelial space and by-passing the enterocytes was not considered. A consequence this misattribution was that later research concentrated almost exclusively on enterocyte mediated secretion, resulting in the concept of luminally directed chloride ion [4,5] driving fluid secretion. Most subsequent research involved elucidating the molecular details of this process and continues to do so [6]. Other directions were investigated that either directly or obliquely [7,8] indicated the paracellular pathway as a route for severe fluid loss but the dominant paradigm remained unchallenged. It seemed self-evidently true, given the large extent of apparently confirmatory data, most not involving measurements of mass transport. More recently, a challenge to the validity of the paradigm has arisen solely because of the properties of the heat stable (STa) form of *E. coli* enterotoxin, involving fluid recovery.

This anti-absorptive toxin meets all *in vitro* criteria for being secretory, i.e. increased short circuit current [9,10] and luminally directed chloride ion flux increases [11] but fails to cause fluid secretion *in vivo* in the perfused small intestinal loop [12]. If STa is not secretory as measured by mass transport of fluid, then there must be doubt about the prevailing *in vitro* methods used to demonstrate secretion; the events attributed to secretion are not secretory and undermine evidence for the enterocyte model of secretion, which becomes a doctrine without proof [13]. An attempt at reconciliation is that STa is indeed not secretory but is an aberrant enterotoxin for reasons that are unclear; the lack of secretion is a distracting, minor feature of intestinal pathophysiology, potentially eventually explicable within the present enterocyte based paradigm. A more robust defence of the paradigm is that contradictory findings originate from one laboratory only [12,14-18] and could represent merely the failure in one laboratory to demonstrate the known fact of secretion. The error must arise through inadequate maintenance of blood pressure, a poor perfusion regime that prevents secretion from manifesting itself or a combination of these and other unrealised factors.

To allay this last critique, small intestine was exposed *in vivo* to *E. coli* LT, *C. difficile* toxin A and osmotic challenge, all known to cause secretion. The normal protocol for *E. coli* STa experiments was adapted to the closed, unperfused loop procedure that demonstrates net secretion with *C. difficile* enterotoxin [19]. STa in combination with a second circumstance such as added theophylline, a luminal osmotic load or pithing was also undertaken to achieve net fluid secretion. STa enterotoxin was re-confirmed as an anti-absorptive but not a secretory toxin. The lack of secretion after STa exposure in a preparation that can detect secretion, were it to occur, must call into question some currently preferred methods for studying secretion and further challenge the concept of enterocyte secretion being the root cause disorder of secretory diarrhoeal disease.

2. METHODS AND MATERIALS

2.1 *In vivo* Perfusion Procedures

All experiments complied with UK (1986) animal research legislation. Absorption of fluid *in vivo* from perfused jejunal loops was measured by a recirculation procedure [20], described in detail elsewhere [12,14], using a fluid recovery method [21]. Adult Sprague-Dawley female rats were anaesthetised (70 mg/Kg i. p.) with sodium pentobarbitone (Sagatal) and tracheotomised. Isolated loops were perfused in the distal direction at 1.8 ml/ min by a peristaltic pump from a reservoir maintained at 37ºC. The entry cannula was of 2 mm internal diameter, with a 4 mm exit cannula, as wide as possible to prevent any increases in luminal pressure as measured by manometer, while still allowing cannulation. After three hours, recirculation was stopped with all perfusate collecting in the reservoir. The loop was excised and remaining perfusate was drained by gravity into the reservoir, assisted by further flushing with air by syringe. Recovered volume was measured to the nearest 0.1ml, this representing 0.4% of a 25 ml volume of perfusate. The perfusate was a solution of 100 mM sodium bicarbonate and 54 mM sodium chloride, unless otherwise stated. Fluid absorption, the difference between the initial added and final recovered volume, is expressed as µl per cm length of intestine per hour, with positive values denoting absorption. In a second series of perfusion experiments, the effect of combination of STa perfusion with an additional perturbing substance was investigated. STa luminal perfusion was supplemented by luminal theophylline and in another set by STa perfusion in the pithed rat preparation. This was done because theophylline or other phosphodiesterase inhibitors are sometimes used to supplement STa action, having assumed that both factors act through the same pathway and elevate cGMP concentrations within the enterocyte. Similarly, the pithed animal does not require long term anaesthesia and also can often show secretion that can be enhanced by luminal STa perfusion, again with the implicit assumption that both are acting on the same enterocyte secretion mechanism.

2.2 *In situ* Loop Preparation

Some experiments were done using *in situ* filled but unperfused loops to confirm that net fluid secretion caused by enterotoxins could be detected using the same duration and anaesthetic regime. Initially, fluid movement *in vivo* was measured using *in situ* closed (i.e. non-perfused) loops, using a protocol that had previously been able to demonstrate *Clostridium difficile* toxin A mediated secretion [19], in animals allowed to recover from short term anaesthesia.

The present *in situ* loop experiments were done under anaesthesia for the duration of the experiment, without recovery. Three loops were formed, approximately 10 cm past the ligament of Treitz. Two 10 cm length jejunal loops were created, separated by a 5 cm empty loop that prevented the transfer of content between loops. Test and control loops was flushed with isotonic saline to remove debris and then with air to expel residual fluid. To test the effectiveness of an enterotoxin, one ml of solution containing either saline or saline plus enterotoxin was introduced through a cannula and the loop end was tied off. The introduced amount was measured by weighing a syringe before and after fluid introduction. Enterotoxins were assigned randomly. Initially, the infusate was 20 mM ethanolamine and 0.2 M NaCl adjusted to pH 7.5 [19], duplicating a protocol that had originally shown secretion with *C. difficile* toxin A. After this initial duplication of results using a successful protocol, the loop infusate was changed to isotonic saline with or without toxin to confirm that *C. difficile* toxin A was secretory even in the absence of an inadvertently imposed isosmotic gradient.

Unless otherwise stated, loops were left for two hours, excised and weighed uncut, then cut but with luminal solution recovered by gravity drainage and weighed. At the end of the experiment, the animals were humanely killed under terminal anaesthesia. Fluid accumulation was expressed as in the Torres protocol [19] as mean ratio of uncut loop weight to loop length; the control ratio was subtracted from the toxin ratio, giving a single difference value for each pair of loops. Ratios were also individually considered. In addition to this, absorption was measured by direct reference to the recovered volume that was weighed at the end of the experiment. Fluid movement estimates were therefore based both on explicit volume measurements derived from recovered volume which is the preferred method in this laboratory and on implied volumes derived from differences in loop weight before and after drainage. It was thought important to do this to check for any systematic difference or difficulties with the

closed loop method of assessing fluid absorption compared with the open perfused loop method used routinely [12,14 -16] in this laboratory.

The length of the loop was measured after laying out the excised loop without stretch, allowing fluid absorption to be expressed as µl per cm length of intestine per hour. Excised loops were weighed and also dried to constant weight in an oven at 100ºC, allowing fluid uptake also to be standardised per milligram dry weight and per gram wet weight. These were calculated but only fluid flows per cm length are presented here as changing the standardisation variable did not alter any statistical inferences.

2.3 Blood Pressure Experiments

Arterial blood pressure was measured via a carotid cannula (Portex, 'pink' o. d. 0.9 mm) using a Harvard pressure transducer, coupled to a Toshiba lap-top computer and recorded using Spike 2 for data capture via the CED 1401 interface (Cambridge Instruments, Cambs, UK). Heart rate and respiration frequency (from the Traube-Hering waves) were also measured from the blood pressure record.

2.4 Administration of Chemicals and Enterotoxins

Synthetic STa (Sigma) was given at a concentration of 80 ng/ml of perfusate. STa from P16 *E. coli* was given as a dose in saline to equal this, as was LT from E49 *E. coli*. *Clostridium difficile* toxin A was added to loops at 5.0 µg protein per 1 ml loop.

2.5 Source of Chemicals

E. coli STa and other chemicals were purchased from Sigma Chemical Co (Poole, UK), Sagatal (sodium pentobarbitone) from Rhone Merieux Ltd (Harrow, UK). For closed loop experiments, STa from a P16 strain of *E. coli* was used (gift of Dr. M. Burgess, Beecham Pharmaceuticals, UK), LT from an E49 strain of *E. coli* (gift from Houghton Poultry Research Institute, Huntingdon, UK) and *C. difficile* toxin A from Professor R. Aitken, University of Glasgow.

2.6 Statistical Analysis of Data

Results are given as the mean and standard error of the mean, with the number of experiments, equal to the number of animals in parenthesis. Implementation of statistical analysis was done using BMDP [22]. Comparison of means was by paired and unpaired "Student's" 't'-values with Dunnett's [23] correction for any multiple comparisons. In addition, significant differences for proportions or frequencies were calculated by Fisher's exact test with Yates' correction for small numbers and confirmed by testing the odds ratio (Sokal & Rohlf, 24).

3. RESULTS

3.1 Data and Observations on Methods of Measuring Intestinal Fluid Transport *in vivo* by the Recovered Volume Protocol

Fluid recovery for measuring fluid transport, involving three hours perfusion *in vivo*, was preferred to the alternative but widespread method of calculation of volume using assumedly unabsorbable marker substances. These are now known [25] to introduce gross errors as loss of label by absorption gives false positive indications of secretion both in short duration experiments and when low absorption rates occur. A critique of the fluid recovery protocol used in this laboratory is that the *E coli* STa induced intestinal secretion that assumedly should occur, cannot be shown. This lack can be attributed to a lengthy perfusion period compared with most *in vitro* procedures and other accessory conditions, including perfusate content, poor maintenance of blood pressure, differing responses between male and female rats, paradoxical responses to anaesthesia or simply just an unspecifiable inability to respond to secretory stimuli. Since all observations on the failure to secrete after exposure to STa enterotoxin seemed to arise only when the fluid recovery technique was used and that in one laboratory only, this could be taken to imply a technique mediated failure to show secretion rather than an absence *per se* of secretion. Consequently, it was necessary to consider these concerns.

Failure to maintain an adequate blood pressure and heart rate does not occur in the chosen protocol. An initial heart rate of 394 ± 22 (5) beats per minute (Table 1) did not vary throughout perfusion and ended at 379 ± 4.6 (5) beats per minute. Mean arterial blood pressure of 132 ± 9.0 (5) mmHg at the onset, remained steady and ended at 125 ± 4.0 (5) mmHg. Falls in pressure were inducible by i.v. perfusion with

2.8 mg/Kg/hr cromakalim, from 108 ± 11.7 (5) to 62.4 ± 7.0 (5) mmHg. Hence, while barbiturate anaesthetised animals did not suffer from deleterious blood pressure changes that might have prejudiced the detection of secretion, falls in blood pressure were detectible when these were deliberately induced. Sex of the animals was also not relevant to maintenance of blood pressure since initial blood pressure in three female animals of comparable weight to the males was 109.3 ± 12.2 (3) mmHg and rose to 124.7± 4.9 (3) mmHg by the end of the recirculation period.

Intravenous infusion of glucose-containing saline is sometimes advocated as mandatory for maintenance of adequate blood pressure. In the absence of intravenous fluid infusion, blood pressure is well maintained in the present protocol. In two male animals, given i.v. isotonic saline at 1ml/hour, blood pressure did not differ (Table 1) from unsupplemented animals. Perfusate bicarbonate anion concentrations resembling those that arise in the lumen during pancreatic secretion, promote fluid absorption [26]. Intestinal fluid uptake of 5 - 6 mls over a three hour period already approximates commonly used hourly rates of i.v. fluid infusion. Hence, additional i.v. infusion will overhydrate and expand the plasma volume which is known to predispose to fluid secretion. Glucose addition to the intestinal perfusate might be deemed to be desirable to counteract purportedly inadequate tissue nutrition during a three hour perfusion but plasma glucose concentrations did not decline over the experimental period. Inclusion of glucose in any intestinal perfusate would be a severe complicating factor as it would stimulate fluid absorption despite the presence of STa and may obscure any secretion taking place. The experiments would therefore be on intestine subjected to enterotoxin but also simultaneously to a form of oral rehydration solution, as occurs with most, if not all, *in vitro* and some *in vivo* experiments.

3.2 Detection of Secretory Action of Toxins and Circumstances Known to Cause Net Secretion of Fluid in the Small Intestine

With *E. coli* STa in the absence of any adverse osmotic gradient (Fig. 1), STa, as expected, reduced absorption in the bicarbonate perfusate from 84.4 $\pm$ 8.4 (4) µl/cm/hr to 11.4 $\pm$ 6.6 (4) ul/cm/hr, not significantly different from zero net absorption but not indicating net secretion. With isotonic saline, a lesser rate of normal absorption of 51.5 $\pm$ 5.2 (5) µl/cm/hr was reduced significantly ($p < 0.01$) to a value of 9.4 $\pm$ 10.4 (5) µl/cm/hr by STa, not significantly different from zero net fluid absorption. Where a hypertonic perfusate was additionally recirculated as in the Torres protocol [19], net fluid absorption was 25.3 $\pm$ 9.4 (5) µl/cm/hr, becoming net secretory through the combination of STa exposure and an adverse osmotic gradient to - 34.4 $\pm$ 3.6 (5) µl/cm/hr, significantly different from normal absorption ($p < 0.01$) but also significantly ($p < 0.01$) secretory (Table 2). Exposure of the loop to LT enterotoxin from *E. coli* reduced net absorption in saline significantly ($p < 0.01$) from 47.9 $\pm$ 4.4 (9) µl /hr/cm to -27.1 $\pm$ 11.0 (9) µl/cm/hr, which was a significantly ($p < 0.05$) net secretory value. *Clostridium difficile* toxin A changed net fluid absorption of 35.0 $\pm$ 1.7 (5) µl/cm/hr to net fluid secretion of -10.3 $\pm$ 4.3 (11) ul/cm/hr into the lumen. Every loop exposed to *C. difficile* toxin A had a final loop weight that exceeded the initial injected weight and also had

Table 1. Mean arterial blood pressure and heart rate at the start and end of a three hour perfusion period in normal male, normal female rats and i.v. infused normal male rats undergoing Sagatal (70 mg/Kg body weight) anaesthesia *in vivo*

Mean arterial Blood pressure (mmHg)	Heart rate beats per minute (bpm)	Experimental circumstances
		Without i.v. perfusion
132 ± 9.0 (5)	394 ± 22.0 (5)	Male rats, at onset of experiment
125 ± 4.0 (5)	379 ± 5.0 (5)	Male rats, at end of experiment
109 ± 12.0 (3)	363 ± 16 (3)	Female rats, at onset of experiment
125 ± 5.0 (3)	375 ± 15.0 (5)	Female rats, at end of experiment
		With i.v. perfusion
134 (2)	400 (2)	Male rats, at onset of experiment
105 (2)	355 (2)	Male rats, at end of experiment

a higher recoverable volume, thus demonstrating that net fluid secretion can be detected if an enterotoxin is *capable* of causing net fluid secretion. It could also be detected using a non-secretory toxin if an adverse osmotic gradient was also present. Hence, where circumstances would allow secretion to be occur, this could be observed in the barbiturate anaesthetised animal preparation protocol used in this laboratory.

3.3 Joint Exposure to Luminal STa Enterotoxin and a Second Perturbing Factor

In the case of theophylline perfusion, the results are self-evident in that STa reduces net fluid absorption (Table 2) but does not lead to net secretion. Only when theophylline is additionally perfused is there net secretion that is significantly different ($p< 0.01$) from zero net fluid absorption, all six experiments leading to more fluid recovered than was added to the reservoir.

In a pithed group of animals, a combination of STa and pithing led to net fluid secretion that was statistically significant when a one sided test was used i.e. that the secretory values were below zero net movement. The likelihood that an additional, ancillary condition made STa seem secretory was tested by considering the numbers of secreting and absorbing intestines within each experimental series. It should be noted that where net secretion occurred in STa treated populations, this was a very marginal event that small errors in protocol or measurement could have induced. To estimate the probability of secretion having occurred due to chance, the numbers of secreting animals where there was a second perturbing factor e.g osmotic load was used. The numbers of expected secretory events was used, using the least favourable ratio in the normal population to test the null hypothesis that there was no secretion when STa was co-perfused with theophylline, an additional luminal burden of osmolarity was imposed or the animals were additionally pithed. The least favourable ratio for the null hypothesis was used i.e. that for the saline and STa group where 1 out of 10 was secretory making a likely probability of 0.1.

Using Fisher's exact probability test for a binary event (secretion or absorption) as described above, the probability of a second deleterious circumstance and STa not causing net secretion is very improbable ($p < 0.0001$). This indicated that pithing, additional luminal osmolyte and theophylline with STa leads to secretion while STa on its own does not.

4. DISCUSSION

A crucial goal of the investigation of secretory diarrhoeal disease is to establish the means by which fluid is made to enter the small intestine in life threatening quantities. Secretory diarrhoea is widely held to result from enterotoxins causing the epithelial cells (enterocytes) of the villus to secrete fluid at abnormally high rates [1, 2]. Since the inception of this concept, various methods have been used to measure secretion, including *in vitro* electrical measurements, *in vitro* radioactive tracer transport measurements or flawed *in vivo* methods [27]. This has led to the situation where the proxy methods of detecting secretion demonstrate the features associated with exposure to enterotoxin, yet the enterotoxin does not cause net fluid secretion. A particularly clear expression of this dichotomy is the undoubted elevation in short-circuit current after exposure of small intestinal tissue to heat stable *E. coli* STa [9], whereas no net secretion is found *in vivo* in a perfused loop [12] when luminal fluid is physically recovered. A contestable explanation for the lack of net fluid secretion after STa perfusion of the jejunum of the barbiturate anaesthetised rat is that in some way this preparation cannot demonstrate secretion after STa enterotoxin exposure but would do so if other protocols were used - hence, a failure to demonstrate secretion is both local and limited to laboratories using prolonged recirculation *in vivo* techniques with accompanying barbiturate anaesthesia. However, maintenance of blood pressure and other vital signs are not compromised with barbiturate anaesthesia. The suitability of this preparation is also confirmed by the fact that net secretion is found when known secretory toxins are perfused. STa enterotoxin in contrast has the appearance of causing secretion when perfusion occurs in the presence of a second perturbing circumstance. This observation helps to define more clearly how net secretion should be measured.

In order to establish whether net fluid secretion has occurred, the chosen method should unequivocally measure mass transport of fluid. This is only achievable by minimising the possibilities for volume measurement error and introducing no assumptions about the variable being measured. These strict criteria rule out the use of 'unabsorbable' markers because calculating volume from concentration change is

compromised by their known absorption, which violates Fick's principle [25] and falsely indicates secretion. Recirculation of fluid with recovery of all volume at the end of the experiment is therefore the required *in vivo* technique. Any increase in luminal volume can then only be attributed to net secretion. In contrast, any method that measures total loop weight necessarily measures luminal volume plus interstitial and vascular fluid volumes as well [28-30]. Weight changes cannot in these preparations unequivocally be attributed to fluid secretion because vascular events can increase interstitial fluid weight, without any fluid entering the lumen [31,32]. The weighed loop can be seen from any schematic of the apparatus [28–29] to include vascular and interstitial fluid volume to the total volume; the technique will misattribute expansion of the interstitial space after vasodilatation to the further, unproven step of movement of fluid into the lumen. This may explain the many discordant findings between the Gothenberg loop preparation and recovery protocols when luminal volume alone is measured and not total loop weight. Using the weighed loop technique, fluid secretion is claimed on exposure *in vivo* to *Salmonella* bacteria [33], to cholera toxin [34] and to *E. coli* STa [35]. In all cases, hexamethonium can apparently inhibit the rate of secretion whilst atropine cannot. In contrast, using recovered luminal volume to assess secretion, *Salmonella typhimurium* infection had little effect on net jejunal fluid absorption [36] while the weighed loop preparation apparently shows [33] copious rates of secretion. Similarly, perfused loop preparations showed [14] that STa prevents absorption but does not exhibit the apparent copious secretion that characterises the weighed loop preparation [35]. Neither atropine nor hexamethonium affects the secretion caused by *Clostridium difficile* toxin A in the perfused loop [7] or the inhibited fluid absorption on perfusion with *E. coli* heat stable STa enterotoxin [18]. In contrast, using the weighed loop protocol, hexamethonium apparently inhibited the anti-absorptive actions of STa [30] and the secretory actions of *C difficile* toxin A [37]. An explanation for these persistently discrepant findings is that the Gothenberg weighed loop preparation unavoidably also measures fluid entry into the interstitial space from the vasculature as well as any expansion of intravascular volume. As a consequence, many pharmacological interventions that are claimed to be anti-secretory may be vascular events leading to the erroneous conclusion that fluid secretion into the lumen has been altered.

In contrast, the recovered luminal volume protocol does not have this defect and should be the preferred procedure for detection of net fluid secretion, for which phenobarbitone is known to be the preferred anaesthetic [38]. The failure to detect widely expected secretion after STa exposure can be shown not to be due to failure of technique in this laboratory or inadequate maintenance of blood pressure under anaesthesia since net fluid secretion can be detected when a loop is exposed to *E. coli* LT,

Table 2. The effect of *E. coli* STa toxin plus a second perturbing factor (10 mM luminal theophylline, pithing or a luminal osmotic load) on jejunal fluid absorption *in vivo* in anaesthetised rats.

Perfusate	Control	Secretion to absorption ratio	Plus STa	Secretion to absorption ratio
150 mM bicarbonate	94.9 ± 8.0 (21)	0/21	18.8 ± 3.2 (10) [d, g]	0/10
100 mM bicarbonate	94.5 ± 5.7 (19)	0/19	24.2 ± 5.1 (17) [d, f]	2/17
100 mM bicarbonate plus 10 mM theophylline	47.2 ± 11.3 (6) [b]	0/6	-25.8 ±4.0 (6) [d, g]	6/0 [h]
154 mM saline	59.7 ± 6.4 (10)	0/10	25.2 ± 5.3 (10) [d, f]	1/9
154 mM saline plus pithing	18.2 ± 4.4 (9) [c]	0/9	-9.1 ± 4.8 (9) [d, e]	7/2 [h]
154 mM saline plus 140 milliosmoles luminal osmotic load	25.3 ± 9.4 (5) [a]	0/5	-34.4 ± 3.6 (5) [d, g]	5/0 [h]

Symbols: a = p<0.02; b = p<0.01; c = p<0.001, for comparison with appropriate control mean; first symbol in STa column d = p<0.001 for comparison of STa v control; second symbol in STa column, e = p<0.05; f = p<0.01; g = p<0.001 STa mean v zero net absorption Symbol h = p< 0.001 for exact probability of differences in secretion to absorption numbers arising by chance.

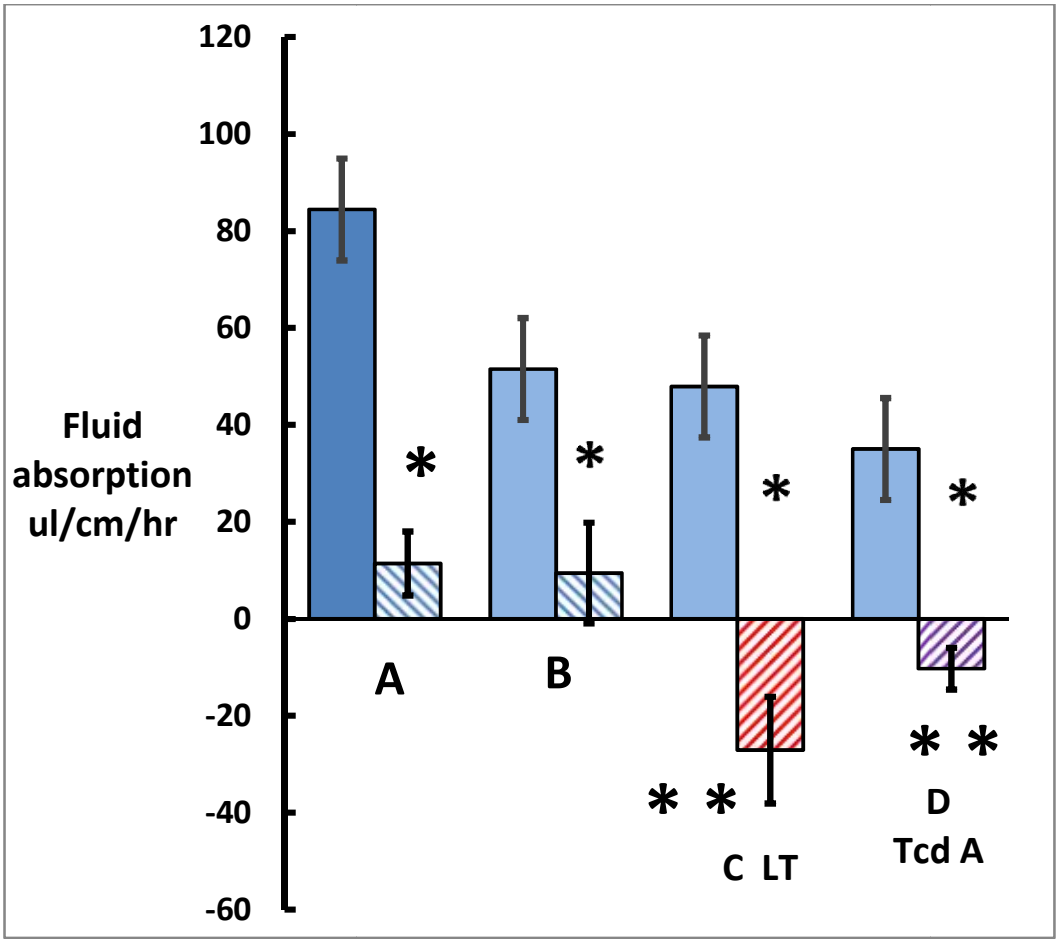

Fig. 1. The effect of A) *E. coli* STa in high bicarbonate (100 mM) perfusate, B) *E. coli* STa in saline, C) *E. coli* LT in saline and D) *Clostridium difficile* toxin A in saline on jejunal fluid absorption (µl/cm/hr).

*Jejunal fluid absorption or secretion assessed by the inoculated non-perfused loop technique in vivo in anaesthetised rats. Statistical significance by paired 't'-test, * = p < 0.01 for all control v toxin means, ** = p< 0.05 for toxin values compared with zero net absorption.*

Clostridium difficile toxin A or adverse osmotic pressure gradients. In this respect, *E. coli* STa is an important enterotoxin for investigating whether a process is secretory or not since STa can almost completely suppress fluid absorption, thus allowing the testing of whether a second procedure or enterotoxin is genuinely secretory.

E. coli STa is not a secretory toxin but it can appear secretory when STa is used in conjunction with an agent such as theophylline that has pressor actions. Similarly, *E. coli* STa can misleadingly be deduced to be secretory when perfused in a pithed animal. Pithing removes spinal nerve control and it is likely that sympathetic vasoconstriction is absent. This makes it likelier that pithing and theophylline are secretory in their own right since they are associated with reduced arterial blood pressure and hence increased capillary pressure, making the jejunum secretory because of altered Starling forces [17]. Secretion under these circumstances would be opposed by normal absorption but would become very evident when fluid absorption was inhibited by STa. Although it is possible that the addition of theophylline or iso-butyl-methyl-xanthine could enhance enterocyte second messenger concentrations or turnover, their actions would not only affect the enterocytes since they also have known actions on smooth muscle. This would explain why some *in vivo* studies seem to show net secretion in the presence of STa but only when IBMX is added to the perfusate [39].

The lack of genuine secretion after STa exposure, when measured by the simplest method of collecting fluid, contrasts starkly with an alleged demonstration of secretion using other methods: weighed loop measurements aggregate interstitial with luminal volume change; net flux measurements showing increased serosal to mucosal chloride ion fluxes must also be discounted since the mathematics of the three compartment model as applied to the Ussing chamber do not permit the simple conclusion that net flux is the result of two independent 'unidirectional' fluxes [40]; short-circuit current elevations have been widely adopted as a reliable method of measuring net fluid secretion yet STa will elevate short-circuit current whilst not causing secretion; markers such as polyethylene glycol can indicate secretion yet the known absorption even of small amounts of marker compromises calculation of volume from concentration since loss of label would be wrongly attributed to net secretion [41].

5. CONCLUSION

The difficulties with misattribution of cessation of absorption to enterocyte secretory processes, as arises when the prevailing methods detailed above are used, are twofold; i) important observations can be misinterpreted and ii) epiphenomena such as elevations in short circuit current are studied almost to the exclusion of fluid movement and buttress a false model of how fluid secretion is achieved. Both these aspects derive from an erroneous model of fluid secretion stemming from the enterocytes, the persistence of which paradigm continues to disappoint [6] and also to prevent the discovery of effective anti-diarrhoeal medications.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per University & UK standard, written ethical permission was sought and has been preserved by the authors

COMPETING INTERESTS

The authors declare that there are no competing interests.

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