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Research Paper

In vitro Interactions Between the Oral Absorption Promoter, Sodium Caprate (C_{10}) and *S. typhimurium* in Rat Intestinal Ileal Mucosae

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Purpose. The medium chain fatty acid, sodium caprate (C_{10}), is a promising oral drug delivery agent that may promote permeability of peptides through increasing paracellular permeability of the intestinal epithelium. One safety concern is that it may permit co-absorption of by-stander agents including pathogens. The purpose of this *in vitro* study was to examine the effects of C_{10} on rat intestinal ileal mucosae in the presence of co-administered *Salmonella typhimurium* in a low volume vertical Ussing chamber.
Methods. C_{10} or *S. typhimurium* was added to rat ileal mucosae mounted in chambers and the flux of the paracellular flux of [^{14}C]-mannitol examined. *S. typhimurium* adherence and uptake by ileal mucosae was also examined by counting. Bacterial growth curves were assessed in the presence of C_{10}. Minimum inhibitory- and bacteriocidal concentrations of C_{10} were determined against a range of bacteria.
Results. Apical addition of either C_{10} or *S. typhimurium* to rat ileal mucosae mounted in modified diffusion chambers significantly increased the flux of [^{14}C]-mannitol in a concentration-dependent fashion. Co-exposure with increasing concentrations of C_{10} attenuated the *Salmonella*-induced increase in mannitol flux. Histological evaluation revealed that C_{10} inhibited both adhesion and invasion of *S. typhimurium* to intestinal mucosae. Short term bacterial growth studies in the presence of C_{10} showed evidence of concentration-dependent inhibition. C_{10} was bacteriocidal in mM concentrations against *S. typhimurium* and selected gram positive and negative bacteria.
Conclusions. C_{10} does not promote the permeation of a common bacterium across isolated intestinal tissue upon acute co-exposure. It prevents *S. typhimurium* attachment to epithelia and impedes growth of a range of different bacterial strains.

KEY WORDS: C_{10}; intestinal bacterial adhesion; medium chain fatty acids; oral peptide delivery; Ussing chamber.

INTRODUCTION

Oral administration of the majority of biotech molecules remains difficult mainly due to poor intestinal epithelial permeability. While lipophilic compounds tend to be able to cross the epithelial layer passively via transcellular passive diffusion, hydrophilic peptides are generally confined to the low capacity, size-restricted paracellular pathway unless apical membrane transporters are available [reviewed in (1)]. Passage through intestinal tight junctions limits bioavailability of peptides to those less than 1,000 Da in molecular weight, and alternative possibilities for increasing absorption include targeting of esterified amino acid prodrugs to transporters in the small intestine, including PepT1 (e.g., valacyclovir) (2), amphiphilic polymer conjugation to alkylated poly(ethylene)glycol oligomers (3), and uptake by a range of polymeric nanoparticles (4). It is also possible that peptide-loaded nanoparticles can be targeted with ligands to Peyer's patch M cells in the follicle-associated epithelium (5). Enhanced paracellular absorption of peptides has been reported using absorption promoters such as medium-chain fatty acids (6), *zonula occludens* toxin derived peptide (7), and chitosan (8).

The medium chain fatty acid, sodium caprate (C_{10}), is found in mM concentrations in milk constituting roughly 2–3% of total fatty acids (9). C_{10} is approved for use as a food additive in several countries and has GRAS (generally regarded as safe) status in that capacity. C_{10} enhanced the intestinal absorption of a decapeptide in rat ileal mucosae (10) and oral delivery promoted absorption of an antisense ologonucleotide in pigs (11). While data from human gut perfusion studies were not suggestive of an absorption promoting effect of C_{10} solutions in respect of delivery of phenoxymethylpenicillin (12), significant oral bioavailability was achieved in Phase I trials following oral delivery of C_{10} tablet formulations containing either low molecular weight heparin, alendronate or desmopressin [reviewed in (13)], as

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well as for tablets loaded with an antisense oligonucleotide targeting tumour necrosis factor (14).

The reversible increase in paracellular permeability to mannitol induced in Caco-2 monolayers by C_{10} is associated with tight junction dilatation occurring within minutes. Associated decreased transepithelial electrical resistance rapidly returns to baseline levels following removal of the agent (15). Part of the mechanism of action of C_{10} is to initiate a signaling cascade via interaction with phospholipase C and reduction in cytosolic ATP levels that culminate in morphological changes in the F-actin peri-junctional ring and cytoskeletal disruption (16). It seems that concentrations of C_{10} above those that have a selective effect on tight junction opening are required for *in vivo* efficacy in man. Rectal administration of ampicillin required 25 mM of C_{10} in a base, a dose that induced hyperosmolarity and temporary abrasion of the epithelium (17). Still, recent reports have indicated that C_{10} has been formulated into capsules and given in repeated oral dosing regimes to more than 300 human subjects without evidence of toxicity (13).

Candidate delivery agents being considered for promotion of intestinal absorption of pharmaceuticals need to enhance membrane permeability without damage to tissue. Several *in vitro* studies assessed potential cytotoxic effects of permeability enhancers through the potential release of lactate dehydrogenase (LDH) from epithelia to the apical compartment. C_{10} was shown not to significantly increase LDH release from Caco-2 cells at the mM concentrations required to increase paracellular permeability (18,19). In contrast to the calcium chelator, EGTA, C_{10} did not increase cellular release of the inflammatory Type 1 mediators, IL-6 and IL-8, in cultured human airway epithelial monolayers, data that were confirmed in mouse lung instillation studies *in vivo* (20). Microscopic evaluation of the mucosal morphology of rat intestine exposed to C_{10} following single pass perfusion also showed an intact appearance of the intestine (21). Since solute movement across the tight junction barrier is based in part on permeant size, repeated temporary openings of tight junctions to permit the passive absorption of biopharmaceutical molecules could theoretically result in co-absorption of pathogens. Ironically, from the desirable standpoint of promoting gene delivery for cystic fibrosis patients, C_{10} facilitated adenovirus-mediated gene transfer across primary cultures of human airway epithelia (22).

As a model pathogen, we selected *Salmonella enterica*. It is a Gram-negative family of bacillus bacteria, whose pathogenicity requires adherence and invasion across villous enterocytes (23). *Salmonella*-induced enteritis is most frequently caused by *S. enterica* serotypes, *typhimurium* and *enteritidis,* and represents one of the most common causes of contaminated food-born disease (24). Diarrhoea, vomiting, and abdominal pain manifest 12–72 h after ingestion, which is associated with mucosal edema, inflammation and infiltration of neutrophils (25). Therefore, our aim was to investigate whether or not the presence of apically administered C_{10} increases the adherence and invasion of *S. typhimurium* in isolated rat ileal intestinal epithelial tissue mucosae mounted in horizontal diffusion chambers. Secondly, we measured the capacity of the fatty acid to alter bacterial viability and growth following short term exposure of selected bacteria to C_{10}.

MATERIALS AND METHODS

Materials

Adult male Wistar rats (210–300 g, n=91) were obtained from the Biomedical Facility, University College Dublin, Ireland. A clinical isolate of *S. typhimurium* was obtained from the Microbiology Department, St. Vincent's University Hospital, Dublin. All *Salmonella* data refers to this strain unless stated. *Escherichia coli* ATCC 10536 and *Micrococcus luteus* ATCC 9341 were a gift from Dr. Siobhan McClean, Institute of Technology, Tallaght, Dublin. *E. coli* equine isolate, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* bovine isolate and *Lactobacillus salivarius* UCC118 were obtained from Dr. Denise Drudy, Centre for Food Safety, University College Dublin. Luria–Bertani agar was purchased from Cruinn Technologies Ltd., Dublin. Tryptic soy broth (TSB), bacteriological agar, and de Man Rogosa and Sharpe (MRS) media were purchased from Sigma, UK. Xylose lysine deoxycholate agar was obtained from LIP Diagnostic Services Ltd., Galway, Ireland. C_{10} (MW 194 g) and FITC-dextran 4000 (FD-4) was purchased from Sigma-Aldrich, UK. 1.5 ml Eppendorf ® polyethylene tubes were obtained from Starstedt, Ireland.

Intestinal Tissue Preparation

Maintenance and welfare of animals along with an approved method of sacrifice were in accordance with the "Principles of Laboratory Animal Care," (N.I.H publication number 85-23, revised in 1985). Rats were sacrificed by cervical dislocation. Intestinal tissue was exposed in each

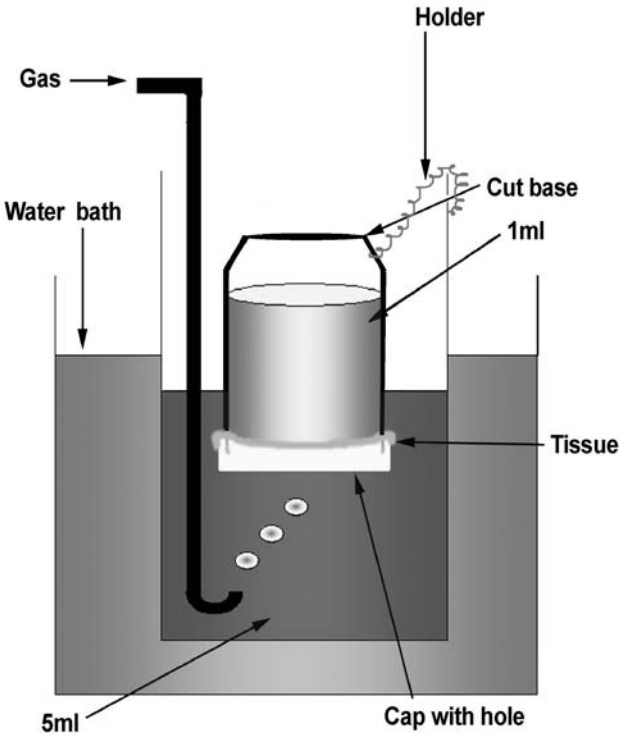

Fig. 1. Schematic of horizontal diffusion chamber. The internal window area was 0.63 cm^2. The internal mucosal-side volume contained 1 ml while the bath contained 5 ml KH.

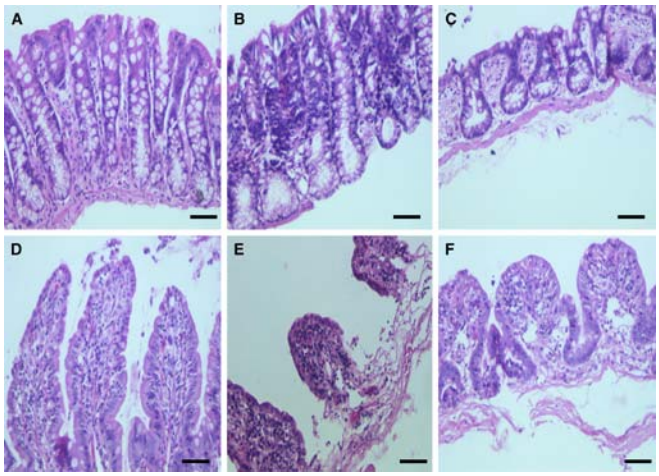

Fig. 2. Haemotoxylin and eosin histology staining of chamber-mounted colonic and ileal mucosae. Colonic mucosae: **A** 0; **B** 60 and **C** 120 min. Ileal mucosae: **D** 0; **E** 60 and **F** 120 min. *Horizontal bars* denote 50 μm.

species via a mid-ventral abdominal incision. Segments were excised and immediately immersed in freshly prepared oxygenated Krebs–Henseleit solution (KH) at room temperature. Proximal ileal and colonic tissue was immediately excised, bisected along the line of the mesentery and washed with warm oxygenated buffer. Four-inch tissue segments were then pinned mucosal side down on a cork-board. The sub-mucosal longitudinal and circular muscle layers were stripped from the mucosa using blunt dissection with a size 5 forceps. Rat ileal mucosal sheets were mounted in the chambers within 10 min of euthanasia.

Horizontal Diffusion Chambers

The system used was a further modification of a recent prototype (26). Inverted 1.5 ml polystyrene Eppendorf ® tubes comprised the apical compartment of the horizontal chamber. The cap of the Eppendorf ® was removed and a hole bored through the center of the cap to give an area of 0.63 cm^2. The tapered base of the Eppendorf ® tube was cut off with a razor blade, removing approximately 0.25 ml capacity. A small hole was then bored very close to the cut edge and a copper wire affixed so that the excess portion of the wire could be bent into a hook to suspend the tube upside-down from the side of a 20-ml capacity Sterilin® tube (Staffordshire, UK), which comprised the basolateral chamber (Fig. 1). When tissue was mounted onto the chamber it was stretched across the inward sealing cylinder of the cap, with the apical surface of the tissue facing up into the interior of the chamber. The cap was then snapped onto the body of the Eppendorf ® tube generating a tissue barrier between the fluid contents of the chamber (apical side) and the fluid contents of the Sterilin ® tube (basolateral side). The apical and basolateral compartments contained 1 and 5 ml KH, respectively. KH was gassed via a flexible polyethylene tube with 95% O_2:5% CO_2 on the basolateral side only, since apical gassing led to evaporation. Temperature was maintained at 37°C by immersing the rack of chambers in a heated water bath. After 5 min equilibration, KH was removed from the apical compartment using a sterile pipette. Because C_{10} precipitates out of solution in the presence of calcium, Ca^{2+}-free KH was used to replace the buffer on the apical side in those studies (15). C_{10} was dissolved in de-ionized water to give a stock concentration of 250 mM, and then diluted to the desired apical concentration (1–30 mM).

Table I. Effects of Apical Addition of C_{10} on the Papp of [^{14}C]-Mannitol Across Isolated Rat Ileal Sheets

[C_{10}] mM	Papp (cm/s) $\times 10^6$	% Absorption
0	6.3±1.3	0.0004
1	5.1±1.8	0.0003
10	26.1±5.4*	0.0012
20	284.3±102.7*	0.013
30	510.3±161.5*	0.024

Flux period was 120 min. N=6 in each case.
*P<0.001 vs. control.

[^{14}C]-Mannitol and FD-4 Fluxes Across Rat Ileal Mucosae

Five microcuries of [^{14}C]-mannitol was added to the apical side of tissues and 100 μl immediately sampled to calculate the donor-side concentration. Basolateral samples (100 μl) were sampled every 20 min for 2 h and replaced with fresh KH to maintain constant volume and sink conditions. The apparent permeability (Papp) for mannitol was calculated from the following equation (27): Papp (cm/s)=$(dQ/dt)/(A \cdot C_o)$, where dQ/dt is the transport rate (mol/s); A is the surface area of the intestinal sheet (cm^2), and C_o is the initial concentration in the donor compartment (mol/ml). In some studies, absorptive fluxes of the paracellular flux marker, fluorescein isothiocyanate-labeled dextran 4000 (FD-4) were measured using a donor side concentration of 0.5 mg/ml according to previous methods (27). In studies in which C_{10} or *Salmonella* were co-incubated on the apical side of the tissues with [^{14}C]-mannitol, all agents remained for the 120 min duration.

Growth of Bacterial Strains and Minimum Inhibitory and Bacteriocidal Concentrations of C_{10}

E. coli ATCC 10536, *E. coli* equine isolate, *S. enteritidis* ATCC 13076, *S. typhimurium* bovine isolate and *M. luteus*

Table II. Effects of C_{10} on the Papp of [^{14}C]-Mannitol Across Isolated Rat Ileal Sheets in the Presence of *S. typhimurium*

Agent	Papp (cm/s) $\times 10^3$	% Absorption
S. typhimurium	4.2±1.0	0.19
1 mM C_{10} with *S. typhimurium*	4.2±1.0	0.10
10 mM C_{10} with *S. typhimurium*	3.4±0.4	0.16
20 mM C_{10} with *S. typhimurium*	2.4±0.3	0.01
30 mM C_{10} with *S. typhimurium*	0.6±0.2*	0.03

Flux period was 120 min. N=6 in each case
*P<0.05 vs. *S. typhimurium* alone (10^8 CFU/ml)

ATCC 9341 were cultured in TSB at 37°C and *L. salivarius* UCC118 was cultured in MRS media at 37°C. The minimum inhibitory concentration (MIC) was calculated as the lowest concentration of C_{10} to completely inhibit growth of the bacterial cultures. The method of MIC calculation was adapted from the microdilution broth dilution procedure outlined in the Clinical and Laboratory Standards Institute (formerly NCCLS) protocol for aerobic bacteria (28). Briefly, bacterial cells were seeded at approximately 5×10^5 CFU/ml per well in a microtitre plate with varying concentrations of C_{10}. Plates were incubated at 37°C for 18 h and cell growth was determined at 600 nm on a UV spectrophotometer. The data was plotted and MICs expressed as CFU/ml.

The minimal bactericidal concentration (MBC) was calculated as the lowest concentration of C_{10} to elicit 99.9% killing of the final inoculum after 18 h growth. C_{10} was considered bactericidal if the MBC was $\leq 4\times$ the MIC concentration (29). The method of MBC calculation was adapted from the microdilution endpoint protocol outlined in the National Committee Clinical Laboratory Standards protocol for determining bactericidal activity (30). Briefly, bacterial cells were grown as before, then after 18 h incubation aliquots were spread on agar plates and incubated at 37°C for 24 h. MBCs were calculated as the lowest concentrations of C_{10} that produced 99.9% reduction in resulting CFUs compared with controls.

Adherence and Uptake of *S. typhimurium* by Rat Ileal Mucosae

Mounted tissues were exposed to *S. typhimurium* (10^8 CFU/ml) and incubated for 5 min at 37°C in the presence and absence of C_{10}. Following the incubation period, tissues were fixed for 24 h in 10% formalin, then preserved in paraffin. Tissues were Gram-stained and viewed under a light microscope. Ten villi from each tissue were visualized. The height, width, and the surface perimeter of each villus were measured and *Salmonella* that had adhered to or invaded the villus were counted. The number of total bacteria that adhered and invaded the whole three-dimensional villus was then extrapolated from these data (31). Total villus surface area was calculated using width and height measurements based on the assumption that villi are roughly cylindrical. Bacterial adhesion and invasion was expressed per villus section surface area by multiplying villus perimeter measurements by the section thickness of 5 μm, yielding bacteria per μm^2 of section. The total villus surface area was divided by the sectional surface area, and multiplied by bacteria per μm^2 of section to yield bacteria per μm^2 of total villus surface area. Values were expressed in mm^2.

Viability of Ileal Mucosal Sheets

Apical fluids were sampled at regular intervals after tissue mounting and extracellular LDH levels were measured using an enzyme assay kit (Sigma, Poole, Dorset, UK) adapted for isolated intestinal tissue enzyme release (32). The percent cytotoxicity was determined by comparing the amount of extracellular LDH observed in the apical chamber to the maximum releasable LDH induced by exposure to 0.01% Triton-X-100. Mean maximum LDH levels for the amount of tissue used in the chambers was determined by manually homogenizing three segments of tissue, of the same size as those used in the horizontal chamber, in a mortar and pestle, then digesting those homogenates with 1 ml 0.5% Triton-X for 5 min. Structural integrity was evaluated by histological examination of rat tissue mounted at zero, 60 and 120 min. Following incubation, tissues were collected in formalin and fixed overnight. Three sections from each formalin-fixed block were collected on glass slides and examined by light microscopy.

C_{10} and *S. typhimurium* Interactions

In a contact study, C_{10} was diluted in PBS and mixed with *S. typhimurium* (10^8 CFU/ml) and incubated for 10 min at room temperature. Standard dilutions of each test were

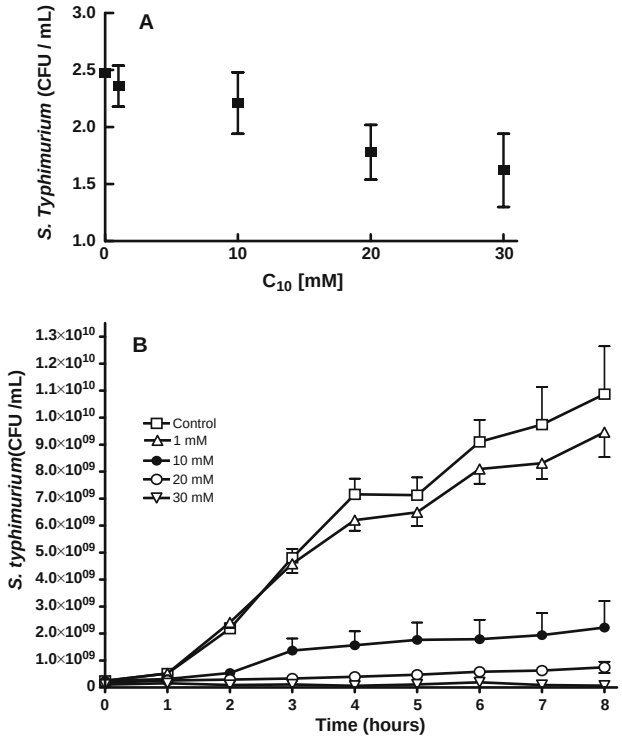

Fig. 3. **A** Effects of a short-term 10 min exposure of C_{10} on the viability of *S. typhimurium* (10^8 CFU/ml). 20 mM and 30 mM C_{10} caused significant reductions in viability. **B** Effects of long-term 8 h exposure of C_{10} on the viability of *S. typhimurium* (10^8 CFU/ml). 10, 20 and 30 mM C_{10} caused significant reductions in viability.

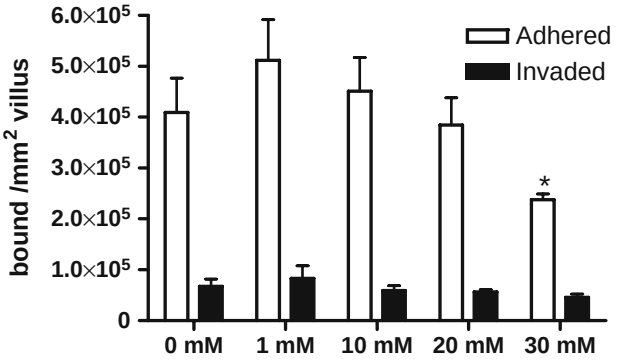

Fig. 4. Effects of a short-term 5 min exposure of C_{10} on the adherence to and uptake of *S. typhimurium* (10^8 CFU/ml) by rat ileum. 30 mM C_{10} caused significant reduction in adherence.

incubated on LB agar for 24 h at 37°C and viable colonies of *S. typhimurium* were then counted in the presence of selected concentrations of the fatty acid. To assess the impact of C_{10} on bacterial growth, C_{10} was diluted in brain–heart infusion broth (BHI) to varying concentrations, inoculated with *S. typhimurium* (10^4 CFU/ml), and incubated with agitation at 37°C for 8 h. The optical density at 600 nm (OD_{600}) of each sample was measured at regular time points on a spectrophotometer (Beckman Instruments Inc., California, USA). Every 2 h, viable colonies of *S. typhimurium* were counted using samples from test vials.

Statistics

Quantitative data are expressed as mean±standard error of the mean. Statistical analyses on group values compared over time were performed by repeated measures ANOVA. Statistical analysis for significant differences between independent groups was calculated using Student's two-tailed paired or unpaired *t* test, as appropriate. P values of <0.05 were considered significant.

RESULTS

Intestinal Tissue Viability in Horizontal Diffusion Chambers

To examine fluxes, it was important to assess the tissue viability in the chamber system over time. This was achieved using two assays, LDH release from tissue into the apical medium and transepithelial fluxes of FD-4. For LDH release, there was an initial 20–30 min transient release of approximately 25% from mounted ileal and colonic mucosae, followed by a stable phase of 120 min. Importantly, tissues that were isolated, muscle-stripped and suspended in KH displayed the same profile, indicating that the initial LDH release profile was not due to edge damage from mounting. Histological evaluation revealed that mounted ileal and colonic mucosae retained good morphology at a macroscopic level for up to 120 min (Fig. 2). It was notable that tissue thickness was reduced at 120 min vs. earlier time points, an affect that was also seen when the study was repeated with tissues from another rat. Since loss of thickness was only seen in muscle-stripped but not unstripped tissue at 120 min, it is possible that it is caused by the stretching that the stripped tissue undergoes during mounting. Nonetheless, the biochemical and functional data seen at 120 min suggests that this structural change is of limited importance.

FD-4 absorptive Papp values were linear and low, consistent with integrity of barrier function over that period. The Papp values across ileum and colon were 2.1×10^6 and 0.2×10^6 cm/s, respectively (n=6), i.e., tenfold lower across colonic than ileal mucosae (P<0.01). Several parameters were examined in an attempt to further optimize the system: replacement of KH with DMEM on the apical and basolateral sides, and switching from unilateral to bilateral gassing. Neither modification altered LDH release profiles (data not shown). Indeed, basolateral gassing was preferable to gassing both compartments since the former reduced the potential for evaporation from the 1 ml apical bath.

[^{14}C]-Mannitol Fluxes Across Rat Intestinal Mucosae: Effects of C_{10} and *S. typhimurium*

To assess the effects of C_{10} on absorptive permeability, ileal tissue segments were prepared and mounted on horizontal chambers. Paracellular flux of [^{14}C]-mannitol from the apical to the basolateral direction was measured over 120 min. Mannitol was selected over FD-4 for these studies since the smaller molecule can be detected with greater sensitivity and yields a more reproducible signal in the presence of absorption promoters. The basal Papp across ileal sheets was 6.3×10^6 cm/s (n=6). The basal Papp across colonic sheets was twofold lower than ileum: 3.1×10^6 cm/s (N=6; P<0.05). [^{14}C]-Mannitol flux across ileal mucosae was measured in the presence of apical additions of 1, 10, 20, and 30 mM C_{10}. Permeability was increased by fourfold with concentrations of C_{10} at 10 mM and above (Table I). The EC_{50} of C_{10} for increasing the flux of [^{14}C]-mannitol was 21 mM. Exposure of ileal epithelial sheets to 10^8 CFU/ml *S. typhimurium* for 120 min resulted in a 650-fold increase in the permeation of [^{14}C]-mannitol across ileal tissue segments mounted in horizontal chambers (P<0.0011, n=6). The Papp was increased to 4.1×10^3 cm/s in the presence of the bacteria, amounting to 0.2% of the donor-side concentration of

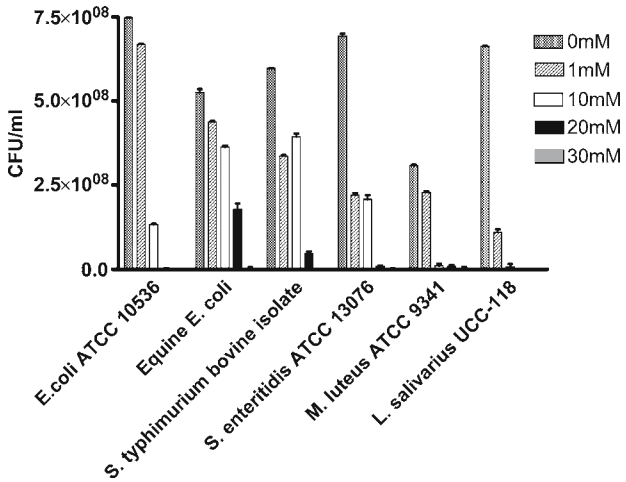

Fig. 5. Growth inhibition as indicated in CFU/ml of six bacterial strains by C_{10} over 18 h. Concentrations of C_{10} were 0, 1, 10, 20 and 30 mM. N=5–6 separate studies in each case.

mannitol. Surprisingly, exposure of ileal tissues to apical additions of C_{10} in the presence of *S. typhimurium* resulted in amelioration of the dramatic increase in flux normally induced by the bacteria (Table II).

Bacterial Viability and Growth in the Presence of C_{10}

To examine if C_{10} acts directly on *S. typhimurium,* 10^8 CFU/ml *S. typhimurium* were exposed to 1, 10, 20, and 30 mM C_{10} for 10 min. Twenty and thirty millimolars of C_{10} caused a statistically significant reduction in viability ($P<0.05$ and 0.0002 respectively). The EC_{50} for cell death in the 10 min period was 15 mM C_{10} (Fig. 3A). In separate experiments the effects of C_{10} on growth of *S. typhimurium*, 10^4 CFU/ml of *S. typhimurium* were exposed to C_{10} in broth for 8 h. C_{10} was found to inhibit the growth of *S. typhimurium* at 10, 20, and 30 mM in a concentration-dependent manner (Fig. 3B). At higher concentrations of C_{10}, the growth rate of *S. typhimurium* became negative, indicating that the population decreased as bacteria fail to reproduce at an equal or greater rate to the rate of death. As an inhibitor of bacterial growth, the EC_{50} of C_{10} was 3.1 mM.

Bacterial Adherence and Invasion of Rat Ileal Mucosae

The effect of C_{10} on adherence and invasion of *S. typhimurium* on ileal epithelial segments after an apical-side exposure of 5 min to both agents was assessed. Bacteria observed on the apical surface following three washes with PBS were designated as having adhered to the epithelium, whereas those that were observed to be within the cytoplasm of epithelial cells were designated as having invaded. Tissues were inoculated with 10^8 CFU/ml *S. typhimurium* and co-incubated with C_{10} at concentrations up to 30 mM. Only a high concentration of 30 mM C_{10} caused a significant reduction in bacterial adherence ($P<0.05$) (Fig. 4). The ratio of adhered to invaded bacteria did not vary significantly with concentration of C_{10}, suggesting that, while C_{10} appears to prevent adhesion of bacteria at 30 mM, once bacteria are adhered, it does not impair their ability to go on to invade the epithelial cells.

Inhibition of Growth and Bacteriocidal Effects by C_{10} on a Range of Bacterial Strains

C_{10} inhibited the growth of six different bacterial species in mM concentrations. The MICs were [mM]: *E. coli* ATCC 10536 (20), *E. coli* equine isolate (30), *S. enteritidis* ATCC 13076 (20), *S. typhimurium* bovine isolate (30), *M. luteus* ATCC 9341 (10) and *L. salivarius* UCC118 (10). The growth inhibition of each strain by C_{10} was concentration-dependent (Fig. 5). The minimal bacteriocidal concentrations (MBC) of C_{10} were also determined. The agent was bactericidal to all six organisms tested. The MBCs were [mM]: *E. coli* ATCC 10536 (30), *E. coli* equine isolate (30), *S. enteritidis* ATCC 13076 (20), *S. typhimurium* bovine isolate (40), *M. luteus* ATCC 9341 (10) and *L. salivarius* UCC118 (10). C_{10} therefore displayed stronger growth inhibition and bacteriocidal effects on the gram-positive organisms *M. luteus* and *L. salivarius* than on the gram-negative organisms.

DISCUSSION

C_{10} is currently being evaluated as a component of solid dose formulations which promote oral absorption of Class III biopharmaceuticals (13). The major issues for absorption promoters include i) demonstration of efficacy in patients with low individual variation at reasonable doses and ii) a propensity to cause abrasion resulting in possible co-absorption of bystander pathogens, especially following repeat dosing regimes. In respect of cytotoxicity of C_{10}, interpretation of data from Caco-2 remains somewhat divided, with reports stating that monolayers tolerate high concentrations well (18,21), or quite poorly (33). *In vivo* however, oral delivery of solid-dose formulations incorporating C_{10} have not shown evidence of intestinal toxicity in pigs (11) or in humans (13,14), although evidence of rectal epithelial abrasion was seen with a C_{10}-containing suppository characterised by high pH and hyperosmolarity (17). Recent urinary lactulose/mannitol data from human subjects following oral administration of C_{10} solution suggest that the absorption-promoting effects of the fatty acid on the epithelium are temporary and are reversed after just 40 min exposure (13). Furthermore, *in situ* evidence of loss of villus tips and cell sloughing from rat jejunal loops in response to a non-ionic surfactant was partially reversed after 60 min (34), so it seems that the capacity of the intestine to rapidly regenerate *in situ* and *in vivo* following exposure to potential abrasives should not be underestimated.

This study focussed on addressing the issue of the possibility of C_{10} induced co-absorption of a common intestinal bacterial pathogen, *S. typhimurium*. We tested whether a single apical-side exposure to a range of concentrations of C_{10} could permit adherence and internalisation of *S. typhimurium* to rat ileal segments mounted in modified horizontal diffusion chambers. Firstly, basal mannitol and FD-4 fluxes were within the normal range of Papp values for small hydrophilic compounds across rat ileal segments (35), data supported by the normal histology over the flux period. Secondly, these fluxes were higher in ileal mucosae than distal colon, reflective of increased paracellular flux capacity in ileum in a range of species (36). Thirdly, apical addition of C_{10} increased the absorptive flux of mannitol by fourfold across ileal mucosae at a concentration of 10 mM, a similar concentration as that required to dilate tight junctions and increase paracellular flux across both Caco-2 monolayers (15,16) and rat ileum mounted in Sweetana–Grass type Ussing chambers (9).

There is considerable debate over calculations of paracellular flux of drugs across different intestinal regions. If the density of tight junctions and total absorptive surface areas in ileum and colon are taken into account, Collett *et al.* (37) have argued that the absolute permeabilities of paracellular flux markers may be similar in both regions. Nonetheless, these data indicate that the modified vertical diffusion chamber system produces reliable flux data across intestinal mucosae over a defined short period. The chamber described here has advantages over the prototype (26) in terms of ease of modification and tissue mounting. In addition, it was easier to change the volume capacity of the basolateral side and it had more efficient gassing and mixing (data not shown). Anderberg *et al.* (15) make the point that concentrations of

the fatty acid above 10 mM irreversibly perturb the apical membrane of Caco-2 monolayers, although it is unlikely to be due to formation of micelles since the critical micellular concentration of C_{10} is approximately 50mM (38). Thus, even though substantial (>50-fold) increases in mannitol fluxes were detected across ileum at concentrations of 20 and 30 mM C_{10}, these are most likely accounted for by a combination of para- and transcellular effects of the agent *in vitro* (15) and *in vivo* (17).

The efficacy of *S. typhimurium* in permitting mannitol absorptive flux across the ileum over 2 h was dramatic and substantially more than that seen following exposure to C_{10}. *S. typhimurium* is known to attach in clusters, as distinct from mass invasion, to cultured human intestinal enterocytes leading to membrane ruffling as an initial stage in infection (23). In rabbit ileal explants, there is a degree of correlation between the potential of different strains of *S. typhimurium* to invade and resulting virulence (39). An additional route into the body is via attachment, invasion and destruction of Peyer's patch M cells, leading to access via the basolateral membrane to adjacent enterocytes (40). There is additional debate over the route of permeation of *Salmonella* strains across enterocytes: *Salmonella typhi* permeated Caco-2-derived monolayers via the paracellular route causing epithelial cell injury and extrusion, while *S. typhimurium* appears to use the transcellular route (41). Our data describing the attachment and invasion of ileal epithelia by *S. typhimurium* support those findings. It seems likely that the bacterium can destabilize the tight junctions as a secondary event, leading to enhanced mannitol flux. Since the bacterial-induced flux was increased several fold above what would be expected from a permeability enhancer using an exclusively paracellular route, a reasonable conclusion would be that the bacteria also increased transcellular flux.

Our hypothesis was that C_{10} increases absorption of *S. typhimurium*. In contrast to expectations, short term exposure to mM concentrations of the fatty acid attenuated the bacterial-induced mannitol flux, reduced bacterial adherence to the epithelium and stymied bacterial growth. Moreover, the data showed that C_{10} was bacteriocidal, most likely due to direct effects on bacterial membranes. Studies to clarify the mechanism(s) of the bacteriocidal effect are ongoing. *In vitro* bactericidal effects of medium chain fatty acids are well-established and appear more potent than effects of short chain fatty acids (42). For example, C_{10} caused a log-fold reduction in viable *Helicobacter pylori* (43). When fatty acids including C_6, C_8, and C_{10} were added to the feed of chicks 5 days prior to oral exposure, there was a decrease in colonisation of ceca and internal organs by *S. enterica* 3 days after infection (44). In addition to reducing invasion, in the same study medium chain fatty acids also appeared to decrease expression of *hilA*, an important regulating gene controlling invasive capacity of *Salmonella*. Using an *in vivo* mouse model of bacterial colonisation, Petschow *et al.* (45) demonstrated that concomitant intra-gastric administration of a mono-glyceride form of C_{10} with *Vibrio cholerae* caused a reduction in recovery of viable bacteria. Prior or subsequent administration of monocaprin® was without effect on viability, suggesting that a direct physical interaction between bacteria and C_{10} is essential. In the current study, growth inhibition by C_{10} was not limited to *Salmonella* and concentration-dependent inhibition of the growth of several unrelated gram positive and negative strains was also detected, suggestive of a common non-specific mechanism of action.

C_{10} and other medium chain fatty acids are not the only type of absorption promoter that may have anti-bacterial properties. Detergents such as SDS (46) and polymers including chitosan (47) have bacteriocidal actions, while methacrylate-based polymers were also found to inhibit the growth and epithelial attachment of *Salmonella* to HT29 cells (48). In addition, the bee venom-derived anti-microbial cationic peptide defensin, mellitin, was recently shown to act as a tight junction opener at similar concentrations to those of its antibiotic effect (49). Therefore, many established and potential formulation excipients are likely to have direct anti-microbial effects, largely based on a combination of non-specific mechanisms including cationic charge, bacterial membrane targeting, and detergent actions. The significance of the anti-microbial effects of excipients and polymers will be most notable in topically applied formulations where sufficiently high concentrations can be maintained locally. Finally, in the case of solid-dose oral formulations of C_{10}, it is likely that initial concentrations being released in the intestine will be considerably higher than the CMC of the fatty acid. All the anti-bacterial effects in this study were present at approximately 30 mM or below since higher concentrations proved very difficult to work with due to solubility issues and the difficulty in assessing free concentrations. Little is known about interactions of C_{10} with bacterial and enterocyte membranes at concentrations higher than the CMC.

CONCLUSION

Using a modified horizontal diffusion chamber to mount isolated rat intestinal mucosae, tissue was presented in a viable functional state for a minimum of 60 min incubation. Permeability coefficients of two paracellular markers were increased in ileum over colon. There was no evidence that C_{10} permits increased epithelial adherence of *S. typhimurium*. While both C_{10} and *S. typhimurium* increased the absorptive flux of mannitol, C_{10} offset the increased flux induced by the bacteria. Evidence was obtained to show that the fatty acid impeded bacterial adherence and reduced growth and that it was directly bacteriocidal in mM concentrations. In summary, our data in an *in vitro* intestinal tissue model supports claims that medium chain fatty acids impede bacterial growth and colonisation in concentrations likely to be present for a period in the intestine in repeated-oral dosing regimes *in vivo*.

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