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The effect of heat-stable *Escherichia coli* enterotoxin, theophylline and forskolin on cyclic nucleotide levels and mucosal surface (acid microclimate) pH in rat proximal jejunum in vivo

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The normally acidic mucosal surface pH of 6.24 ± 0.02(30) in rat proximal jejunum in vivo is effectively neutralised by 30 min exposure to heat-stable *Escherichia coli* (STa) enterotoxin (14 μg/ml) to 6.80 ± 0.07 ($n = 5$) or to a forskolin/theophylline combination (1 mM:20 mM) to 7.10 ± 0.07(7) while perfusion with Krebs-phosphate buffer alone without glucose left the mucosal surface pH unchanged at a pH of 6.21 ± 0.02(9). Forskolin alone had no effect, and 20 mM theophylline moderately elevated the surface pH to 6.52 ± 0.03(5). Theophylline, forskolin and their combination all elevated cAMP levels per mg tissue DNA above control values while STa enterotoxin was without effect. In contrast, all agents elevated cGMP levels per mg tissue DNA above control levels. These findings indicate that surface pH is only moderately affected by changes in cAMP levels and is affected to a much greater extent by altered cGMP levels.

Introduction

Studies using pH electrodes indicate that rat proximal jejunum maintains a mucosal surface more acid than neutral bathing medium in vitro [1–4] and in vivo [5–7]. In addition to direct measurement using electrodes, pH-sensitive dyes have also indicated the presence of an acidic mucosal surface – the so-called acid microclimate – in rat jejunum [8]. The source of mucosal acidity is uncertain, but may be either hydrogen ion secretion or bicarbonate absorption. Many bacterial enterotoxins, including cholera toxin [9], *Salmonella typhimurium* [10] and heat-stable E. coli (STa) enterotoxin [11] interfere with mucosal acidification or alkalinisation mechanisms. Such interference should alter the pH of the mucosal surface and recent studies confirm this view [11]. However, although STa enterotoxin causes a marked elevation of surface pH in vivo in the jejunum, cholera toxin does not. Since STa enterotoxin elevates cGMP [12–14] and cholera toxin elevates cAMP levels [15,16], a simple interpretation of the enterotoxin effects on surface pH is that only enterotoxins which modify cGMP levels can neutralise the acid microclimate.

This view receives support from the fact that exogenous 8-bromo-cyclic GMP resembles STa enterotoxin in neutralising the mucosal surface pH while 8-bromo-cyclic AMP does not [11], nor does forskolin, the potent adenylate cyclase activator [17]. However, when forskolin was used in combi-

Abbreviations: STa enterotoxin, heat-stable *Escherichia coli* enterotoxin; TCA, trichloroacetic acid.

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nation with theophylline, the in vivo surface pH was strikingly elevated, as it was then theophylline was applied after exposure to cholera toxin [11]. The aim of the present study was to measure rat jejunal mucosal cyclic nucleotide content under conditions which neutralised the surface pH in order to determine which cyclic nucleotide was associated with microclimate neutralisation.

Methods

Physiological procedures. The physiological procedure consists of mounting the intestine in vivo in a chamber such that the tissue receives blood from the animal but with the mucosal surface accessible for electrode measurement [18]. A midline incision was made in the abdomen of male Wistar rats (300 g), anaesthetised by intraperitoneal injection (80 mg/kg) of pentobarbitone (Sagatal) and maintained with additional doses (15 mg/kg) as required. The ligament of Treitz was located and a 5-cm segment of jejunum was isolated by ligaturing at each end of the loop to minimise blood loss during preparation. The isolated loop was sectioned along the antimesenteric border and the tissue was pinned, mucosal side upwards, onto the base plate of the perfusion chamber. After clamping on the upper half of the chamber, the mucosal surface was perfused at a rate of 1 ml/min with Krebs-phosphate buffer [19] of pH 7.1 at 37 °C. Clamping prevents blood loss through the sides of the tissue resulting in exposed mucosa with an intact blood supply. Unless at least two or three mesenteric arcades are included within the selected area of jejunum, the mucosa becomes pale and lumen-surface pH differences can no longer be detected.

Electrodes and recording details. After preparation, the tissue was allowed to recover for 15 min before an initial measurement of surface pH was made using a miniature glass pH electrode (SA3, WPI, New Haven, CT., U.S.A.). Previously reported in vivo surface pH measurements [11] were made with the MI506 (Microelectrodes, Londonderry, NH, U.S.A.) electrode. Values for surface pH did not differ from previous estimates when the WPI electrode substituted for the MI electrode. The pH electrode was connected to a Pye-Unicam 9409 electrometer, output from which drove a Linseis paper chart recorder. A Ag/AgCl electrode connected to the chamber via a salt bridge (3% agar/3 M KCl) acted as the reference electrode. The pH electrode was calibrated in buffers of pH 7 and pH 4 before and after each experiment. The pH electrode had a slope of some 55 mV/pH unit and displayed negligible drift over the experimental period. To measure the mucosal pH, the electrode was initially positioned in the buffer perfusate to measure the bulk phase pH. Then, under visual control, the electrode was lowered, using a Prior micromanipulator, to rest on the mucosal surface, where it remained until a stable signal was obtained. The electrode was then removed to the bulk phase of the perfusate. Three control pH measurements were made from different areas of the jejunal mucosa to establish viability. Surface pH was then measured at 15 min and 30 min under each experimental condition.

Perfusion details. The selected procotocol was one of initial pH measurement followed by perfusion with control solution or one of four test solutions which consisted of the same buffer additionally containing either: (a) forskolin (1 mM), (b) theophylline (20 mM), (c) forskolin/theophylline or (d) *E. coli* heat-stable (STa) toxin (14 μg/ml). The surface pH was again measured at 15 min and 30 min after the initiation of the experiment. The stability of the preparations was intermittently checked by ensuring that the control values after 15 and 30 min perfusion resembled the initial values. The effects on surface pH with each test solution could be compared with the appropriate control values. Forskolin and theophylline were obtained from Sigma Inc., U.K. STa toxin was a generous gift from Beechams Pharmaceuticals, Tadworth, U.K. All other chemicals were supplied by BDH.

Radioimmunoassays of cyclic nucleotides. After 30 min perfusion, the jejunal loop was rapidly removed from the chamber. The mucosa was scraped by drawing a glass microscope slide along the length of the loop. The mucosal scrape was immediately placed in 1.0 ml of 5% trichloroacetic acid (TCA) and left overnight at room temperature. The whole procedure required less than 2 min. After high-speed (13000 rpm) centrifugation, a 0.4-ml aliquot of the supernatant solution was

removed leaving a protein pellet. The supernatant was washed four times in water-saturated diethyl ether to remove TCA. Excess diethyl ether was boiled off at 80 °C. Following the addition of 100 µl of 200 mM sodium acetate buffer of pH 6.2, the nucleotides were acetylated by the addition of 5 µl of a mixture of triethylamine and acetic anydride solution (2 : 1, v/v) to increase the affinity for the binding proteins. The nucleotide levels were assayed using commercially available radioimmunoassay kits (Amersham International plc, Amersham, U.K). Duplicate samples of the antibody-nucleotide complex were added to scintillation vials containing 10.0 ml of scintillant (Ecoscint, National Diagnostics, Manville, N.J. U.S.A.) and counted using the ^3H channel of a Packard Tricarb 300CD scintillation counter. The cpm for each duplicate pair was averaged, corrected for background, and the nucleotide level was compared with background-corrected counts of standard nucleotide concentrations.

DNA assay. The DNA content of the precipitated pellet was measured using a fluorescence technique [20]. After dissolving the pellet in 50.0 ml of 1 M NaOH, a 100-µl aliquot was removed for the assay. Ten standard concentrations of DNA (from 5 µg/ml to 50 µg/ml) were made up to 100 µl in NaOH. Following the addition of 40 µl of 2 mM ice-cold diaminobenzoic acid to every standard and unknown, all tubes were incubated at 60 °C for 1 h. After cooling, 0.8 ml of 0.6 M perchloric acid was added to each tube. Following centrifugation at 13000 rpm for 2 min, a 200 µl sample was assayed for fluorescence at 520 nm after excitation at 420 nm, using an Aminco-Bowman spectrophotofluorimeter. The average fluorescence for a duplicate pair of samples corrected using an NaOH blank, was calculated from the standard curve.

Analysis of results. Results from the radioimmunoassays gave estimates of pmol of cAMP and cGMP per sample. These were standardised per cm^2 intestinal perfusion chamber area and per mg DNA. Calculation of means and associated parameters (S.E.) was by BMDP software [21] as was all statistical testing. Significance levels were determined using Students *t*-test assuming a normal distribution, but were confirmed using the Mann-Whitney non-parametric significance test.

Results

pH Measurements

Initial measurements of jejunal mucosal surface pH verified that control values for in vivo surface pH resembled previously obtained values [11]. As in previous experiments, when a pH electrode was placed onto the surface of the jejunum (Fig. 1), there was an immediately discernible change in measured pH which reached a stable level within 1 min. This value was always maintained until the electrode was returned to the bulk phase (the luminal perfusate). A surface pH of 6.24 ± 0.02 ($n = 30$) was detected at the jejunal mucosal surface when the luminal phase pH was 7.1. For comparison, an ileal measurement is presented showing the absence of any acid microclimate. In this typical control experiment, where Krebs-phosphate buffer was perfused, no change in surface pH was detected after 30 min perfusion indicating the stability of the intestinal preparation.

The mean jejunal surface pH of $6.24 \pm 0.02(30)$ consisted of tissues which were to be perfused with either control or test solutions. There were no significant differences in the surface pH at the onset of the perfusion period between treatment groups, and for this reason the initial values of all tissues are presented (Fig. 2) as a combined mean. When the mucosal surface was superfused with Krebs-phosphate buffer or buffer containing 1

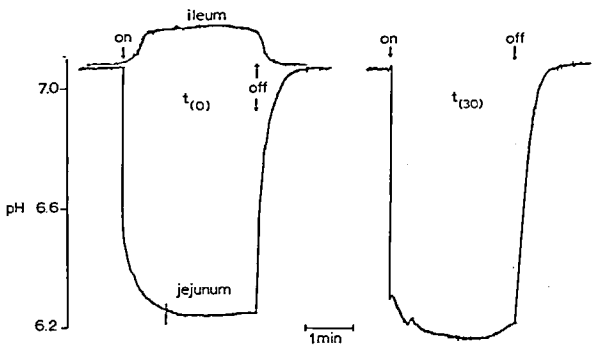

Fig. 1. A typical trace showing the change in measured surface pH when a pH electrode is placed on the surface (on) of rat proximal jejunum and then removed into the bulk phase (off) at the onset (t_0) and after (t_{30}) 30 min luminal superfusion with glucose-free Krebs-phosphate buffer. In this trace, in vivo estimates of jejunal are compared with ileal surface pH.

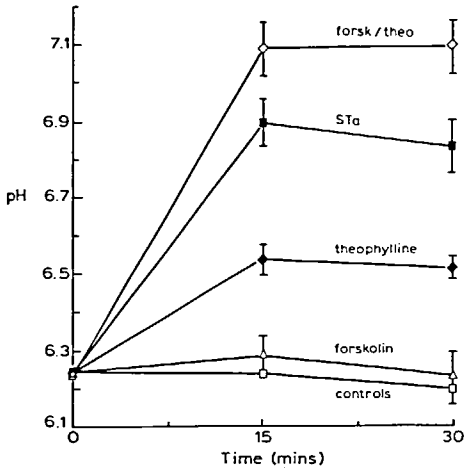

Fig. 2. The mean surface pH of rat proximal jejunum incubated in vivo for 30 min with Krebs-phosphate buffer alone or additionally containing 1 mM forskolin, 14 μg/ml *E. coli* heat-stable (STa) enterotoxin, 20 mM theophylline or a combination of 1 mM forskolin and 20 mM theophylline.

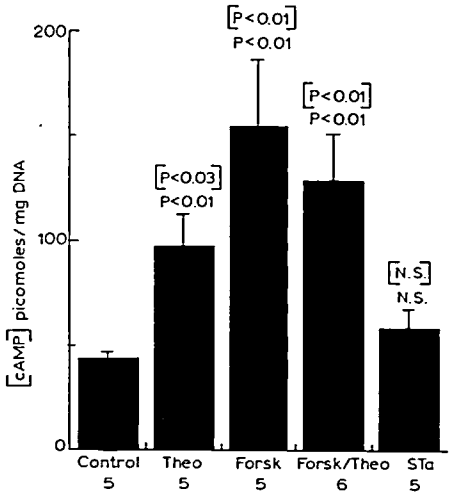

Fig. 3. The effect of perfusion in vivo with Krebs-phosphate buffer (no glucose) containing 20 mM theophylline (theo), 1 mM forskolin (forsk), a combination of 1 mM forskolin and 20 mM theophylline, 14 μg/ml of *E. coli* heat-stable enterotoxin (STa) or perfusion with buffer alone (control) on mucosal tissue cAMP content of the proximal jejunum in pmol per mg tissue DNA. Results are expressed as the mean plus S.E. with the number of in vivo animal experiments given at the base of each column. Statistical significance levels are given for normal distribution statistics with non-parametric levels in parenthesis.

mM forskolin, there was no significant change in the mean surface pH. In contrast, after 20 mM theophylline, the mean surface pH rose modestly, but significantly ($P < 0.001$) after 15 min to 6.54 ± 0.04(5) and remained elevated above control values. As previously reported [11], STa enterotoxin in the perfusate strikingly elevated surface pH ($P < 0.001$) to 6.90 ± 0.07(5) after 15 min as did the forskolin/theophylline combination ($P < 0.001$). These results show that STa enterotoxin and forskolin/theophylline effectively neutralise the acid microclimate, confirming previous findings [11] from a larger study not designed to investigate cyclic nucleotide levels.

Cyclic nucleotide levels

Cyclic nucleotide levels were standardised per tissue DNA content. This was preferred to standardisation with reference to sample protein con-

TABLE I

SURFACE pH, CYCLIC NUCLEOTIDE AND DNA CONTENT OF RAT PROXIMAL JEJUNUM

Results are shown after 30 min in vivo perfusion with Krebs-phosphate buffer alone or additionally containing either 20 mM theophylline, 1 mM forskolin, a combination of both theophylline and forskolin (F/T) or 14 μg/ml heat-stable *E. coli* enterotoxin (STa). Results are expressed as means ± S.E.M. with the number of observations (equals number of in vivo experiments, one observation per experiment) in parentheses. Statistical significance: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

	pH_{30}	cAMP (pmol/cm²)	cGMP (pmol/cm²)	DNA (mg)
Control	6.21 ± 0.01(9)	23.1 ± 1.8(9)	2.33 ± 0.41(8)	1.32 ± 0.04(5)
Theophylline	6.52 ± 0.03(5) ***	47.3 ± 5.0(5) ***	9.90 ± 1.99(5) ***	1.50 ± 0.11(5)
Forskolin	6.23 ± 0.07(5)n.s.	68.7 ± 12.9(5) ***	6.54 ± 0.89(5) ***	1.34 ± 0.08(5)
F/T	7.10 ± 0.07(6) ***	58.8 ± 11.2(6) **	8.53 ± 2.70(6) *	1.36 ± 0.10(5)
STa	6.80 ± 0.07(5) ***	30.0 ± 3.5(5)n.s.	5.89 ± 0.89(6) **	1.61 ± 0.20(5)

tent, because of the in vivo nature of the experiment. Altered rates of mucus production or changes in blood flow and, hence, blood content within the tissue sample could occur after experimental treatment. In contrast, these factors would not alter DNA content appreciably, since blood and mucus contain much protein but little DNA. The estimated DNA content did not vary between the tissue samples (Table I) and was consistent with reported values [24].

When cyclic AMP levels were standardised per mg DNA (Fig. 3), the pattern of values corresponded exactly to those standardised per mucosal area (Table I). In particular, cAMP levels at 57.6 $\pm$ 9.7(5) pmol/mg DNA after STa enterotoxin exposure resembled the control levels of 43.5 $\pm$ 3.2(5). In contrast, theophylline significantly ($P <$ 0.01) elevated levels to 96.9 $\pm$ 15.1(5), forskolin significantly ($P < 0.01$) elevated levels to 153.9 $\pm$ 32.5(5) and the forskolin/theophylline combination significantly elevated levels to 128.0 $\pm$ 23.2(5) pmol cAMP/mg DNA. The combination of forskolin and theophylline did not significantly elevate cAMP levels above those detected in the presence of either agent alone. When cyclic GMP levels were investigated, values expressed per mg DNA were identical to values normalised per area (Table I). STa enterotoxin, as expected, doubled the cGMP content (Fig. 4) significantly ($P < 0.03$) from 4.95 $\pm$ 1.27(5) to 11.20 $\pm$ 2.10(5) pmol/mg DNA. Theophylline alone significantly ($P < 0.01$) elevated cGMP levels to 20.3 $\pm$ 4.4(5) and forskolin alone significantly ($P < 0.01$) elevated cGMP levels to 14.6 $\pm$ 2.3(5) pmol/mg DNA. The increase to levels of 17.1 $\pm$ 4.1(5) pmol/mg DNA in cyclic GMP after forskolin/theophylline treatment was significantly higher ($P < 0.03$) than control values, but did not differ from the effect of forskolin or theophylline given separately.

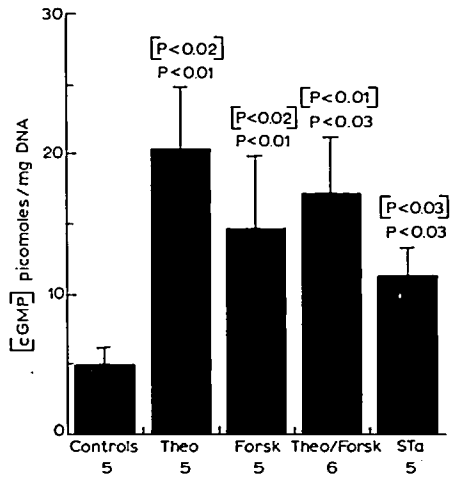

Fig. 4. The effect of in vivo perfusion with Krebs-phosphate buffer (no glucose) containing 20 M theophylline (theo), 1 mM forskolin (forsk), a combination of 1 mM forskolin and 20 mM theophylline 14 μg/ml of *E. coli* heat-stable enterotoxin (STa) or perfusion with buffer alone (control) on mucosal tissue cGMP content of proximal jejunum in pmol per mg tissue DNA. Results are expressed as the mean plus S.E. with the number of in vivo animal experiments given at the base of each column. Statistical significance levels are given for normal distribution statistics with nonparametric levels in parenthesis.

Discussion

The striking effect of *E. coli* heat-stable (STa) enterotoxin on the acid microclimate, raises the question of through which second messenger system STa enterotoxin acts in order to neutralise the surface pH. Until recently, few observations had been made of the effect of secretory agents and cyclic nucleotides on mucosal surface pH, apart from a minor study indicating that dibutyryl cAMP could modestly elevate the surface pH in vitro [25]. Since then, two larger studies have indicated that the maintenance of surface pH is mediated through cyclic nucleotides [11,26]. The neutralisation of the acid microclimate by STa enterotoxin, which elevates intracellular cGMP but not cAMP levels [22,23], is also achieved by perfusion with 8-bromo-cGMP [11]. Yet cholera toxin, 8-bromo-cAMP and forskolin have only moderate effects on surface pH. Since the greatest effects were achieved with cGMP-acting agents, it was proposed that maintenance of the acid microclimate was disturbed predominantly by changes in intracellular cGMP levels, possibly by influencing Na^+–H^+ exchange and that only minor modulation of surface pH could be caused by changes in the levels of cAMP [11].

A recent Japanese study presented findings consistent with the minor role of changes in intracellular cAMP levels. Using pH microelectrodes to measure jejunal mucosal surface pH in vitro, similar values for the acid microclimate have

been detected [4,26]. Low concentrations of both dibutyryl cAMP and dibutyryl cGMP elevated the surface pH (in the presence of sodium ion-containing buffers only) by about 0.3 pH units, i.e., much more modestly than after STa enterotoxin treatment. Theophylline and prostaglandin E_2 similarly elevated jejunal surface pH but the calcium ion ionophore A23187 was ineffective, as others have found (Daniel, H., personal communication). It was concluded that both cAMP and cGMP regulate surface pH, probably by controlling the rate of Na^+–H^+ exchange which is sensitive to amiloride [35], a compound which can elevate surface pH [7]. It seems likely that on perfusion with higher concentrations of nucleotides for longer than 5 min, the same differential effect of cAMP and cGMP would have been detected. Indeed, when one compares these results with those obtained in this laboratory, it is evident that over a short incubation period both nucleotides elevate surface pH to the same extent, but that cGMP continues after 5 min to exert a larger effect on surface pH (see Fig. 6 in Ref. 11).

In this study, STa enterotoxin, while neutralising the acid microclimate, elevates cGMP, but not cyclic AMP levels. Conversely, the adenylate cyclase activator forskolin elevates cAMP, but has no effect on surface pH. It therefore, seems unlikely that the dramatic effect of STa enterotoxin on surface pH is mediated through altered cAMP levels. A curious aspect of previous work [11] was that when combined with theophylline, the cyclic AMP agonists cholera toxin and forskolin did manage to neutralise the acid microclimate. In the present study, the combination of forskolin and theophylline increased cAMP levels as expected, but also elevated cGMP levels, consistent with a role for that nucleotide in influencing surface pH. The forskolin/theophylline combination did not further increase cAMP to levels above those seen when these agents were given separately. For this reason, the idea of acid microclimate neutralisation being caused by supramaximal levels of cAMP induced by the combination of forskolin and theophylline can also be excluded. Since STa enterotoxin acting exclusively through cGMP and externally applied cGMP neutralise the microclimate, it is evident that cGMP is the principle second messenger causing changes in the system which maintains a low surface pH.

This view requires some refinement, since forskolin alone elevated cGMP levels with little effect on surface pH. The concentrations used were high, but seem to be required in vivo unlike in in vitro experiments (R. Bridges, personal communication). The dissolving vehicle for forskolin was ethanol, but the final concentration to which tissues were exposed was 0.5%, far less than that required to elevate surface pH [25]. This discrepant effect of forskolin may be explained by the division of enzyme activity [27] into a soluble cytosolic fraction and a particulate membrane fraction (through which STa enterotoxin exclusively acts). Some substances act specifically on one pool only, e.g., sodium nitroprusside on cytosolic guanylate cyclase. If the forskolin/theophylline combination acts on particulate guanylate cyclase, whereas forskolin alone does not, then this may explain the resemblance of forskolin/theophylline to STa enterotoxin in its effects on surface pH. However, the elucidation of the detailed involvement of particulate guanylate cyclase and its putative connection with Na^+–H^+ exchange awaits further study.

The present findings also have implications for the interpretation of previous work, in which theophylline was used in combination with bacterial enterotoxins, pre-treated or diseased tissue. When theophylline is used in combination with cholera toxin, net fluid secretion increases above the level detected with cholera toxin alone [28]. Invasion by *Salmonella* species [29] or transmissible gastroenteritis [30] changes undirectional sodium and chloride fluxes which can be further altered by theophylline addition [31,32]. Prostaglandin E_1 which is known to increase cAMP levels [33] is occasionaly used in combination with theophylline [34]. In all these cases, the combination of theophylline with agents which may elevate cAMP levels on their own, may, nevertheless, be altering cGMP levels, as the forskolin/theophylline combination was shown to do. This renders pharmacological experiments which attempt to reduce net fluid or sodium chloride secretion difficult to interpret.

A simplified view of intestinal acid-base movement in the rat and its control by second messenger systems is that cyclic GMP modulates Na^+–H^+ exchange in the proximal jejunum and that cAMP modulates anion exchange. Sodium–

hydrogen ion exchange processes seem to be present along the length of the intestine [35] whereas bicarbonate–chloride ion exchange appears more distally. The mediation of HCO_3^-–Cl^- exchange by cAMP would explain why cAMP angonists have only a minor or no effect on surface pH in the jejunum. STa enterotoxin, acting through cGMP, prevents Na^+–H^+ exchange, but does not elevate surface pH above luminal pH values, which is what might be expected if anion secretion were promoted. In the ileum in which HCO_3^-–Cl^- exchange is present, STa enterotoxin, by inhibiting Na^+–H^+ exchange, unmasks the anion exchanger, resulting in mucosal alkalinisation. It may be anticipated that cAMP agonists would affect ileal surface pH and that the ability to detect cAMP-mediated surface pH change reflects the extent to which tissue samples are taken from regions other than the immediately proximal jejunum.

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