THE EFFECT OF BACTERIAL ENTEROTOXINS ON INTESTINAL WEAK ELECTROLYTE ABSORPTION

by

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DECLARATION

This thesis comprises my own original research. No part of this work has previously been submitted as a thesis in any form.

SUMMARY

Weakly dissociable compounds partition into lipid membranes by non-ionic diffusion. The 'microclimate hypothesis' predicts that the rate of weak electrolyte absorption from the small intestine is determined by the pH prevailing at the mucosal surface. This pH will determine the proportion of unionised:ionised species presented to the brush border membrane. In rat and human jejunum, the mucosal surface pH is significantly lower than neutral luminal fluid and is relatively resistant to bulk pH changes. This tends to favour weak acid absorption but hinders the uptake of weak bases. There are indications that some types of small bowel derangement might disrupt the mechanisms maintaining this low mucosal surface pH, or 'acid microclimate' resulting in its dissipation. If the microclimate hypothesis applies, any change in the mucosal surface pH should be accompanied by alterations to weak electrolyte absorption. To test this the present study undertook to find a method of altering the intestinal microclimate pH in rats *in vivo* with a view to investigating the effects of a surface pH shift on the absorption of a number of weakly dissociable drugs.

Using an *in vivo* perfusion chamber, the pH at the intestinal mucosal surface could be measured with ease confirming previous findings that a low pH exists in the jejunum while a neutral value was observed in the ileum. Measurements with liquid ion exchange (LIX) pH microelectrodes *in vitro* demonstrated that a pH gradient exists along jejunal but not ileal villi. In the

jejunum, the lowest pH values were detected in the upper villus region below the villus tips. Exposing the intestinal mucosa to E.coli STa enterotoxin or its synthetic, peptide analogue, STh(6-19), resulted in a rapid, reversible elevation of the intestinal microclimate pH to values significantly higher than those measured in unchallenged tissue. In the jejunum, this alkalinisation was most prominent in the upper villus region which had previously demonstrated the lowest pH values. The observation that 8-bromo cGMP has very similar effects on mucosal surface pH to STa suggests that this mucosal surface alkalinisation is mediated through cGMP. The additional finding that theophylline, a phosphodiesterase inhibitor, prevented the surface pH from returning to control values after enterotoxin removal provided further evidence for this. The 8-bromo analogue of cAMP induced a considerably lesser surface pH elevation than its cGMP counterpart. Forskolin and cholera toxin, both potent adenylate cyclase activators, ellicited similar small intestinal surface pH elevations. This suggests that cGMP-dependent secretory processes are more capable of inducing surface pH changes than those dependent on cAMP. However, in the presence of theophylline both forskolin and cholera toxin elevated the mucosal surface pH to values similar to those attained after STa challenge. Theophylline alone had no effect on the mucosal surface pH.

Since STa was the most potent and reliable effector of surface pH changes it was used as a tool to test the microclimate

hypothesis. Solutions of five weakly dissociable drugs were perfused through *in vivo* loops of rat jejunum in the presence or absence of STa. Two weak acids (salicylic acid and phenytoin) and three weak bases (amphetamine, morphine and lignocaine) were investigated. In these experiments STa reduced the absorption of both weak acids while enhancing the absorption of all three weak bases. These changes were confirmed by similar alterations to drug appearance in the peripheral blood indicating that total drug transfer was affected by STa. Enhanced weak base absorption was despite unfavourable net fluid secretion. These results are in accordance with prediction using the microclimate hypothesis in that the alterations to weak electrolyte absorption are consistent with an STa-induced elevation of the intestinal microclimate pH. This conclusion was strengthened by the observation that a combination of forskolin and theophylline, which had very similar effects to STa on mucosal surface pH also caused a similar enhancement of lignocaine absorption. Therefore the present experiments provide evidence in favour of the microclimate hypothesis, that the microclimate pH is a primary determinant of weak electrolyte absorption rate. The similarities between rat and human intestine suggest that these observations may have direct relevence to considerations of drug absorption in human small intestinal disease.

ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
cAMP	Adenosine 3':5'-cyclic monophosphate
cGMP	Guanosine 3':5'-cyclic monophosphate
DDS	Dichlorodimethylsilane
ESCR	External standard channels ratio
Km	Michaelis-Menten constant
LIX	Liquid ion exchange
log P	Logarithm of octanol:water partition coefficient
LT	Heat-labile enterotoxin of Escherichia coli
mw	Molecular weight
PCO_2	Partial pressure of carbon dioxide
PEG	Polyethylene glycol
pH	Negative logarithm of hydrogen ion activity
pKa	Negative logarithm of dissociation constant
PO_2	Partial pressure of oxygen
STa	Heat-stable enterotoxin of Escherichia coli
TDDA	Tri-n-dodecylamine

1 INTRODUCTION

1.1 The pH partition hypothesis

All orally administered drugs encounter one common problem which must be overcome if there is to be benefit from their specific therapeutic effects. This problem is one of traversing the gastrointestinal epithelium. The mucosal membrane of this epithelium is essentially lipoid in nature and hence, within limits, the more lipophilic a molecule is, the better it will permeate this barrier. This feature of cell permeation was first recognised eighty five years ago by Overton (1902). Later work (Hober & Hober, 1937) specifically demonstrated the importance of lipophilicity for the absorption of foreign organic compounds from the intestinal lumen.

Many drugs are weak electrolytes and therefore, depending on the pH of the medium and the dissociation constant of the compound, exist either as the ionised or unionised species. The unionised species of a weakly dissociable compound is more lipid soluble than the ionised species since the latter forms a hydration shell rendering it lipophobic. The result of this is that it is only in the undissociated state that organic weak electrolytes can partition into the cell membrane.

The first indication of this was a report (Meltzer, 1896) that the weakly basic strychnine was not toxic when it was administered into the stomachs of rabbits whose pylorus had been ligated. However, with the pylorus open the rabbits quickly died.

Later, Travell (1940) noted that the absorption rate of several weak bases varied with the pH of the stomach contents. From this it was concluded that the rate of penetration of a weak base through the cell membrane varies in general with the concentration of undissociated weak base in solution. By investigating the absorption characteristics of several weakly acidic and basic drugs from rat stomach (Schanker, Shore, Brodie & Hogben, 1957) it was demonstrated that the dissociation constant of the drug is an important determinant of the gastric drug absorption rate. From this study and from another on drug absorption from the human stomach (Hogben, Schanker, Tocco & Brodie, 1957) the conclusion arose that drugs traverse the gastric epithelium by passive diffusion of their lipid soluble undissociated form. This permeation of weak electrolytes across lipid membranes is known as 'non-ionic diffusion' (Milne, Scribner & Crawford, 1958).

A consequence of non-ionic diffusion is the 'pH partition hypothesis' (Shore, Brodie & Hogben, 1957). This assumes that there is negligible permeability to the ionised species and hence the pH of the medium and the dissociation constant of the molecule will determine the concentration of the undissociated species available to partition into the membrane. Provided there is a pH difference across a membrane, weak acids or bases will be concentrated on one side. This concentration gradient will have occurred by a purely physicochemical diffusion process since it is only dependent on the greater permeability of the membrane to

the unionised fraction. No active transport process and no energy other than that required to maintain the hydrogen ion gradient is involved. Weak bases would tend to accumulate in the more acidic medium, weak acids in the alkaline medium.

1.2 The microclimate hypothesis

Due to its extremely large surface area, the small intestine is the major site for drug absorption along the gastrointestinal tract. When the drug absorption studies described in the previous section were extended to the rat small intestine (Schanker, Tocco, Brodie & Hogben, 1958) it was observed that the absorption of weakly dissociable drugs from the small intestine also conformed to the pH partition hypothesis, the rate of absorption being related to the dissociation constant of the drug. However, it was noted that relatively strong acids such as salicylic and benzoic acid were absorbed at faster rates than would be predicted by their dissociation constants. This was difficult to reconcile with the pH partition hypothesis since, at the pH of the intestinal contents these compounds would be predominantly in the ionised form. Such anomalies led the investigators to predict the existence of a zone at the intestinal mucosal surface which is more acidic than the bulk phase of the luminal contents (Hogben, Tocco, Brodie & Schanker, 1959). The pH of this zone, or 'acid microclimate' was proposed to be, within limits, independent of the pH of the bulk solution in the lumen. A value of pH 5.3 at the mucosal surface of the upper small intestine was

calculated to explain the findings at the time, namely that weak acids with pKa values below 3 and weak bases with pKa values above 7.8 were not absorbed.

This hypothesis, whereby the rate of absorption of organic weak electrolytes is determined by the pH prevailing at the absorptive surface rather than the pH of the bulk solution is known as the 'microclimate hypothesis'. In the subsequent sections, evidence in favour of the microclimate hypothesis will be presented along with other alternative hypotheses for the intestinal absorption of weak electrolytes.

1.3 Acid-base changes in the small intestine

The maintenance of a region of low pH at the mucosal surface of the jejunum would require a local secretion of hydrogen ions or conversely absorption of bicarbonate anions. A consequence of either of these situations would be acidification of the luminal contents. The first indication of this came from early studies looking for a possible link between rickets and intestinal acid-base balance. It was observed that the luminal contents of the proximal small intestine of rats (Redman, Willimot & Wokes, 1927; McRobert, 1928; Oser, 1928) and dog (Grayzel & Miller, 1927; Graham & Emery, 1928) were more acid than those of the distal small intestine. This pH differential between jejunum and ileum was later associated with a high PCO_2 in the jejunum and a low PCO_2 in the ileum of the dog (Robinson, Luckey & Mills, 1943).

Experiments on everted sacs of rat jejunum (Wilson, 1953; Wilson, 1956) demonstrated that the observed fall in luminal pH was due to a net transport of bicarbonate into the serosal compartment where a rise in pH was noted. This was confirmed by the findings of an *in vivo* study (Parsons, 1956) which examined changes in pH and PCO_2 on perfusing bicarbonate-saline solutions through rat small intestine *in situ*. As before the jejunum acidified its luminal perfusates whilst absorbing bicarbonate, whereas bicarbonate accumulated in the ileal lumen and the perfusate remained alkaline. It was proposed that bicarbonate absorption in the jejunum occurs as a consequence of hydrogen ion secretion into the lumen, the hydrogen ions reacting with the bicarbonate to liberate water and CO_2 which would then diffuse back into the blood stream. This hypothesis has in its favoure the high CO_2 levels observed in the jejunum.

It was noted that when the intestine was was pretreated with a carbonic anhydrase inhibitor (Parsons, 1956) luminal acidification was reduced in parallel with the absorption of Na^+, Cl^- and fluid. This suggested the possibility that hydrogen ion secretion may be linked to Na^+ absorption. This Na^+-H^+ exchange process was also proposed by Wilson & Kazyak (1957), who suggested the hydrolysis of water as a possible source of hydrogen ions. Evidence for an electro-neutral Na^+-H^+ exchange process was supplied by studies on the mechanisms of sodium and bicarbonate absorption in the human small intestine (Turnberg, Fordtran, Carter & Rector, 1970). A Na^+-H^+ exchange mechanism has

also been demonstrated in the rat jejunum *in vivo* (Hubel, 1973; Podesta & Mettrick, 1977). In these studies bicarbonate was shown to stimulate Na^+ absorption while Na^+ deletion inhibited bicarbonate absorption, reduced luminal acidification and lowered luminal PCO_2 levels. This was also demonstrated in the rat jejunum *in vitro* (Lucas, 1976). Additional evidence for Na^+-H^+ exchange came from studies on brush border membrane vesicles prepared from rat small intestine (Murer, Hopfer & Kinne, 1976; Liedtke & Hopfer, 1977). In these experiments, when sodium was added to the outside solution protons were ejected from the vesicles. Additionally, the creation of a pH gradient across the vesicles ($pH_{in}<pH_{out}$) was shown to stimulate sodium accumulation in the vesicles. An alternative to this mechanism for electro-neutral hydrogen ion secretion has been proposed in which K^+ rather than Na^+ is exchanged for hydrogen ions (Lucas, 1976). Aminophylline, a phosphodiesterase inhibitor, reduced luminal acidification in the jejunum while causing a reversal of K^+ transport. However there was no discernible effect of aminophylline on Na^+ flux.

Studies on everted sacs of rat jejunum (Blair, Lucas & Matty, 1975) demonstrated that acidification can occur in the absence of glucose but is increased when the glucose concentration exceeds 1mM. However acidification is not a consequence of the active transport of hexoses since galactose and 3-O-methyl glucose, which are both actively transported but not metabolised, had no effect on acidification. It has been proposed (Blair et al.,1975)

that acidification is due to the absorption of metabolisable sugars since mannose, which is not actively transported, increased acidification. Exogenously applied ATP induced the largest increase in acidification of all agents tested despite the fact that ATP was shown not to enter the intestinal cells (Blair et al., 1975). These findings led to the conclusion that acidification in the jejunum occurs as a result of the hydrolysis of ATP produced from the aerobic metabolism of sugars.

1.4 Intestinal surface pH studies

The above acidification studies provided indirect evidence for the existence of an acid microclimate in the jejunum, however, the first direct evidence came from measurements of the mucosal surface pH of everted sacs of rat jejunum using pH-microelectrodes (Lucas, Schneider, Haberich & Blair, 1975). In this study when the pH of the incubation medium was 7.14 the pH at the intestinal mucosal surface was 6.30. This acid pH region was extracellular and was not due to cell penetration by the electrode since tangential measurements also yielded acid values. The finding of an acid microclimate in the rat jejunum in vitro has since been confirmed in several studies using pH-microlectrodes (Rechkemmer, Wahl, Kuschinsky & von Engelhardt, 1978; Daniel, Neugebauer, Kratz & Rehner, 1985; Shimada, 1987) and also using larger surface electrodes when cell penetration is no longer feasible (Lucas & Blair, 1978; Lucas, Lei & Blair, 1980; Shiau, Fernandez, Jackson & McMonagle, 1985; Daniel &

Rehner, 1986). An acid mucosal surface in in vitro rat jejunum has also been demonstrated using pH sensitive dyes (Shiau et al., 1985) although a previous attempt to demonstrate this failed (Lucas et al., 1975).

The presence of D-glucose in the incubation medium has consistently been shown to lower the microclimate pH in vitro (Lucas & Blair, 1978; Lucas et al., 1980; Daniel & Rehner, 1986; Shimada, 1987). A Lineweaver-Burk plot of the change in surface pH with glucose concentration demonstrated an apparent Km of 2mM (Lucas et al., 1980). This was very similar to the Km obtained for glucose-enhanced luminal acidification in the jejunum (Blair et al., 1975). Similar to luminal acidification (Blair et al., 1975) it has been shown that the metabolisable sugars fructose and mannose, as well as glucose, lower the surface pH in vitro whereas the non-metabolisable sugars galactose and 3-O-methyl glucose have no effect (Blair, Hilburn, Lucas & Said, 1982; Shiau et al., 1985; Daniel & Rehner, 1986). Intragastric and intravenous administration of glucose, fructose and mannose was also shown to lower surface pH in the jejunum (Daniel & Rehner, 1986). This would suggest that the low surface pH in the jejunum is related to sugar metabolism. It has been suggested that hydrolysis of the ATP produced by the aerobic metabolism of these sugars could be a source of hydrogen ions which would diffuse out into the microclimate region. The presence of a mucus layer at the mucosal surface probably helps to maintain the low surface pH in the jejunum by providing a resistance to the diffusion of

hydrogen ions (Blair et al., 1975). Evidence for this was provided by the finding that DL-dithiothreitol, a drug which is known to reduce surface mucus (Lucas & Cannon, 1983), elevated the rat jejunal surface pH in vitro (Shimada, 1987). Reducing the mucus layer by spinning also elevates the jejunal surface pH (Shiau et al., 1985). This latter finding led the investigators to propose a mechanism for the maintenance of an acid microclimate in the jejunum in which hydrogen ion secretion plays no part. Instead, it was postulated that the low mucosal surface pH is maintained by the ampholyte properties of mucus. Mucus is positively charged at low pH and negatively charged at high pH. Therefore, depending on the pH of the bulk solution in the lumen, hydrogen ions will either be attracted or repelled by the surface-adhering mucus layer thereby maintaining the pH relatively constant. Lowering of the microclimate pH in the presence of glucose was explained by increased mucus production. A recent finding that glucose did not cause a thickening of the microclimate region despite lowering the pH (Shimada, 1987) is inconsistent with glucose increasing mucus production. Therefore, although conclusive evidence is lacking, it would appear that while mucus probably does play an important role in helping to maintain a low surface pH, a source of hydrogen ions is probably also necessary.

It has been suggested that an acid surface pH (or luminal acidification in general) is a physiological artefact of tissue anoxia (Flemstrom, Nylander, Hurst & Heylings, 1982). This was disproved by the observation that when anoxia was induced in *in vitro* preparations the surface pH was elevated (Lucas et al., 1980). Therefore an anoxic preparation has an alkaline surface rather than an acidic one. However it is difficult to prove conclusively that an *in vitro* preparation is receiving a full oxygen supply. This doubt was dispelled by the measurement of a low mucosal surface pH in rat jejunum *in vivo* (Lucas, 1983; Hogerle & Winne, 1983; Iwatsubo, Yuasa, Iga & Hanano, 1986) when the tissue has a full blood supply. In these preparations anoxia was out of the question and indeed, when anoxia was induced deliberately by occluding the afferent blood vessels supplying the tissue (Lucas, 1983) the surface pH became alkaline as in the previous *in vitro* study (Lucas et al., 1980). When glucose was administered to the anoxic tissue the acid surface pH returned (Lucas, 1983). However, applying glucose to healthy *in vivo* tissue had no significant effect on surface pH (Lucas, 1983; Iwatsubo et al., 1986). It was proposed that the *in vivo* preparation would be receiving a full supply of glucose from the systemic circulation and hence adding more glucose would have no additional effect. On the other hand, when the tissue is made anoxic it becomes akin to an *in vitro* preparation: the presence of glucose, therefore, maintains the low surface pH (Lucas & Blair, 1978; Lucas et al., 1980; Daniel & Rehner, 1986; Shimada,

1987).

There is evidence for an electroneutral Na+-H+ exchange process in the rat jejunum (Hubel, 1973; Lucas, 1976; Murer et al., 1976; Podesta & Mettrick, 1977; Cassano et al., 1984). It has been suggested that this process is the mechanism for the secretion of hydrogen ions into the microclimate region. As well as the above evidence from acidification studies it has been shown that reducing the sodium concentration in the bulk solution elevates the mucosal surface pH *in vitro* (Lucas et al., 1980; Shimada, 1987) and *in vivo* (Iwatsubo et al., 1986). However one study (Shiau et al., 1985) has failed to show any effect of sodium concentration on surface pH *in vitro*. Additionally it has been shown that ouabain (Lucas & Blair, 1978; Iwatsubo et al., 1986), amiloride and chlorpromazine (Iwatsubo et al., 1986) elevate jejunal surface pH. Since all of these treatments affect sodium transport it was concluded that this was evidence for Na+-H+ exchange being a source of protons for the jejunal acid microclimate. However, an alternative hypothesis, also invoking sodium, has been proposed to account for the generation of hydrogen ions. The presence of hydrogen ions in the microclimate region is attributed to lactate derived from glycolysis (Daniel & Rehner, 1986). It is proposed that a small proportion of this metabolite, existing as lactic acid, penetrates the brush border membrane. The lactic acid would then dissociate to give lactate and hydrogen ions. The lactate is reabsorbed by an electroneutral lactate-Na+ cotransport system which has been demonstrated in the

brush border membrane of small intestinal epithelial cells (Hildemann, Haase, Barac-Nieto & Murer, 1980). The protons would accumulate in the microclimate region. In support of this an extensive lactate transport by jejunal epithelial cells has been observed (Wilson & Wiseman, 1954) and it was postulated that hydrogen ions are eliminated mucosally and lactate serosally. However, another study (Blair et al., 1975) has demonstrated that only a small percentage of the hydrogen ions secreted into the jejunal lumen can be attributed to lactate.

In all of these studies the elevated surface pH was still significantly lower than the bulk solution pH inferring that there must be some other source of hydrogen ions. An ATP driven H+ pump has been demonstrated in renal brush border membranes (Kinne-Saffran, 1985). It is unknown whether this type of proton pump exists in the intestinal brush border however the observation that mucosally administered ATP causes a large increase in luminal acidification in the rat jejunum (Lucas & Blair, 1978) is consistent with the existence of such a pump.

Whatever mechanism might be responsible for producing luminally directed hydrogen ions, a recent study (Daniel et al., 1985) has localised the site of the acid microclimate in the jejunum to the upper third part of the villus, in the region just below the villus tip. Using antimony microelectrodes a pH gradient was demonstrated along the jejunal villus-crypt axis, alkaline values being measured in the crypt region and the pH falling to a

minimum approximately 50um below the villus tip. Another recent study (Shimada, 1987) using liquid ion exchange pH microelectrodes failed to demonstrate a difference in pH between the crypt and the villus tip. However it is unclear if the position of the electrode tip was observed in this latter study.

The phosphodiesterase inhibitor, aminophylline, caused an elevation of rat jejunal surface pH in vitro to near neutral values (Lucas & Blair, 1978). It was proposed that elevated intracellular cAMP levels causing back diffusion of hydrogen ions into the cell might be the reason for this. However, aminophylline inhibits both adenylate and guanylate cyclase (Quill & Weiser, 1975) and therefore cGMP may also have caused the observed mucosal alkalinisation. Also, since several bacterial enterotoxins eg cholera toxin, E.coli heat labile enterotoxin and E.coli heat stable enterotoxin are known to elevate intracellular cyclic nucleotide levels there is a possibility that these agents may elevate the microclimate pH.

There is also direct evidence for the existence of an acid microclimate in the human jejunum. This was first obtained from measurements on human jejunal biopsy samples in vitro using miniaturised pH electrodes (Lucas & Blair, 1978; Lucas, Cooper, Lei, Holmes, Johnson, Blair & Cooke, 1978; Kitis, Lucas, Bishop, Sargent, Schneider, Blair & Allan, 1982). These studies demonstrated that, like the rat, the human jejunum maintains an acid surface pH in vitro when incubated in neutral buffer

solutions. The values obtained were very similar to those obtained in the rat. Confirmation of this acid surface pH in human jejunum has been supplied by the results of a recent study where the human jejunal surface pH was measured *in vivo* (Rawlings, Lucas & Russel, 1987). Using specially constructed plastic pH electrodes (Rawlings & Lucas, 1985) attached to a Crosby jejunal biopsy capsule the pH at the mucosal surface could be easily measured *in situ*. As with the rat studies described above these *in vivo* measurements dispelled any doubts as to the validility of measurements on isolated tissue, the values obtained being consistent with the values measured *in vitro*.

Although the bulk of surface pH studies stem from the rat jejunum, measurements of ileal and colonic surface pH have also been reported. In the rat ileum *in vivo* the pH at the mucosal surface was 7.2 (Lucas, 1983) whereas the colonic mucosa had a pH of 6.7 (McNeil, Ling & Wager, 1987). As a comparison between omnivores and herbivores the mucosal surface pH of *in vivo* guinea pig proximal jejunum was shown to be slightly alkaline (Lucas, 1983). This clearly indicates that there may be a difference between the microclimate pH of omnivores and herbivores.

1.5 Alternatives to the microclimate hypothesis

(i) The alkaline microclimate

Before direct evidence for the existence of an acidic microclimate in the jejunum had become available, an alternative hypothesis was put forward to explain deviations from the pH partition theory. In this system an alkaline compartment located in the subepithelial extracellular space was proposed to account for anomalies in intestinal weak electrolyte absorption (Jackson, Shiau, Bane & Fox, 1974). Understanding that an intermediate compartment need only have a different pH to that of the two end compartments for alterations in weak electrolyte transport to occur, Jackson (1974) derived a three compartment mathematical model with which to describe weak electrolyte permeation across the intestinal epithelium. In this model three aqueous compartments are arranged in series. The intermediate compartment has a different pH to that of the end compartments. This pH difference provides the driving force for the net transport of weak electrolytes from one end compartment to the other. The direction of this transport is determined by the relative permeabilities of the two membranes bounding the intermediate compartment to the ionised and unionised species of the particular weak electrolyte. The flux ratio between the two end compartments was expressed by the follwing equation (Jackson et al., 1974).

$$\frac{J_{ms}}{J_{sm}} = \frac{(1 + (P_i/P_{ni})_I 10^{\alpha 1})(1 + (P_i/P_{ni})_{II} 10^{\alpha 2})}{(1 + (P_i/P_{ni})_I 10^{\alpha 2})(1 + (P_i/P_{ni})_{II} 10^{\alpha 1})}$$

where J_{ms} = mucosal-to-serosal flux

J_{sm} = serosal-to-mucosal flux

$\alpha 1 = (pH_1 - pKa)$; $\alpha 2 = (pH_2 - pKa)$

P_i = permeability to ionised form

P_{ni} = permeability to unionised form

pH_1 and pH_2 refer to the end and intermediate compartment pH

pKa is that of the molecule under investigation

the subscripts I and II refer to the first and second membrane of the model

Applying this model to a series of flux ratio measurements from in vitro barbiturate absorption experiments (Jackson & Airall, 1978) it was reported that there was a unique solution to the model and that the best fit for the data was achieved when a pH value of 8.1 was assigned to the intermediate compartment. The observation that changing the bulk solution pH had no effect on the absorption (Jackson & Airall, 1978) rate led the investigators to conclude that the pH at the mucosa could not be controlling weak electrolyte permeation and that the intermediate compartment must be located in the subepithelial space. However, the existence of an extracellular microclimate which is resistant to changes in buffer pH (Lucas et al., 1980; Hogerle & Winne, 1983; Shiau et al., 1985; Iwatsubo et al., 1986) would account for the absorption rate remaining unchanged. Utilising the same data set, Lucas (1984) demonstrated that the above model does not

have a unique solution and that a wide range of values for the pH of the intermediate compartment, both acid and alkaline, are equally probable. The previously estimated intermediate compartment pH of 8.1 was shown not to provide the least squares best fit for the data, the residual sum of squares for this value being greater than the minimum. In the light of this evidence and also in view of the evidence for the existence of of an acid microclimate in the jejunum the alkaline subepithelial compartment hypothesis has very little in its favour.

(ii) Ionised permeation

The main condition of the pH partition hypothesis is that the ionised species of weak electrolytes will not traverse the intestinal epithelium to any great extent. However ionised permeation has been postulated as an alternative to the microclimate hypothesis to explain experimentally observed deviations from the pH partition theory (Nogami & Matsuzawa, 1961; Nogami & Matsuzawa, 1962; Crouthamel, Tan, Dittert & Doluisio, 1971; Bridges, Parke, Shillingford & Upshill, 1976). In these studies, drug absorption was investigated over a range of pH values, the bulk solution pH being used to determine the ratio of the unionised to the ionised species presented at the intestinal surface for absorption. The existence of an acid microclimate, however, makes this type of analysis futile since the bulk pH would not necessarily be that prevailing at the mucosal surface. In fact the deviations from the pH partition

theory observed in these studies can be explained if a microclimate is taken into consideration. Hence if, as the current evidence suggests, a microclimate does exist, there is little evidence for ionised permeation across the cell membrane.

The intestinal epithelium is known to be a 'leaky' tissue and therefore the paracellular pathway through the 'tight junctions' should be considered as a possible route for ionised drug permeation. However, even taking this into consideration, on the basis of in vitro unidirectional flux ratio measurements in the rat small intestine, the permeability of the ionised form has been shown to be extremely low, less than 1/1000 that of the ionised form (Jackson & Airall, 1978). Therefore the role of ionised permeation in the overall drug absorption process can probably be regarded as being of little consequence.

There is, however, a possibility of drug molecules being entrained in the fluid flux through paracellular aqueous channels. Since fluid can be either absorbed or secreted by the intestinal epithelium changes in net fluid transport could alter the rate of drug absorption. This will depend largely on the size of the individual molecule, smaller molecules being affected to a greater extent. Evidence for fluid entrainment, or solvent drag, affecting intestinal drug absorption has been presented for the weak acids, salicylic acid and benzoic acid (Ochsenfahrt & Winne, 1974; Karino, Hayashi, Horie, Awazu & Hanano, 1982; Karino, Hayashi, Awazu & Hanano, 1982) and the weak bases, antipyrine and

amidopyrine (Ochsenfahrt & Winne, 1974; Karino et al., 1982; Karino et al., 1982). However, it should be noted that these are relatively small drug molecules. A larger drug molecule, cephalothin, was unaffected by changes in fluid movement (Karino et al., 1982). Therefore, although fluid entrainment will affect drug absorption it is probably only important for small molecules.

(iv) Unstirred water layer

A stagnant or unstirred water layer exists at the mucosal membrane of the intestinal lumen (Dietschy, Sallee & Wilson, 1971). This layer is unaffected by turbulence in the bulk phase of the luminal solution unless it is being rigorously stirred. Diffusion across this layer will cause discernible alterations in the absorption rate if transport of the molecule across the mucosal membrane is faster than the rate of movement across the unstirred layer. Because of this the unstirred water layer has been invoked as a possible explanation for the anomalies observed in the absorption of weakly dissociable drugs (Dietschy et al., 1971; Winne, 1977, Thomson & Dietschy, 1984). Theoretically, reducing turbulence and hence increasing the effective unstirred layer thickness would diminish the permeation rate and would shift the pH absorption curve sideward (Winne, 1977). However, it has been demonstrated that the unstirred layer is not the main factor causing the deviations from the pH partition theory in the rat jejunum (Hogerle & Winne, 1983). In this study reducing unstirred layer thickness increased the permeation rate for both

aminopyrine and benzoic acid although the deviations from the predicted pH absorption curves remained. In parallel experiments the mucosal surface pH was measured at the different bulk pH values used to determine the pH absorption curves. The values obtained were then compaired with values calculated from the drug absorption data. The close similarities between the observed and predicted values led to the conclusion that the permeation rate of organic weak electrolytes is determined primarily by the microclimate pH. Therefore although the unstirred layer can contribute to alterations in the intestinal permeation of drugs it only presents a resistance to diffusion and therefore is probably not responsible for weak electrolytes not conforming to the unmodified pH partition hypothesis in the small intestine.

Obviously there are other physiological factors which can affect drug absorption from the small intestine eg intestinal blood flow, motility, intestinal transit time etc. However these are outwith the scope of this study and while they must be acknowledged they will not be dealt with in any detail.

1.6 Drug absorption in intestinal disease - the microclimate revisited

To date very little is known about the way in which intestinal disease affects drug absorption. If the microclimate hypothesis does apply to weak electrolyte absorption, then any disease process disrupting the mechanisms maintaining the jejunal acid microclimate would cause an elevation of the mucosal surface pH

resulting in alterations to weak electrolyte transport and other pH dependent processes. Weak electrolyte absorption would be affected to the extent that weak acids would be malabsorbed while weak bases should have their absorption enhanced. Supporting this view, an elevated jejunal mucosal surface pH has been measured in human coeliac and Crohn's disease (Lucas et al., 1978; Rawlings et al., 1987) where increased plasma levels of the weak base, propranolol have been detected after oral administration (Schneider, Mitchard, Hoare & Hawkins, 1976; Parsons, 1978). Similarly, elevated serum levels of the weak base antimalarials quinine and trimethoprim were observed in coeliac disease (Matilla, Jussila & Takki, 1973). It is difficult to explain this increased weak base absorption in disease states which are normally characterised by malabsorption without recourse to the intestinal microclimate pH. Of course many other factors are involved in human disease states and these will unavoidably complicate the interpretation of any results obtained.

In an attempt to eliminate these complications an attempt has been made to simulate human intestinal disease in rats with a view to investigating drug absorption under these conditions (Lynch, 1986). In this study various agents were used to induce a malabsorptive state in the rat jejunum *in vivo*. Of these, the heat stable enterotoxin of *Escherichia coli*, STa, was observed to induce a mild fluid secretion in perfused jejunal loops while also causing a reversal of the normal luminal acidification to alkalinisation of the perfusing buffer. Under these conditions

the weak acids folic acid and salicylic acid were malabsorbed while the weak bases propranolol and trimethoprim were absorbed at a faster rate. It was proposed that the changes in luminal acid-base balance in the presence of STa would change the pH at the jejunal mucosal surface and that this predicted surface pH change caused the alterations to weak electrolyte uptake. As with the human studies it is difficult to explain an elevation of weak base absorption during unfavourable fluid secretion unless the microclimate pH is taken into consideration.

In the light of these observations the objectives of the present project were proposed. These were as follows:-

1/ To find possible alternatives to E.coli STa enterotoxin for the induction of secretory states in the in vivo rat intestinal loop preparation.

2/ To measure the mucosal surface pH of rat jejunum and ileum in vivo and to investigate the effect of E.coli STa enterotoxin and other secretory agents.

3/ To measure the intestinal villus pH profile in rats in the presence of E.coli STa enterotoxin to ascertain which villus region is affected by STa.

4/ To study the absorption of weakly dissociable drugs in the presence of STa.

It was hoped that by completing these objectives the microclimate hypothesis would be tested hence providing a better understanding of drug absorption in general.

2 MATERIALS AND METHODS

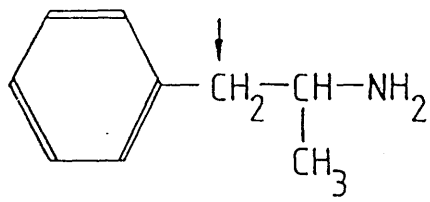
2.1 SOURCE OF CHEMICALS

Salicylic acid, polyethylene glycol 4000, theophylline, 8-bromoguanosine 3':5'-cyclic monophosphate, 8-bromoadenosine 3':5'-cyclic monophosphate were purchased from Sigma Chemical Company, Poole, Dorset, England; standard pH buffers from British Drug Houses, Poole, Dorset, England; Instagel and Soluene 350 from Packard Instrument Company, Caversham, Berks, England; Ecoscint from National Diagnostics, New Jersey, USA; isopropanol from Koch-Light Ltd, Haverhill, Suffolk, England; hydrogen peroxide from Fisons plc, Loughborough, Leics, England; Dorbanex Forte from Riker Laboratories, Loughborough, Leics, England; Sagatal from May & Baker Ltd, Dagenham, Essex, England; dichlorodimethylsilane and TDDA-based ion exchanger from Fluka, Neu-Ulm, FRG.

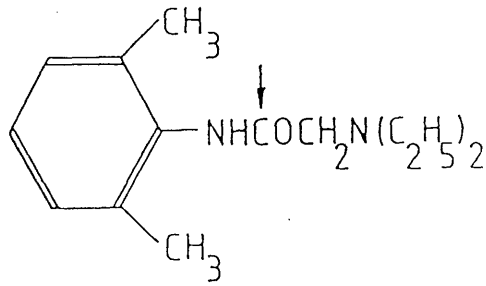
Phenytoin was a gift from May & Baker Ltd; lignocaine from Boots plc, Nottingham, England; amphetamine and morphine from Dr W. Wilson, Glasgow University, Glasgow, Scotland; forskolin from Professors Rummel and Bridges, University of Homburg, Homburg, FRG; synthetic *E.coli* STh(6-19) peptide from Professor Y. Shimonishi, Osaka University, Suita, Japan.

All other chemicals used were of analytical reagent grade and were supplied by Sigma or British Drug Houses.

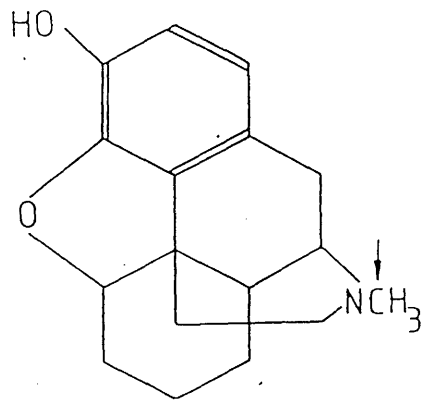
Figure 2.1 (a) Structural formulae and specific activities of labelled compounds. Arrows indicate location of label.



[14C]-Amphetamine (S.A. 49mCi/mmol)

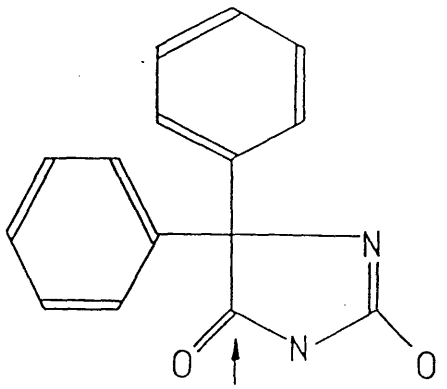


[14C]-Lignocaine (S.A. 52mCi/mmol)

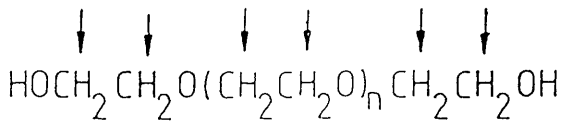


[14C]-Morphine (S.A. 56mCi/mmol)

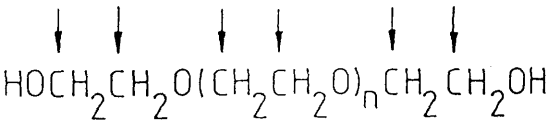
Figure 2.1(b). Structural formulae and specific activities of labelled compounds (contd).



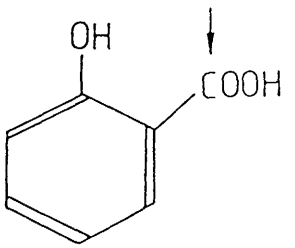
[14C] - Phenytoin (S.A. 58mCi / mmol)



[3H] - Polyethylene glycol 4000 (S.A. 1·2mCi/g)



[14C]- Polyethylene glycol 4000 (S.A. 0·5mCi /g)



[14C] - Salicylic acid (S.A. 52mCi / mmol)

2.2 SOURCE OF ISOTOPES

[^{14}C]-Morphine and [^{14}C]-Phenytoin were purchased from Amersham International plc, Amersham, Bucks, England; [^{14}C]-Lignocaine, [^{14}C]-Salicylic acid, [^{14}C]-Polyethylene glycol 4000 and [^{3}H]-Polyethylene glycol 4000 from New England Nuclear, Dreieich, FRG; [^{14}C]-Amphetamine was purchased from Commissariat a l'Energie Atomique, Gif-sur-Yvette, France.

For structural formulae and specific activities of labelled compounds refer to figure 2.1.

2.3 SOURCE OF BACTERIAL ENTEROTOXINS

Purified heat-stable (STa) *Escherichia coli* enterotoxin from *E. coli* strain P16 was a gift from Dr M.N. Burgess, Beechams Pharmaceuticals, Animal Health Research Centre, Walton Oaks, Surrey, England; purified *Staphylococcus aureus* d toxin from Dr H. Birkbeck, Dept. of Microbiology, Glasgow University; purified *Clostridium perfringens* type A enterotoxin was a gift from Dr P.E. Granum, Norwegian Food Research Institute, Aas, Norway. Purified *Vibrio cholera* toxin was purchased from Sigma.

2.4 PERFUSED LOOP EXPERIMENTS

(a) Anaesthesia

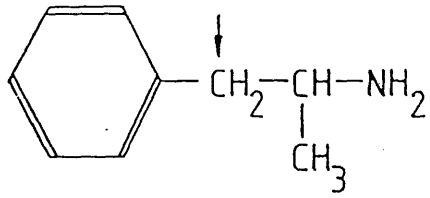
Adult male Wistar rats (245-255g) were anaesthetised initially by an intraperitoneal injection (80mg/kg body weight) of pentobarbitone (Sagatal). Additional 6mg aliquots of pentobarbitone were administered into the peritoneal cavity as required. Body temperature was maintained at 37°C by a rectally placed thermistor controlling a heating table assembly.

Following the onset of anaesthesia the animals were trachaeotomised. In the event of respiratory failure due to accidental anaesthetic overdose the trachaeal cannula was connected to a respirator (55-3438; Harvard Bioscience, MA, USA) allowing the animal to be artificially ventilated with air (stroke volume - 2.5ml; rate - 60 strokes/minute) until spontaneous respiration resumed.

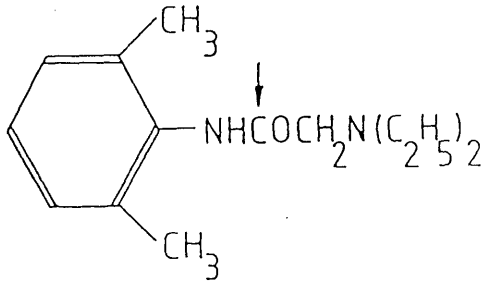
(b) Surgical procedure

Following a midline abdominal incision, the proximal jejunum was exteriorised and the ligament of Treitz (the duodeno-jejunal flexure) was located. The intestine was ligated through the mesentery ten centimetres distal to this point and a small incision was made in the antimesenteric border immediately distal to the ligature. A plastic cannula (length - 3cm; outside diameter - 3mm; internal diameter - 1mm) was inserted into the incision and secured by another ligature. Approximately fifteen

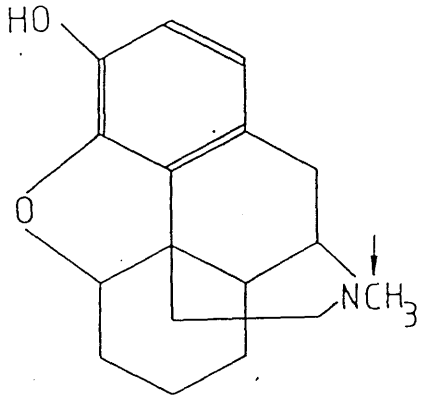
Figure 2.1 (a) Structural formulae and specific activities of labelled compounds. Arrows indicate location of label.



[14C]-Amphetamine (S.A. 49mCi/mmol)

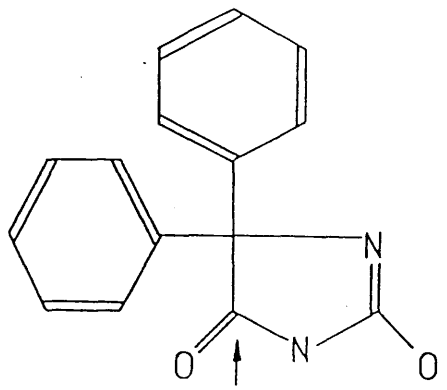


[14C]-Lignocaine (S.A. 52mCi/mmol)

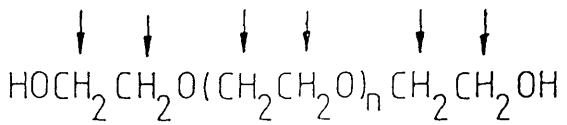


[14C]-Morphine (S.A. 56mCi/mmol)

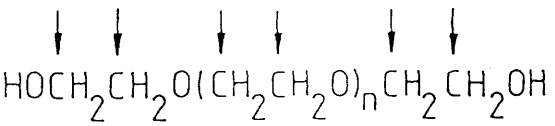
Figure 2.1(b). Structural formulae and specific activities of labelled compounds (contd).



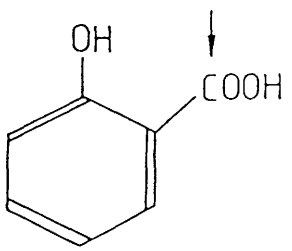
[14C] - Phenytoin (S.A. 58 mCi / mmol)



[3H] - Polyethylene glycol 4000 (S.A. 1·2mCi/g)



[14C] - Polyethylene glycol 4000 (S.A. 0·5mCi /g)



[14C] - Salicylic acid (S.A. 52 mCi / mmol)

centimetres distal to this entrance cannula the intestine was ligated again and a further incision was made in the antimesenteric border just proximal to this. Ten millilitres of an isotonic saline solution (prewarmed to 37°C) was flushed via the entrance cannula through the now isolated intestinal loop to remove any faecal material. The loop was then flushed with air to remove as much fluid as possible. A second plastic cannula (length - 3.5cm; outside diameter - 5mm; internal diameter - 3mm) was inserted through the incision and tied into place. This cannula (the exit cannula) was made as large as possible so as to minimise the risk of blockage by intestinal debris. The completed isolated loop of proximal jejunum with intact mesenteric vasculature was returned to the peritoneal cavity, after which the abdominal wall was closed by loose sutures.

Loops of distal ileum were prepared as described above for the jejunum, the exit cannula being inserted ten centimetres proximal to the ileo-caecal valve.

(c) Perfusion details

The entrance and exit cannulae were connected to the perfusing solution reservoir by flexible silicon tubing (internal diameter - 2mm). The length of tubing used was minimised to reduce the amount of dead space in the system. With the rat disconnected from the circuit the volume of the system was 2.2ml. The perfusate reservoir was situated in a water bath at 37°C and was covered to minimise errors due to evaporation of the perfusing

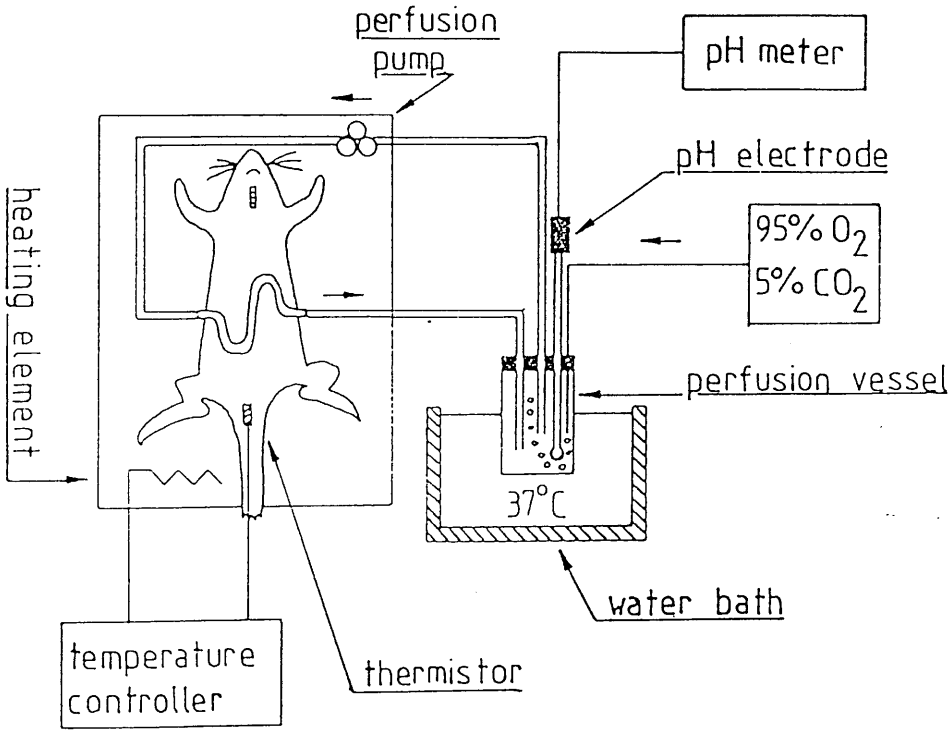
solution. A small peristaltic perfusion pump (82 344; Crouzet, England), incorporated into the perfusion circuit (Figure 2.2), recirculated perfusing solution through the intestinal loop at a flow rate of 1ml/minute.

(d) Perfusing solution

The perfusing solution used in these experiments was Krebs-bicarbonate buffer (Krebs & Henseleit, 1932) additionally containing 5mg/ml of polyethylene glycol, average molecular weight approximately 4000 (PEG 4000) with 1uCi [^{14}C] PEG 4000 as a non-absorbable marker for fluid transport. In experiments designed to investigate the effects of the following compounds these were added to the above solution in the concentrations indicated: E.coli STa enterotoxin - 56ug/ml; C.perfringens type A enterotoxin - 50ug/ml; forskolin - 0.1mmol/l. The buffer was gassed continuously with 95:5% O_2:CO_2 (v/v) to maintain buffer PCO_2 levels.

In experiments designed to investigate the effects of the commercial laxative preparation, 'Dorbanex Forte', the surfactant nature of the product caused excessive frothing when the suspension was gassed. Because of this, the perfusing solution used in these experiments was Krebs-phosphate buffer (Krebs & Henseleit, 1932), which does not require gassing. 'Dorbanex' was added to this solution to give a final danthron (the active ingredient of Dorbanex) concentration of 5mmol/l.

Figure 2.2. Diagram of in vivo perfused loop apparatus.



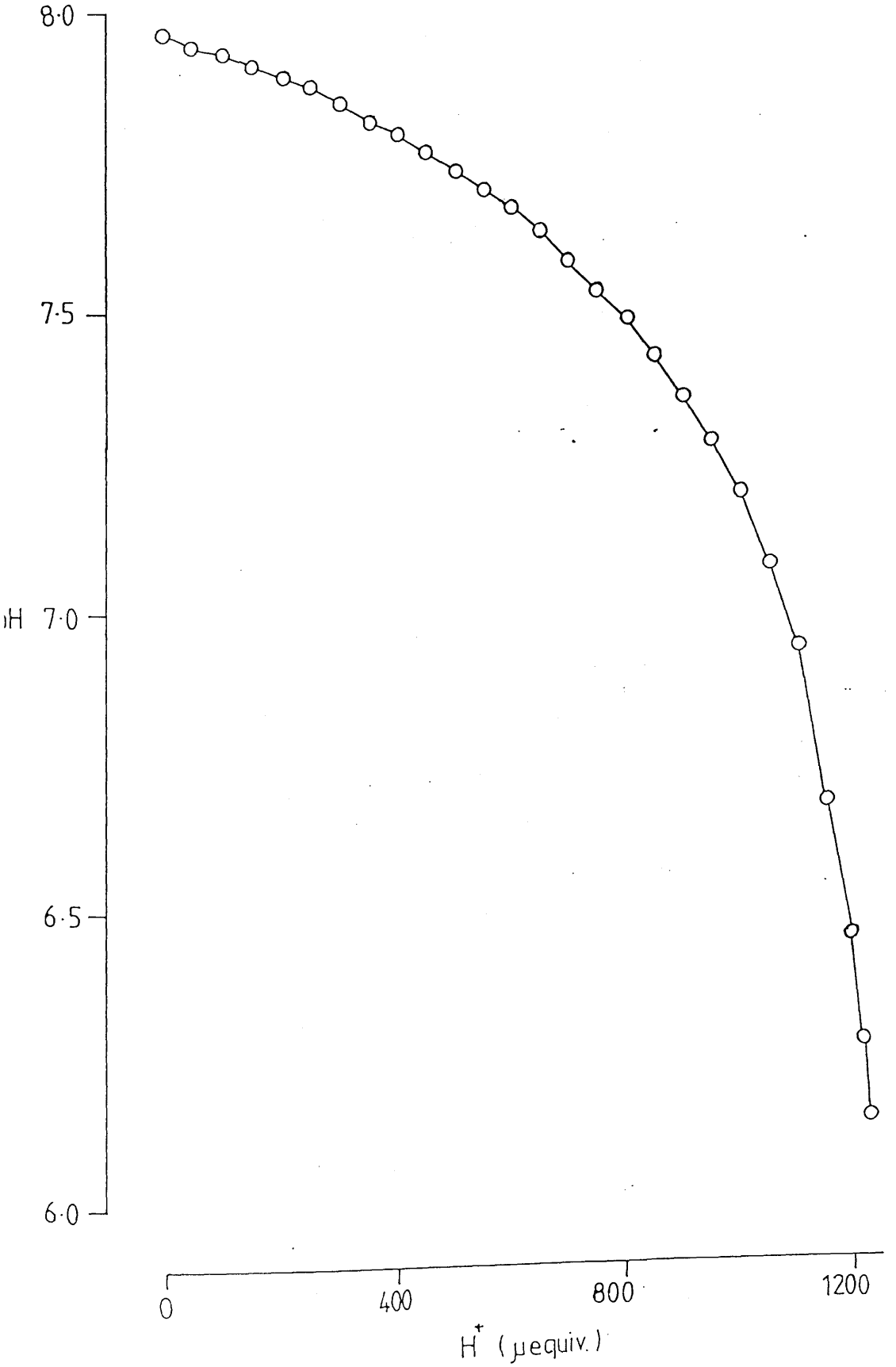
(e) pH measurement

pH was measured using a long-stemmed, glass combination pH electrode (Corning Ltd, Halstead, Essex, England) connected to a digital pH/voltmeter (Pye Unicam 9409). Before and after each experiment the electrode was calibrated using standard buffers at pH 7.0 and 4.0.

(f) Experimental protocol

Before the experiment 15ml of the appropriate perfusing solution was added to the reservoir and the pH was adjusted to 7.40. This solution was then pumped through the intestinal loop for fifteen minutes to equilibrate with residual fluid in the loop and to allow for any initial adsorption of the PEG 4000 onto the mucosal surface. At the end of this equilibration period the 'zero-time' pH was measured and a 50ul sample was withdrawn from the reservoir with an automatic pipette. This and all subsequent perfusate samples were dissolved in 10ml of Ecoscint (commercial emulsifier-type liquid scintillation fluid) in polythene scintillation vials. Perfusate samples and pH measurements were then taken at fifteen minute intervals for the duration of the three hour perfusion. At the end of the experiment the animals were killed by anaesthetic overdose and the intestinal loop was removed. The tissue was then dried to constant weight in an oven at 100°C and all measurements were standardised for a loop dry weight of 100mg.

Figure 2.3 pH titration curve: 15ml Krebs bicarbonate buffer, gassed continuously with 95:5% O_2:CO_2 (v/v) titrated with M NaOH and M HCl.



(g) Liquid scintillation counting of perfusate samples

The [^{14}C]-activity of the perfusate samples (due to [^{14}C]-PEG 4000) was counted in a Packard Tri-carb 2425 liquid scintillation spectrophotometer. Details of liquid scintillation procedures are given in section 2.7(e).

(h) Calculation of perfusate volume changes

Assuming negligible absorption of PEG 4000 across the intestinal mucosa the following equation (2.1) was used to calculate the fluid volumes at different sample times.

$$V_t = \{(C_o/C_t) \times 15\} \text{ ml} \qquad (2.1)$$

where: V_t = perfusate volume at time, t

C_o = activity of [^{14}C]-PEG 4000 in 'zero-time' sample

C_t = activity of [^{14}C]-PEG 4000 at time, t

Equation 2.1 was used to calculate the perfusate volume at fifteen minute intervals for the entire three hour experimental period. The line of best fit was obtained for the data by linear regression allowing the rate of net fluid absorption or secretion to be calculated for each experiment. These rates were standardised for a tissue dry weight of 100mg.

(i) Calculation of apparent net hydrogen ion secretion

Molar solutions of hydrochloric acid or sodium hydroxide were titrated against 15ml of the perfusing solution and the pH titration curve obtained (Figure 2.3) was used to convert the pH

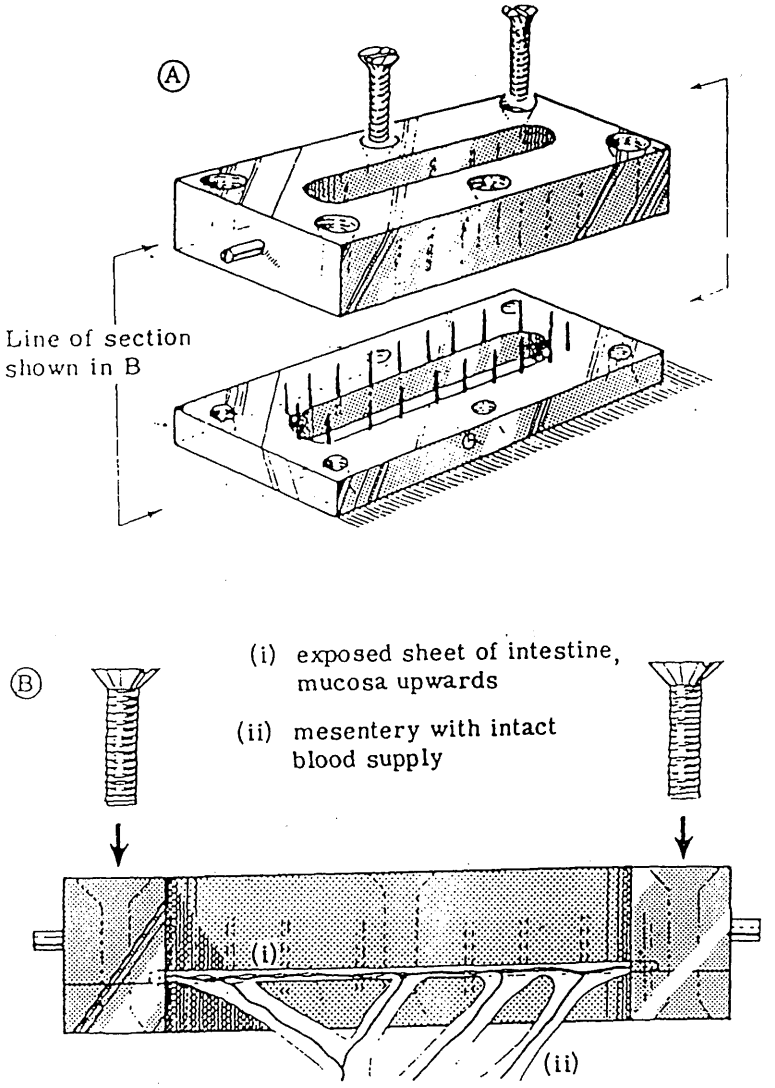
changes measured during these experiments to rates of apparent net hydrogen ion secretion or absorption.

2.5 MUCOSAL SURFACE pH EXPERIMENTS

(a) Surgical procedures

Adult male Wistar rats (300-400g) were anaesthetised and maintained at 37°C as described in Section 2.4(a). Following tracheotomy, a midline abdominal incision was made and a section of small intestine was located either (a) ten centimetres distal to the ligament of Treitz in jejunal experiments or (b) ten centimetres proximal to the ileo-caecal valve in ileal experiments. A five centimetre length of intestine was isolated from the remainder of the intestine by ligating through the mesentery and sectioning. The base plate of a perfusion chamber (Figure 2.4) was then placed above the abdomen. The isolated section of intestine, with its mesenteric blood supply intact was passed through a longitudinal opening in the centre of the chamber and carefully opened along the antimesenteric border. The opened intestine was mounted (mucosal surface up) onto the pins surrounding the opening. The top section of the chamber was then clamped down onto the cut edges of the intestine by six screws which served to secure the top and bottom sections of the chamber together. This clamping also prevented any blood loss from the tissue.

Figure 2.4 Diagram of *in vivo* perfusion chamber. The chamber (A) has a base plate with pins and a top plate, secured to the base plate by six screws. Perfusion ports allow perfusion of the chamber at desired flow rates. The intestine is opened and passed through the base plate prior to pinning. Mesenteric vasculature is intact (B) with the mucosa, after pinning, providing a base for the upper chamber.



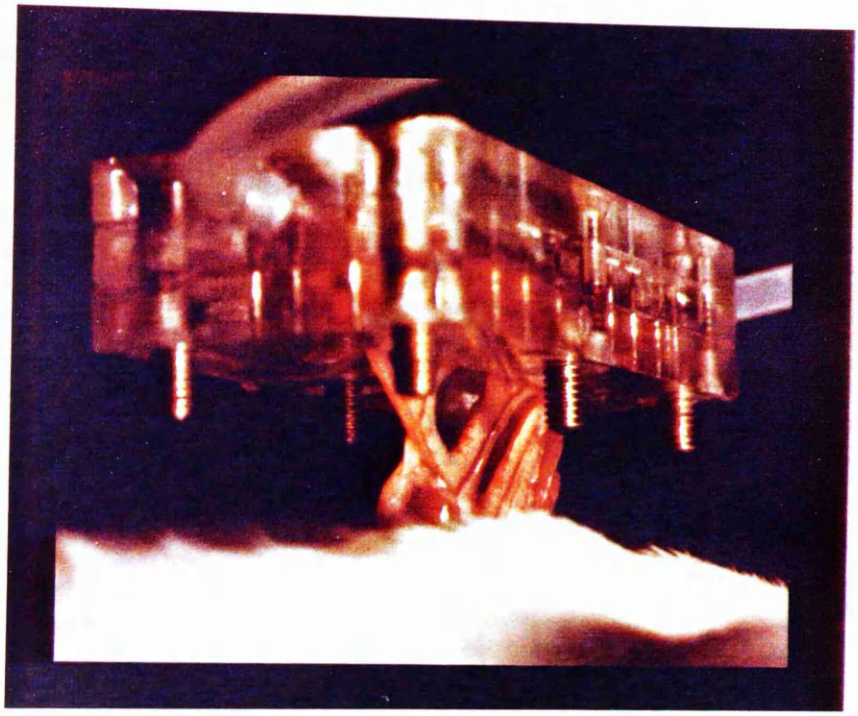
Prepared in this manner, this chamber provided access to the mucosal surface of a length of intestine with undisturbed mesenteric vasculature (Figure 2.5).

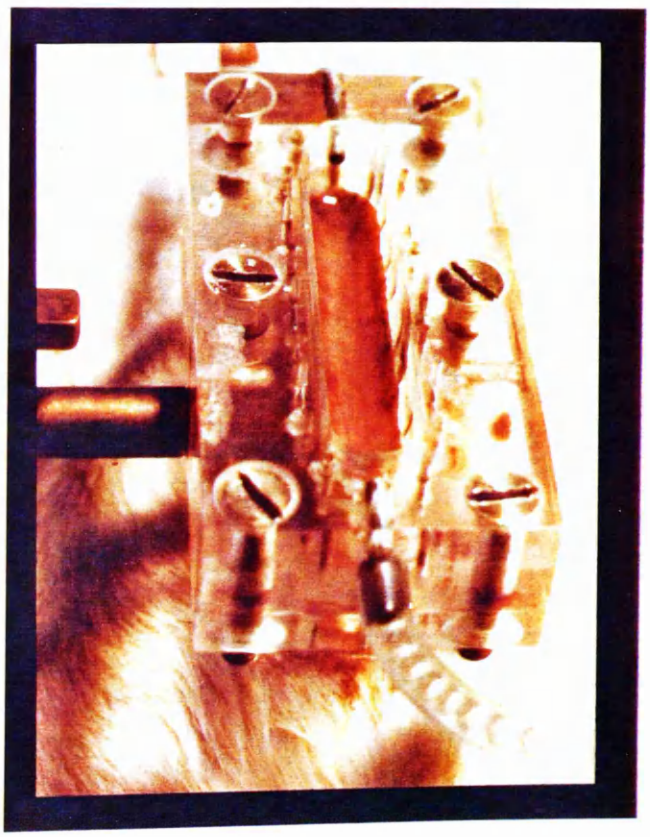
In experiments designed to investigate the effects of pretreatment with cholera toxin on mucosal surface pH, rats were anaesthetised as above and a one centimetre abdominal incision was made. Through this incision the proximal jejunum was located and 100ug of cholera toxin in an isotonic buffered solution was injected five centimetres distal to the ligament of Treitz. The intestine was returned to the abdomen and an antibacterial agent was introduced into the peritoneal cavity. The wound was then sutured and the animal was allowed to recover. Seventeen hours later, the animal was reanaesthetised and a five centimetre section of proximal jejunum was mounted in the perfusion chamber as before.

(b) Perfusion details

Flexible silicon tubing (internal diameter - 2mm) was connected to the perfusion ports on the perfusion chamber (figure 2.4). Two small peristaltic pumps (82 344; Crouzet, England) served to pump perfusing solution from a reservoir (located in a water bath at 37°C) into the chamber and from the chamber back to the reservoir. This system resulted in perfusing solution being recirculated through the chamber and thus over the intestinal mucosal surface, at a flow rate of 1ml/minute.

Figure 2.5 Photographs of *in vivo* perfusion chamber preparation.





(c) Perfusing solution

The perfusing solution was Krebs-phosphate buffer (Krebs & Henseleit, 1932) to which the following compounds were added in the concentrations indicated: E.coli STa enterotoxin - 14ug/ml; theophylline - 20mmol/l; forskolin - 1mmol/l; 8-bromo cGMP - 1mmol/l or 20mmol/l; 8-bromo cAMP - 1mmol/l or 20mmol/l; cholera toxin - 10ug/ml; synthetic E.coli STh(6-19) peptide - 250ng/ml; S.aureus d enterotoxin - 1mg/ml.

(d) Surface pH measurement

A miniaturised glass pH electrode (MI-506; Microelectrodes Inc., Londonderry, N.H., USA) with a 1mm convex tip was connected to a Pye Unicam 9409 digital voltmeter with output to a paper chart recorder (Linseis, FRG). The reference electrode was a Ag/AgCl half-cell connected via 3% agar/3M KCl bridging to the perfusion chamber. The pH electrode had a slope of approximately 57mV/pH unit over the pH 6-8 range and had a response time (90% of final value) of less than 15 seconds. The electrode displayed negligible drift over a period of six hours and as the duration of the surface pH experiments was only one hour, drift was not a source of measurement error. The electrode was always calibrated in standard buffers at pH 6 and 8 before and after each experiment.

(e) Experimental protocol

Prior to experiment, Krebs-phosphate buffer, prewarmed to 37°C was perfused through the chamber at 1ml/minute. With the aid of a

Prior micromanipulator, the pH electrode was introduced into the chamber and the bulk pH of the perfusing solution was measured. Then, under direct visual control, the micromanipulator was used to place the tip of the electrode onto the intestinal mucosal surface. When a stable signal was obtained the electrode was withdrawn into the bulk phase of the perfusing solution where the pH was remeasured. Three measurements of surface pH were carried out in this manner to check for stability and reproducibility. Following this the chamber was emptied and the test solution was perfused through the chamber for sixty minutes. Measurements of surface pH were taken, as described above, at five minute intervals up to thirty minutes and then at fifteen minute intervals until the end of the perfusion period. At this point the test solution was removed from the chamber and replaced with fresh Krebs-phosphate buffer which was perfused for fifteen minutes before the surface pH was finally measured again. After the experiment the animal was killed by anaesthetic overdose.

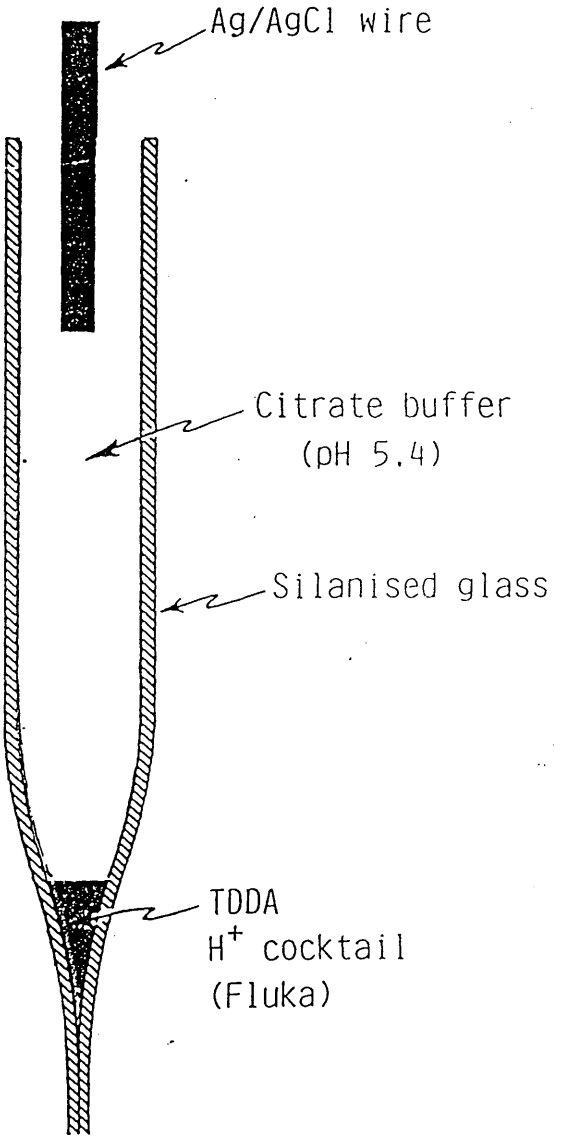
2.6 MEASUREMENT OF pH PROFILE OF INTESTINAL VILLI

(a) pH microelectrode manufacture

Tri-n-dodecylamine (TDDA)-based, liquid ion exchange (LIX) pH microelectrodes (Figure 2.6) were prepared by a modification of the method first described by Ammann and co-workers (1981) and based on the prototype design of Walker (1971). Glass

Figure 2.6

Diagram of TDDA-based pH microelectrode



Electrode characteristics

Tip diameter : 2-20μM

Slope : 55-60mV/pH unit

Response time : <15 seconds

micropipettes were drawn from borosilicate glass capillaries (1103232; Hilgenberg GmbH; Malsfeld, FRG) to give a tip diameter of 1um and a shank length of 5mm. The micropipettes were then baked in an oven at 100°C for one hour. The inner wall of the micropipettes was made hydrophobic by silanisation with a solution which consisted of 1.75ul dichlorodimethylsilane (DDS)/ml chloroform (achieved by mixing equal volumes of the following two solutions (1) 2ul DDS/ml chloroform and (2) 1.5 ul DDS/ml chloroform). The base of the hot micropipettes were dipped into this solution three times for one second and were then returned to the oven to bake for a further hour at 180°C. After this period the micropipettes were removed to a dessicator to cool.

A glass capillary was drawn in a bunsen flame so that the tip was very fine. TDDA-based ion-exchanger (82500; Fluka, Neu-ulm, FRG) was drawn up into the capillary which was then placed into the electrode until the tip was down to the electrode tip (being careful not to scratch the silanised electrode wall). The ion exchanger was blown out of the capillary until approximately 2-3mm of the electrode shank was filled. The electrode was then allowed to sit, tip down, until the cocktail had migrated to the tip without any air bubbles forming. Finally the electrode was back-filled from the shank with 10mM citrate buffer (pH 5.4) making sure that no air space existed at the buffer/cocktail interface.

(b) pH microelectrode characterisation

A Ag/AgCl pellet was inserted into the completed electrode and this was connected via coaxial cable to a high input (10^{13} Ohm) digital pH meter in mV-mode (model 741, Knick, Berlin, FRG) with connected paper chart recorder (Knauer, Berlin, FRG). The reference electrode used for all pH measurements was an external macro Ag/AgCl reference electrode (374-M8, Ingold GmbH, Frankfurt, FRG). The electrode assembly was located in an earthed Faraday cage to eliminate any extraneous electrical signals. Prior to experimentation the electrodes were calibrated in phosphate buffers at pH 5.5, 6.0, 6.5, 7.0 and 7.4. The mean resistance of the electrodes was $5\text{-}10 \times 10^{10}$ MOhm. If the electrode response was greater than 20 seconds (90% of final value) electrodes were bevelled to a tip diameter of 20um. Only electrodes (Figure 2.7) with a response time of less than 15 seconds and a slope between 55 and 60mV/pH unit in a pH range of 5.5 to 8.0 were used. To make the microelectrode tips clearly visible the electrodes were dipped into an indelible blue ink (T 1000; Edding & Co, Ahrensburg, FRG) up to a maximum height of 1mm. This colouring procedure did not affect electrode slope or response time.

(c) Surgical procedures and tissue preparation

Adult male Wistar rats (250g) were anaesthetised and maintained at 37°C as described previously (Section 2.4(a)). A midline abdominal incision was made and the proximal jejunum or distal ileum was located. Closed five centimetre *in vivo* sacs of either

jejunum or ileum were prepared by passing two ligatures through the mesentery five centimetres apart. The distal ligature was tightened, closing off the bottom end of the sac and a small incision was made in the antimesenteric border just proximal to the proximal ligature. Using a syringe fitted with a blunt needle 1ml of Dulbecco phosphate buffer or buffer additionally containing 14ug/ml of purified P16 E.coli STa enterotoxin was injected into the sac through the incision. The second ligature was closed and the completed sac was returned to the peritoneal cavity.

After thirty minutes incubation in situ the sacs were excised and the animals were killed by anaesthetic overdose. One centimetre sac sections were cut and mounted, mucosa up, over a hollow, dome-shaped holder (Figure 2.8). A rubber ring gently stretched the tissue over the dome facilitating access to the villi.

The holder, with the tissue mounted on it, was then placed into a perspex chamber (Figure 2.9) perfused with glucose-free, oxygenated Dulbecco-phosphate buffer (pH 7.40). The perfusion slits in the tissue holder (Figure 2.8) permitted oxygenated buffer to perfuse the serosal, as well as the mucosal, surface of the tissue. The temperature of the perfusing buffer was maintained at 25^{o}C to reduce mucus production and minimise villus motility. Turbulence induced by oxygenation of the perfusing buffer served to reduce 'unstirred layer' thickness.

Figure 2.8 Diagram of tissue holder.

Diagram of Tissue Holder

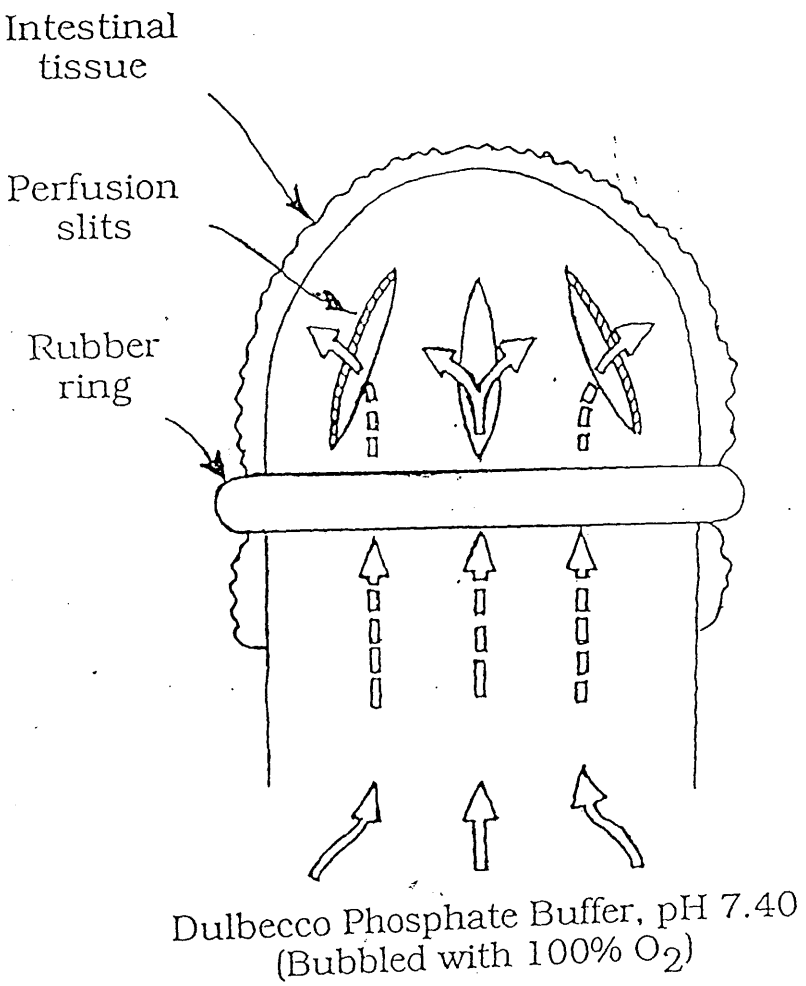
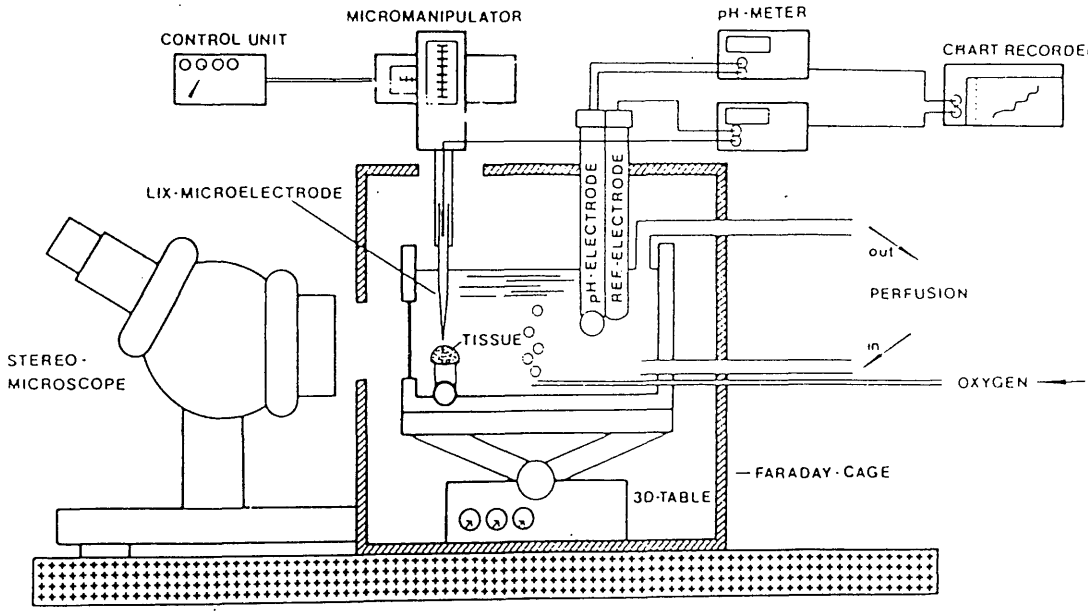


Figure 2.9 Diagram of apparatus for in vitro measurement of villus pH profile. See text for details.



A stereo-microscope (Zeiss, Oberkochem, FRG) (magnification x40-x60) mounted in a horizontal position was used to view the intestinal mucosal surface through a quartz glass disc incorporated into the chamber. By placing the chamber onto a 3-D multipositional table it was possible to orientate the tissue into the position where the villi were most accessible. The chamber was illuminated by a 3-arm fibre optic light (Gossen, FRG). The assembly was completely surrounded by the earthed Faraday cage which eliminated any extraneous electrical signals and this was secured onto a marble slab to minimise vibration.

A motor-driven remote-control micromanipulator (model MM35; control unit ST35E; Brinkman GmbH, FRG) was mounted in a horizontal position above the incubation chamber outside the Faraday cage. The pH microelectrode was fixed in a teflon holder mounted on the x-axis motor and was immersed in the incubation medium in the chamber. The microelectrode was connected via a Ag/AgCl pellet and a coaxial cable to a digital pH meter (model 741, Knick, Berlin, FRG), used in the mV-mode. The reference electrode, which was located in the perfusion chamber, was also connected to this pH meter. Additionally a small, glass combination pH electrode (U 402-M8; Ingold GmbH, Frankfurt, FRG) connected to a second pH meter (model 741, Knick, Berlin, FRG) was introduced into the incubation medium to monitor changes of perfusing buffer pH. Both pH meters were connected to a two channel paper chart recorder (Knauer, Berlin, FRG).

(d) Measurement of villus pH profile

With the aid of the stereo-microscope the micromanipulator was used to position the electrode tip at the base of a villus (Figure 2.10). When a stable signal was obtained the pH was recorded and the electrode position on the micromanipulator was noted. The electrode was then withdrawn vertically in 50um steps, the pH being recorded at the end of each step. When the villus tip was reached the exact villus length and villus tip-pH were recorded. This procedure was repeated until all accessible villi in the section had been measured.

2.7 DRUG ABSORPTION EXPERIMENTS

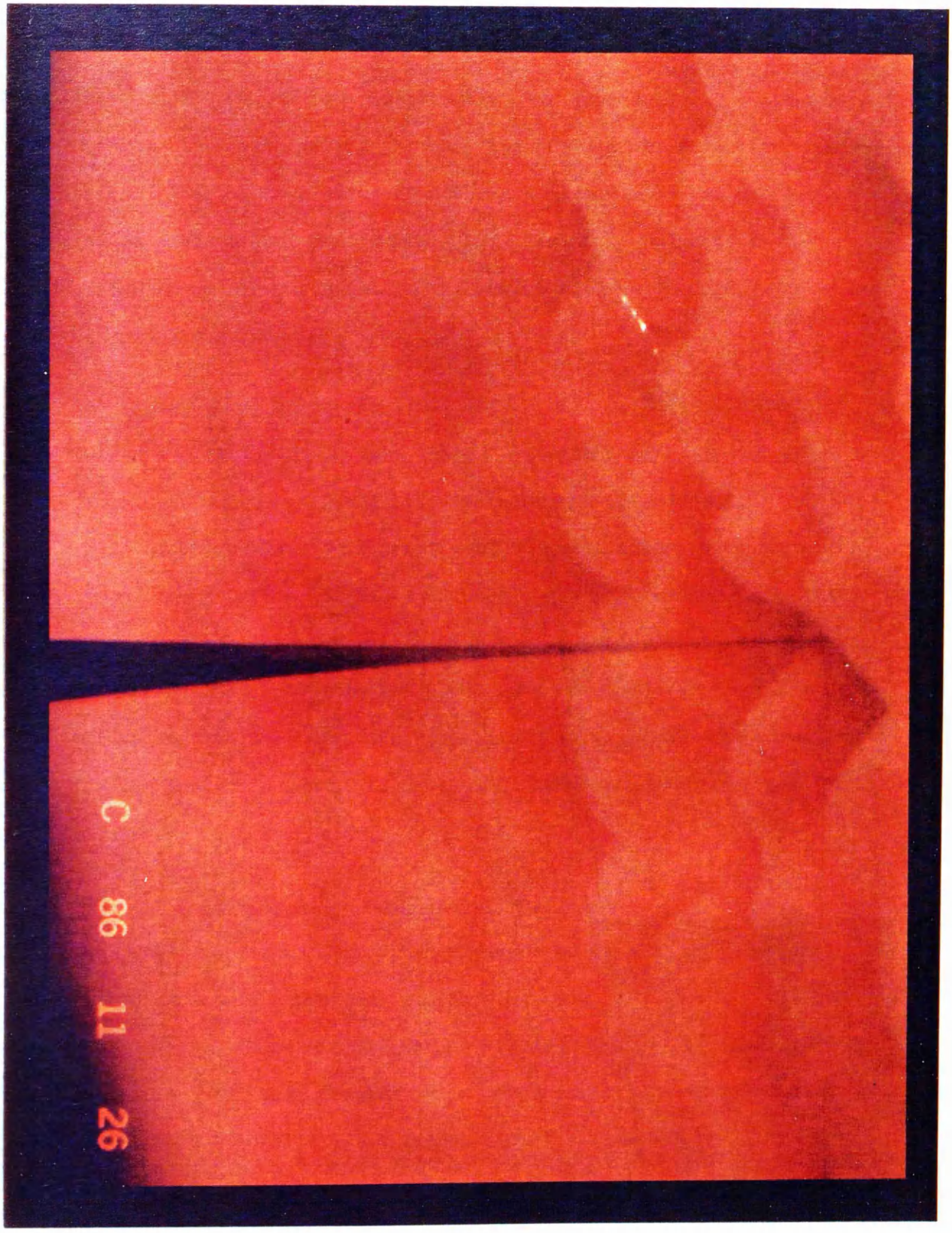
(a) Anaesthesia and surgical procedures

Adult male Wistar rats (250g) were anaesthetised and maintained at 37°C. Fifteen centimetre *in vivo* loops of proximal jejunum were prepared as described (Section 2.4(b)). To obtain peripheral blood samples, the left carotid artery was cannulated with a cannula connected via a three-way tap to a syringe filled with a heparinised 0.9% saline solution.

(b) Perfusing solution

The perfusing solution used in these experiments was Krebs-bicarbonate buffer (Krebs & Henseleit, 1932) additionally containing 5mg/ml of PEG 4000 with 1uCi [^{3}H]-PEG 4000 as a non-absorbable marker for fluid transport. Individual drugs were added to this solution in a concentration of 1mmol/l with 0.5uCi

Figure 2.10 Photomicrograph of LIX pH microelectrode in position at rat jejunal villus.



[^{14}C]-labelled drug as a marker for drug absorption. In experiments designed to investigate the effects of E.coli STa enterotoxin, purified toxin from E.coli strain P16 was added to the perfusate in a concentration of 56ug/ml. In separate experiments investigating the combined effects of forskolin and theophylline on drug absorption these were added to the perfusate in concentrations of 1mmol/l and 20mmol/l respectively. The perfusing buffer was gassed continuously with 95:5% O_2:CO_2 (v/v) to maintain buffer PCO_2 levels.

(c) Experimental protocol

The protocol used in the drug absorption experiments was as described for the perfused loop experiments (Section 2.4) with the exception that 100ul carotid blood samples were withdrawn at 'zero-time' (after fifteen minutes of perfusion) and at subsequent thirty minute intervals for the duration of the three hour perfusion. The withdrawn blood was replaced by an equal volume of an isotonic 0.9% heparinised-saline solution injected via the carotid cannula. All results were standardised for a tissue dry weight of 100mg.

(d) Liquid scintillation counting of samples

The 50ul perfusate samples were dissolved in 10ml of 'Ecoscint' (a biodegradable commercial emulsifying-type liquid scintillation fluid) in polythene scintillation vials. The [^{14}C] and [^{3}H] activities in the samples were measured in a Packard Tri-carb 2425 liquid scintillation spectrophotometer with the facility to

count in two channels simultaneously.

Since the energy spectra for [^{14}C] and [^{3}H] overlap there is an inevitable contribution from [^{14}C] in the [^{3}H] channel and vice versa. However, counting 'windows' were selected to give minimum 'spillover' of either isotope into the other's channel whilst maintaining a satisfactory counting efficincy for both isotopes. Both spillover and counting efficiency are affected by the degree of 'quench' in the sample. Quenching is caused by anything which prevents the blue light energy emitted by the scintillant molecules in the vial reaching the counter's photomultiplier tubes. As a consequence of this, as the degree of quench in the sample (due to e.g., water molecules, colour, etc.) increases, the counting efficiency decreases. The scintillation counter provides an index of sample quench, the external standard channels ratio (ESCR). By counting a series of vials containing a known activity of either [^{14}C] or [^{3}H] with varying degrees of quench a standard curve can be prepared by plotting counting efficiency (detected counts/actual counts x 100%) against ESCR (Figure 2.11). These curves allow the actual activity of [^{14}C] or [^{3}H] in any sample to be calculated irrespective of quench. Using the same quenched standards it is also possible to produce standard curves to correct for the spillover of [^{14}C] into the [^{3}H] channel and [^{3}H] into the [^{14}C] channel (Figure 2.12). The equations of these curves were incorporated into a computer programme (written specially for this project by Jim Beck, Computing Service, Glasgow University) on an ICL 2988 mainframe

Figure 2.11 Quench correction curve for liquid scintillation counting. ^{14}C (O) and ^{3}H (□) counting efficiencies are plotted against the external standard channels ratio (ESCR) at varying levels of quench.

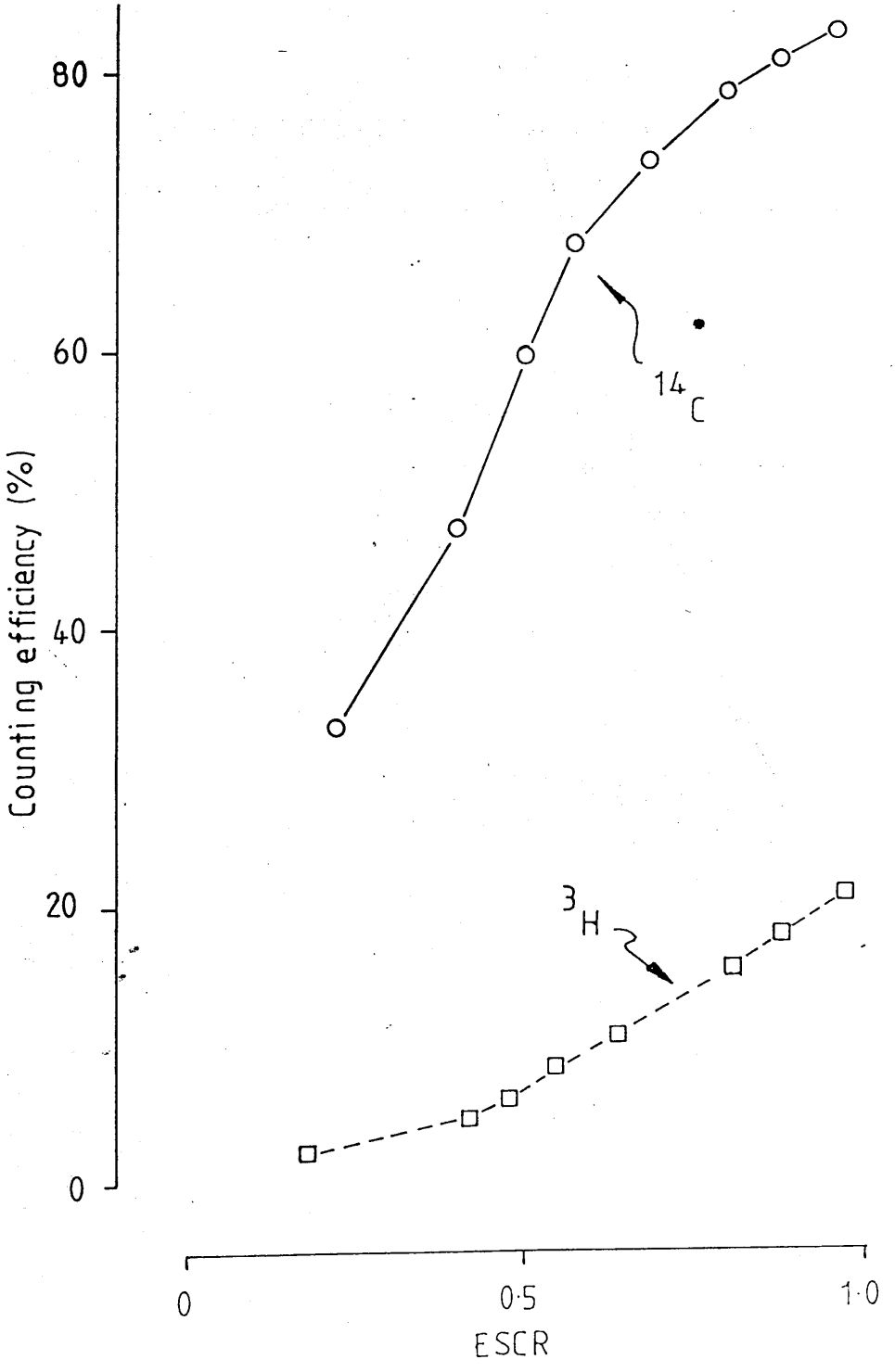
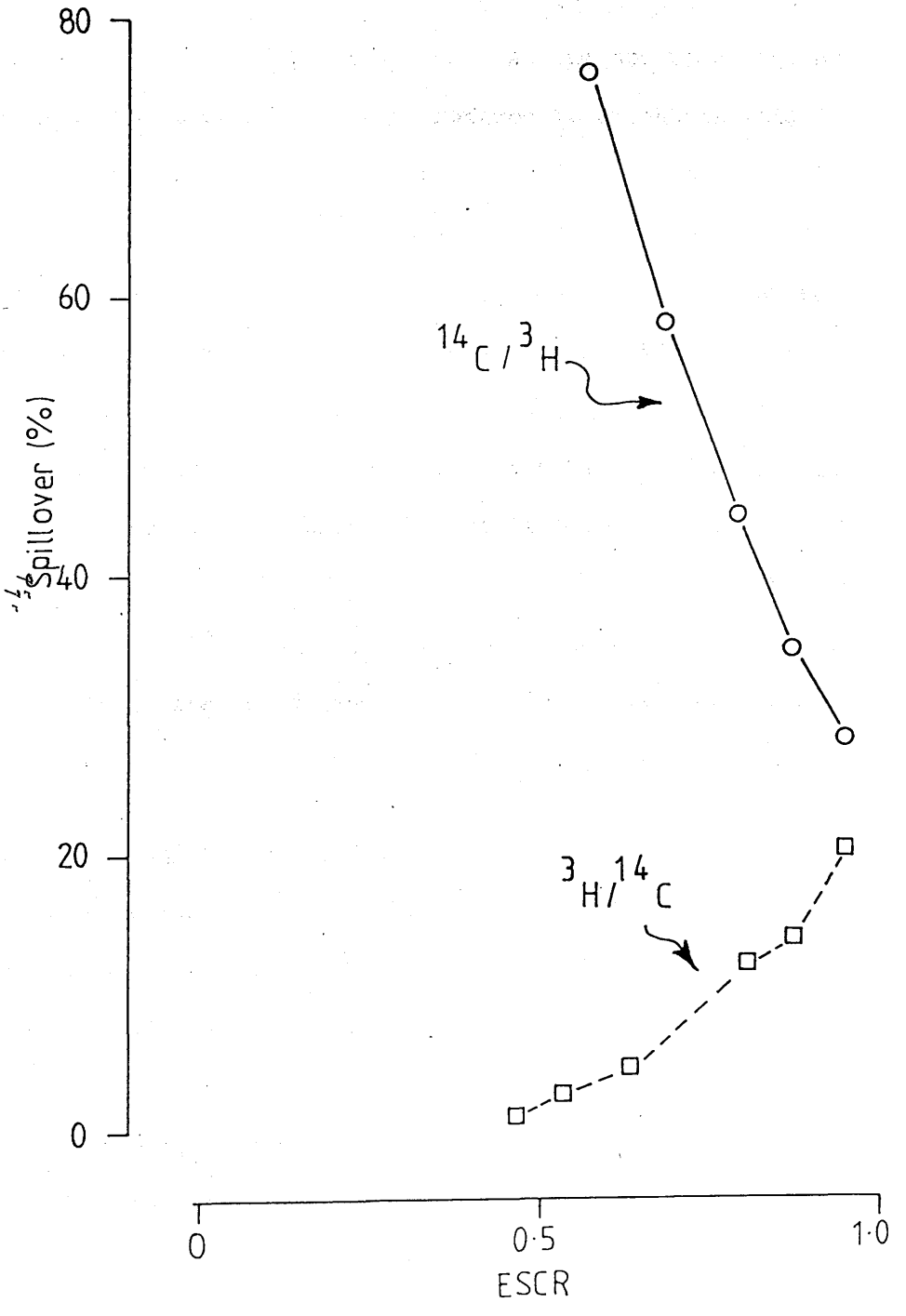


Figure 2.12 Spillover correction curve for ^{14}C and ^{3}H dual channel counting. Percentage ^{14}C counted in the ^{3}H channel ($^{14}C/^{3}H$) (O) and ^{3}H counted in ^{14}C channel ($^{3}H/^{14}C$) (□) are plotted against external standard channels ratio (ESCR) at varying levels of quench.



computer which converted the raw data obtained from the scintillation counter to the actual activity contained in each sample due to the respective isotope.

(e) Blood sample preparation for liquid scintillation counting

Peripheral blood samples were prepared for liquid scintillation counting by a modification of the method described by Moore (1981). The 100ul samples were removed to polythene scintillation vials containing 750ul of a mixture (2:1) of 'Soluene 350', a commercial tissue solubiliser, and isopropanol. The vials were incubated at 40°C for one hour. The solubilised blood was then bleached by the addition of 500ul of a 30% solution of hydrogen peroxide. This bleaching was performed at room temperature for fifteen minutes followed by thirty minutes at 40°C. Finally 15ml of 'Instagel', a commercial scintillation fluid acidified (1:9v/v) with 0.5M hydrochloric acid, was added to the vial contents. The [^{14}C]-activity in the samples was then counted under the same conditions described above for the perfusate samples.

(f) Data handling and statistics

The disappearance of drug from the intestinal lumen and its subsequent appearance in the peripheral blood was calculated using a modification of the method first described by Shanker and co-workers (1959). This method assumes that (1) changes in the [^{14}C]-activity from the labelled drug in the perfusate represents the movement of the unlabelled drug and (2) changes in the

[^{3}H]-activity from the labelled PEG 4000 are representative of changes in the perfusate volume. The following equations were used to calculate changes in the denoted variables with time:-

Drug concentration at time, t $D(t) = (C(t)/Co) \times Do$

Perfusate volume at time, t $V(t) = (Ho/H(t)) \times Vo$

Drug amount at time, t $A(t) = D(t) \times V(t)$

where: Co = [^{14}C]-activity in 'zero-time' sample

C(t) = [^{14}C]-activity in sample at time, t

Do = initial drug concentration in perfusate

Ho = [^{3}H]-activity in 'zero-time' sample

H(t) = [^{3}H]-activity in sample at time, t

Vo = initial perfusate volume

Peripheral blood drug concentrations were calculated using the following equation:

Peripheral blood drug concentration at time, t,

$$Db(t) = (Cb(t)/2Co) \times Do$$

where: Cb(t) = [^{14}C] counts in blood sample at time,t

The line of best fit for perfusate drug amount against time was calculated, which, by definition, provided the rate of luminal disappearance of drug was obtained. Following standardisation for a loop dry weight of 100mg the mean rate of drug absorption was calculated for each series of experiments.

Similarly, the line of best fit was calculated for fluid volume against time and, again, following standardisation for a loop dry weight of 100mg, the mean rate of fluid absorption or secretion was obtained for each series of experiments. Additionally, the

mean peripheral blood drug concentration over the whole experimental period was calculated for each individual experiment and, as before, the mean value was calculated for each series of experiments.

The above treatment of the data provided an empirical analysis of the amount of drug disappearing from the intestinal lumen and reappearing in the peripheral blood. However, in an attempt to derive some further information about the kinetics of drug absorption two pharmacokinetic models were applied to the mean luminal concentration data from each series of experiments. This analysis required regression procedures available as an option in the BMDP package of statistical software, accessible via the Glasgow University ICL 2988 mainframe computer. The programme adjusts an initial series of parameter estimates simultaneously until the least squares best fit to the data has been obtained.

Firstly, a first order model incorporating two parameters was applied, of the form

$$C(t) = Ae^{-kt}$$

where: $C(t)$ = drug concentration in lumen at time, t

A = initial concentration of drug in lumen

t = time

A first order differential equation describing loss of material from one compartment has a solution of the above form. If drug absorption is by diffusion across a single barrier the above equation should apply since, under these circumstances, luminal

concentration should decline exponentially.

However, if a second compartment is involved, exchange between two compartments is described by two first order differential equations and has a similar form of solution requiring two exponential terms. The solution for a two compartment model of the intestinal transport process as represented (Figure 2.13) has the following derivation.

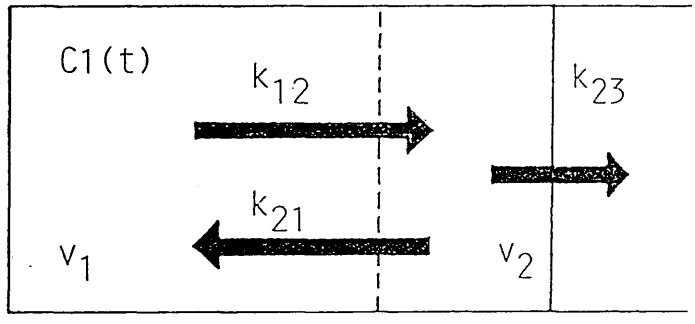
In this model, drug is introduced into the lumen at time, $t_{(o)}$ to give an initial concentration $C_{1(o)}$. Diffusion into (k_{12}) and back out of (k_{21}) a surface layer (microclimate) occurs but absorption into the cell (V_3) from the microclimate (V_2) is unidirectional (k_{23}), there being no corresponding k_{32} term.

This system can be described by a set of three first order differential equations in accordance with Fick's Law:-

$$\left.\begin{aligned} \frac{dQ_1}{dt} &= V_1\frac{dC_1}{dt} = -k_{12}C_1 + k_{21}C_2 \\ \frac{dQ_2}{dt} &= V_2\frac{dC_2}{dt} = k_{12}C_1 - (k_{21} + k_{23})C_2 \\ \frac{dQ_3}{dt} &= V_3\frac{dC_3}{dt} = k_{23}C_2 \end{aligned}\right\} \quad (1)$$

A solution of these equations will provide a description of the change in concentration of drug in each compartment with respect to time as a function of the as yet undetermined rate constants. This set of equations is amenable to solution by Laplace transformation (Simon, 1977).

Figure 2.13 Diagramatic representation of exchange compartment pharmacokinetic model. See text for details.



$$Cl(t) = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t}$$

After transformation, the equations become:

$$\left.\begin{aligned} sg_1 - C_1(o) &= (-k_{12}/V_1)g_1 + (k_{21}/V_2)g_2 \\ sg_2 - C_2(o) &= (k_{12}/V_2)g_1 - (k_{21}/V_2 + k_{23}/V_2)g_2 \\ sg_3 - C_3(o) &= (k_{23}/V_3)g_2 \end{aligned}\right\} \quad (1a)$$

Collecting $g_1, g_2 \& g_3$ terms, the equations become:

$$\left.\begin{aligned} (s + k_{12}/V_1)g_1 - (k_{21}/V_1)g_2 + 0 &= C_1(o) \\ (-k_{12}/V_2)g_1 + (s + (k_{21} + k_{23})/V_2)g_2 + 0 &= 0 \\ 0 + (-k_{23}/V_3)g_2 + sg_3 &= 0 \end{aligned}\right\} \quad (2)$$

Solving this set of equations for g_1, gives:

$$g_1 = \frac{C_1(o)s(s + (k_{21} + k_{23})/V_2)}{s((s + k_{12}/V_1)(s + (k_{21} + k_{23})/V_2) - k_{12}k_{21}/V_1V_2)} \quad (3)$$

Let $\lambda_o = (k_{21} + k_{23})/V_2$, $\alpha = k_{12}/V_1$, $\beta = k_{12}k_{21}/V_1V_2$

Substituting in (3) gives

$$g_1 = C_1(o)(s + \lambda_o)/(s + \lambda_o)(s + \alpha - \beta) \quad (4)$$

or

$$g_1 = C_1(o)(s + \lambda_o)/(s + \lambda_1)(s + \lambda_2) \quad (5)$$

where λ_1 and λ_2 are the roots of $(s^2 + s(\lambda_o + \alpha) + \lambda_o\alpha - \beta)$

expanding (5) gives

$$g_1 = \frac{C_1(o)s}{(s + \lambda_1)(s + \lambda_2)} + \frac{C_1(o)\lambda_2}{(s + \lambda_1)(s + \lambda_2)} \qquad (6)$$

the antitransform of which gives

$$\frac{C_1(t)}{C_1(o)} = \frac{\lambda_1 e^{-\lambda_1 t} - \lambda_2 e^{-\lambda_2 t}}{\lambda_1 - \lambda_2} + \frac{\lambda_o e^{-\lambda_2 t} - \lambda_o e^{-\lambda_1 t}}{\lambda_1 - \lambda_2} \qquad (7)$$

from which

$$\frac{C_1(t)}{C_1(o)} = \frac{(\lambda_1 - \lambda_o)}{(\lambda_1 - \lambda_2)} e^{-\lambda_1 t} + \frac{(\lambda_o - \lambda_2)}{(\lambda_1 - \lambda_2)} e^{-\lambda_2 t} \qquad (8)$$

Consequently, this model can be represented by the sum of two exponentials, i.e. is of the form

$$C_{(t)} = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t}$$

Using the BMDP non-linear regression programme to fit a second order exponential curve to the luminal concentration data, estimates of the four parameters A, B, λ_1 and λ_2 were obtained. These parameters were then used to calcalulate λ_o since, from equation (8)

$$\frac{\lambda_1 - \lambda_o}{\lambda_1 - \lambda_2} = A \quad \text{and} \quad \frac{\lambda_o - \lambda_2}{\lambda_1 - \lambda_2} = B$$

It can be shown that

$$\lambda_1 + \lambda_2 - \lambda_o = k_{12}/V_1$$

$$\lambda_1\lambda_2/(\lambda_1 + \lambda_2 - \lambda_o) = k_{23}/V_2$$

$$\lambda_o - (k_{23}/V_2) = k_{21}/V_2$$

Unfortunately, it is not possible to extract the individual rate constants further than this. However the ratio k_{23}/k_{21} can be calculated. Since this ratio compares absorption into the cell with back diffusion out of the surface compartment (Figure 2.13) it provides an index of how well the drug is taken up and hence allows a quantitative comparison between control and test conditions.

Additionally, the luminal drug concentration data were analysed by simple linear regression as a comparison with a more empirical zero order model.

To provide an index of the degree of fit of each model to the individual data sets, correlation coefficients were calculated for each regression analysis. These were calculated using the

equation:

$$r = (1-(RSS/TSS))^{1/2}$$

Where r = correlation coefficient

RSS = residual sum of squares

TSS = total sum of squares

All statistical comparisons between control and test conditions throughout this study were performed using "Student's" 't'-test, values of $P<0.05$ being considered significant.

3 RESULTS

The experiments carried out in this study fall into four general categories and these will be dealt with in the following order:

1. Perfused loop experiments
2. Surface pH experiments
3. Measurement of pH profile of intestinal villi
4. Drug absorption experiments

Unless stated otherwise the presented data represent the mean $\pm$ estimated standard error of the mean for the number of experiments shown in parentheses ($x \pm esex(n)$).

In view of the diversity of the experiments undertaken in this study, explanations and references, which might be deemed more appropriate for a discussion section, have been included under the results heading. This has been necessary to make clear the reasons for the types of experiments that were done and it is hoped that this treatment will place the experimental work in perspective.

3.1 PERFUSED LOOP EXPERIMENTS

An initial series of experiments investigated the characteristics of fluid transport, the changes in acid-base balance occurring in *in vivo* loops of rat small intestine and how these parameters would be affected by exposure to the various secretory substances tested.

(a) Effect of E.coli STa enterotoxin on fluid transport and acidification in the jejunum

When in vivo loops of rat proximal jejunum were perfused with Krebs-bicarbonate buffer at pH 7.40 (Krebs & Henseleit, 1932) a significant ($P<0.001$) net absorption of fluid by the tissue was observed (Figure 3.1). Regression analysis of perfusate volume changes demonstrated that fluid uptake was essentially linear with time (mean correlation coefficient, $r = 0.94 \pm 0.01(6)$). The mean rate of fluid absorption in these experiments was $21.17 \pm 3.06(6)$ ul/min/100mg dry weight. This amounts to approximately 25% of the total perfusate being absorbed over the experimental period.

To test whether this fluid absorption could be accounted for by tissue oedema, the tissue was weighed before and after drying to constant weight in an oven at 100°C. The mean wet weight:dry weight ratio after three hours of perfusion was $4.78 \pm 0.10(6)$. This ratio was significantly ($P<0.001$) greater than that obtained for unperfused tissue where the mean wet:dry ratio was $3.85 \pm 0.10(6)$ indicating that there was indeed some tissue oedema in the perfused intestinal loops. However, the mean difference between wet and dry tissue weights was $0.87 \pm 0.10(6)$ g (the mean tissue dry weight was $249 \pm 27(6)$ mg). Therefore tissue oedema can only account for approximately 9% of the total fluid uptake observed. The estimated fluid absorption rates measured in this study correspond well with published estimates from other

laboratories (Parsons, 1956; Powell et al., 1971).

In contrast when the jejunal mucosa was challenged by 56ug/ml of E.coli STa enterotoxin (Figure 3.1) the net fluid absorption measured under control conditions was reversed ($P<0.001$) to a net secretion of $2.61\pm1.57(6)$ ul/min/100mg dry weight. This secretion did not differ significantly from zero net fluid movement and thus the effect of STa on fluid transport is antiabsorptive rather than secretory. This is in agreement with previously reported findings (Newsome, Burgess & Mullan, 1978).

As well as investigating changes in fluid transport the pH of the perfusing buffer was monitored over the three hour experimental period. In control experiments (Figure 3.2), as expected, there was a significant ($P<0.001$) apparent net secretion of hydrogen ions into the lumen of $0.75\pm0.05(6)$ uequiv/min/100mg dry weight (as measured by back-titration of the perfusing buffer).

This acidification could only be achieved with living tissue. When loops of jejunum from dead rats were perfused in situ under identical conditions to those adopted for control experiments, the mean rate of hydrogen ion secretion was measured to be $0.01\pm0.01(7)$ uequiv/min/100mg dry weight. This was not significantly different from zero net hydrogen ion secretion and indicates that there is no acidification under totally anoxic conditions. Therefore acidification is not an anoxic artefact. A possible source of error in calculating acid-base alterations was that the tubing might be changing the pH of the perfusate.

Figure 3.1 Effect of E.coli STa toxin on fluid transport in perfused in vivo loops of rat proximal jejunum. Values are given as mean ± S.E.M.for number of experiments shown, one observation per animal.

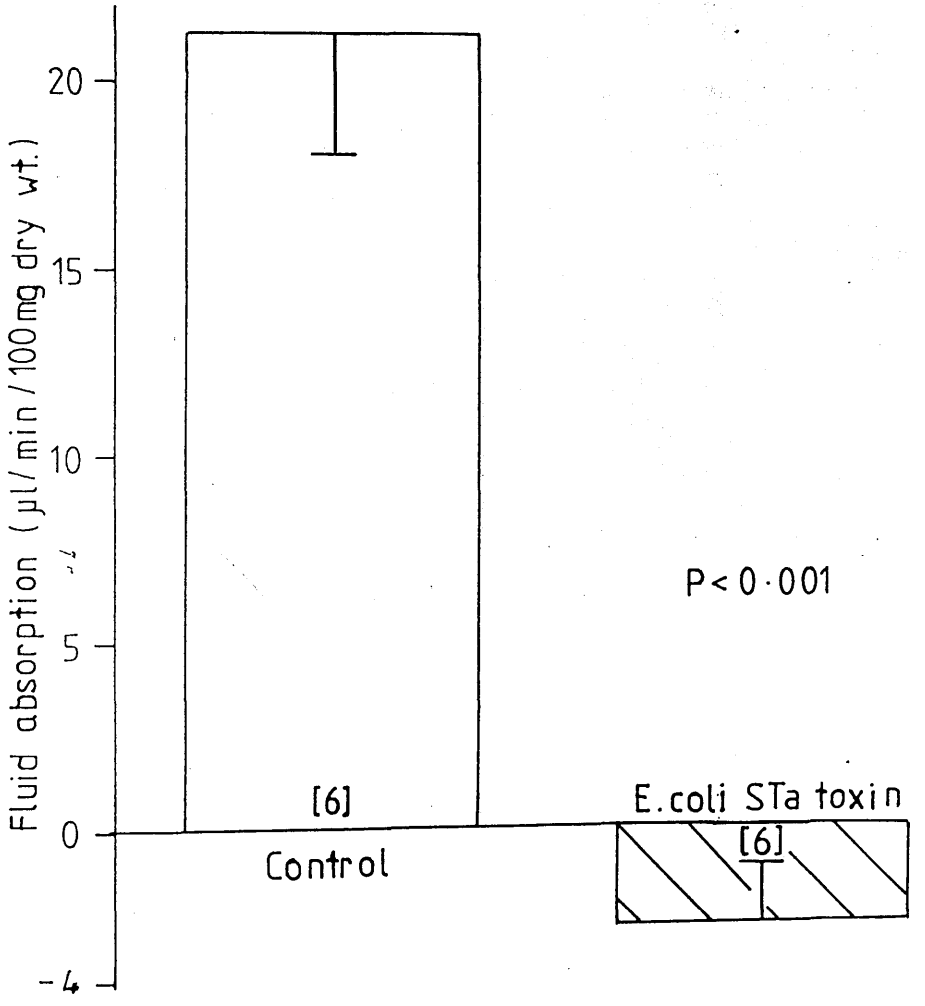
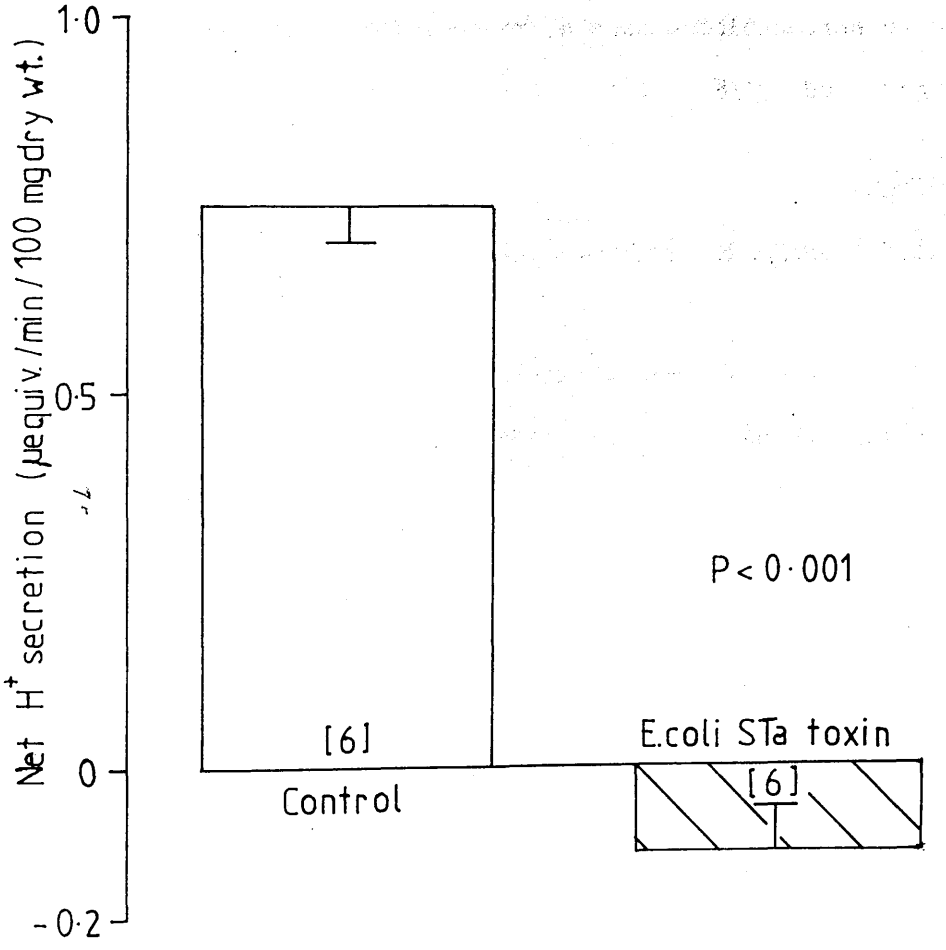


Figure 3.2 Effect of E.coli STa toxin on luminal acid-base balance in perfused in vivo loops of rat proximal jejunum. Details as for Figure 3.1.



However, no pH change was recorded on perfusing Krebs-bicarbonate buffer through the silicon tubing of the perfusing circuit.

When the tissue was exposed to E.coli STa enterotoxin (Figure 3.2) this acidification was reversed ($P<0.001$) to an apparent net absorption of hydrogen ions by the enterocytes of $0.12\pm0.06(6)$ uequiv/min/100mg dry weight. Again this apparent hydrogen ion absorption was not significantly different from zero net hydrogen ion secretion. This abolition of jejunal acidification by STa has been reported previously (Lynch & Lucas, 1983), but has since received scant attention.

(b) Effect of forskolin on fluid transport and acidification in the jejunum.

A series of experiments investigated the effect of forskolin, a potent adenylate cyclase activator (Seamon & Daly, 1981) which has been shown to elevate cAMP levels in the intestine (Boige, Amiranoff, Munck & Laburthe, 1984), on fluid transport and luminal acidification in the jejunum. When the jejunal mucosa was exposed to forskolin (0.1mmol/l) (Figure 3.3) net fluid absorption was abolished ($P<0.001$) to an insignificant net secretion of $1.54\pm3.53(10)$ ul/min/100mg dry weight. Thus, as with E.coli STa enterotoxin, at this concentration in vivo, forskolin appears to be antiabsorptive rather than secretory in its action. Similar to STa, forskolin inhibited ($P<0.01$) the normal acidification observed in control experiments (Figure 3.4), although the reduced rate of $0.27\pm0.11(10)$ uequiv/min/100mg dry

Figure 3.3 Effect of forskolin (0.1mM) on fluid transport in perfused <u>in vivo</u> loops of rat proximal jejunum. Details as for Figure 3.1.

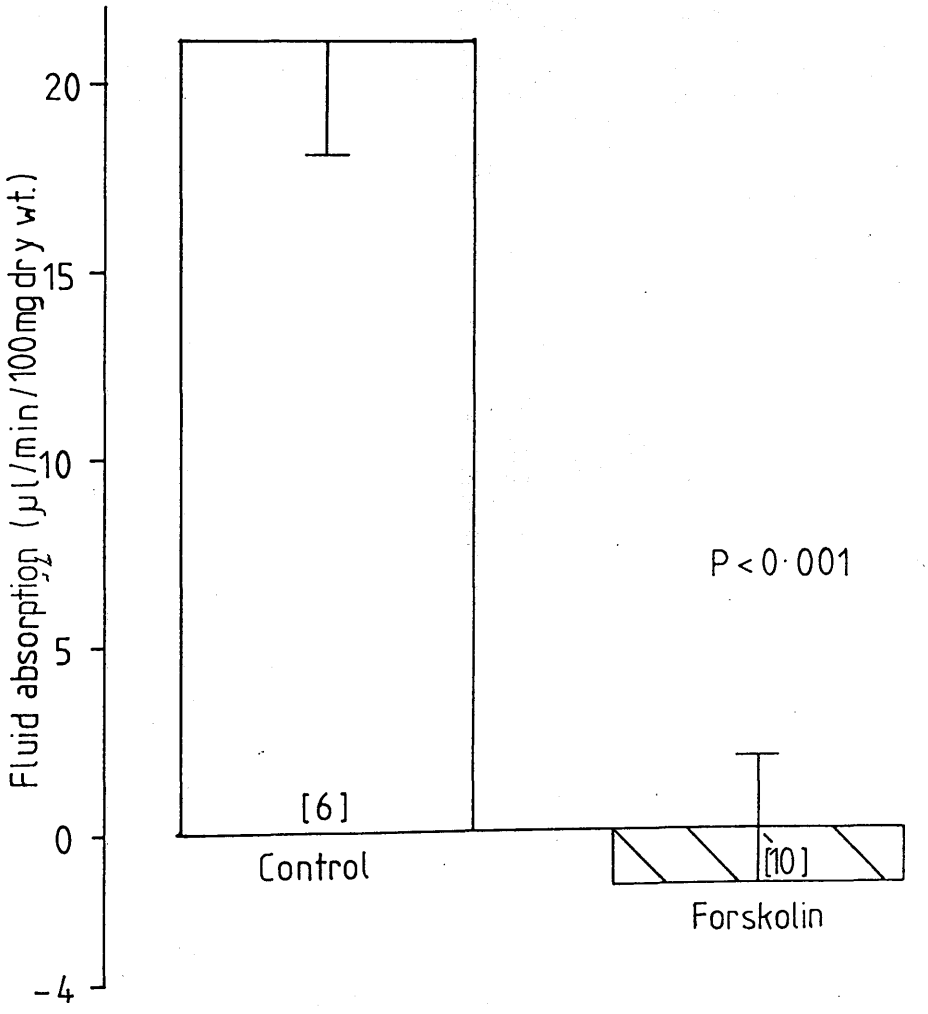
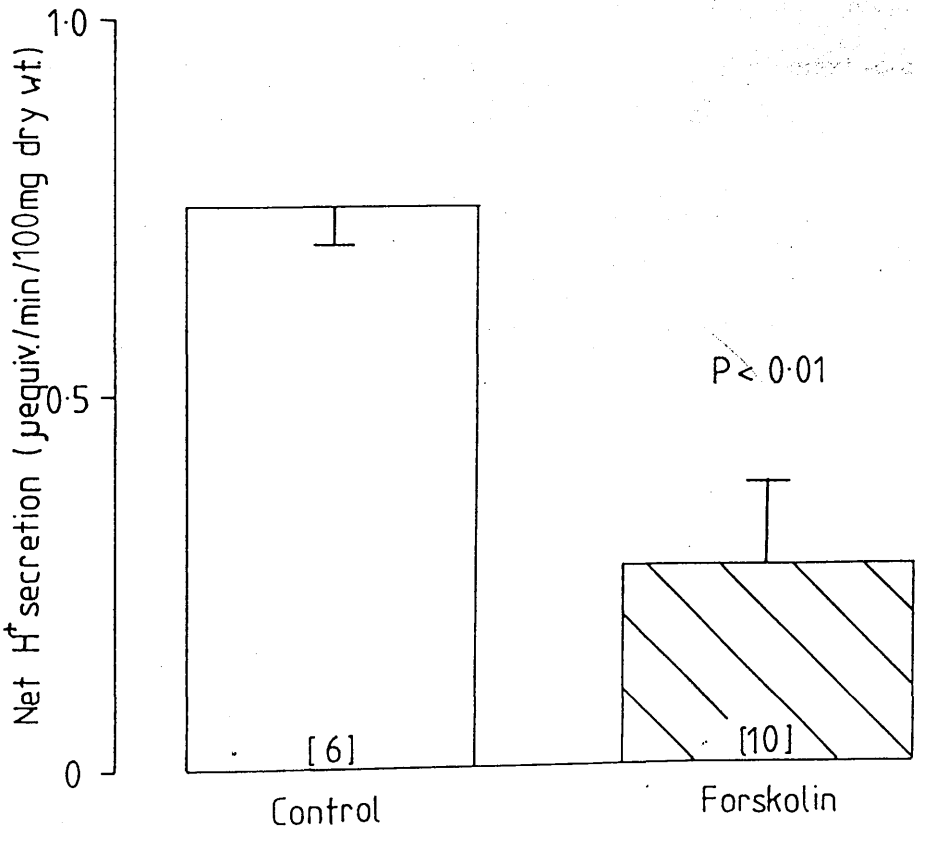


Figure 3.4 Effect of forskolin (0.1mM) on luminal acid-base balance in perfused <u>in vivo</u> loops of rat proximal jejunum. Details as for Figure 3.1.



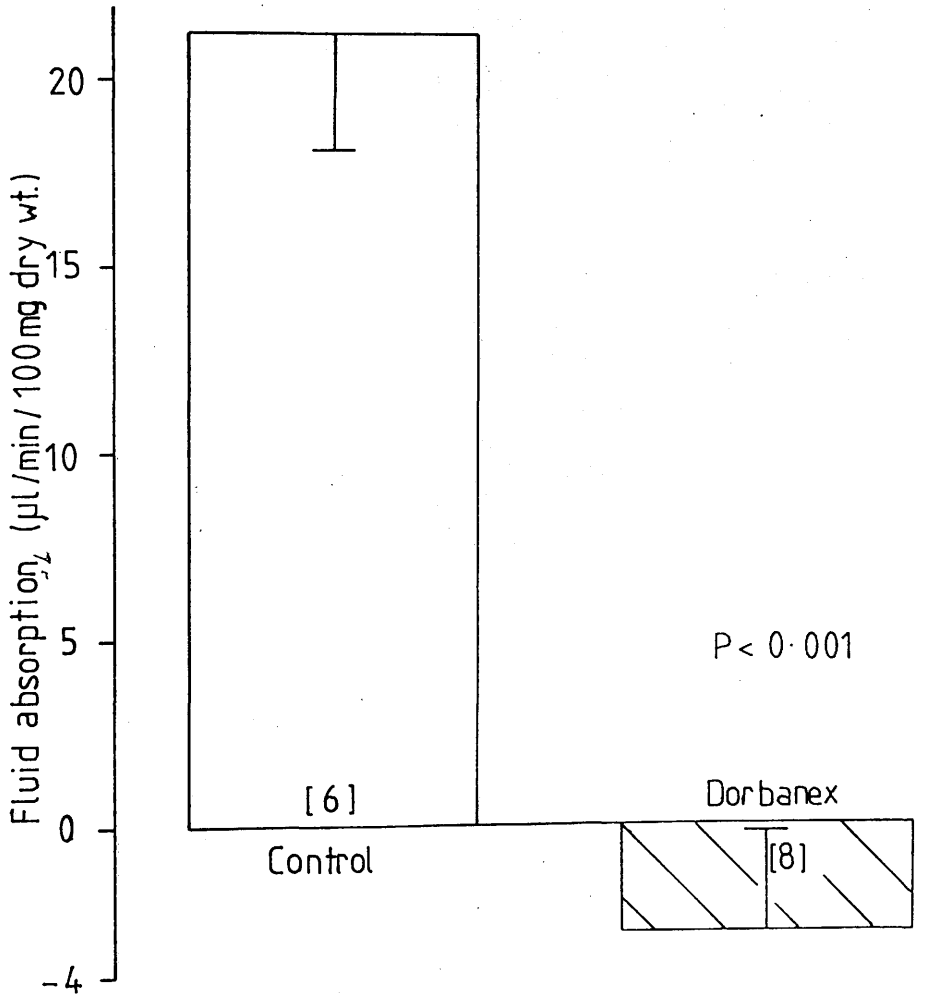
weight still represented a significant ($P<0.05$) apparent hydrogen ion secretion into the lumen.

(c) Effect of 'Dorbanex' on fluid transport and acidification in the jejunum.

'Dorbanex' is a commercial laxative preparation containing the anthraquinone derivative, danthron, as its active ingredient. This class of compounds inhibits fluid and ion transport in the intesine and therefore there was a possibility that this compound might have similar effects to STa. The following experiments investigated the effects of 'Dorbanex' on perfused loops of rat proximal jejunum. The viscosity of the 'Dorbanex'-containing perfusate made it impossible to gas the buffer (because of excessive effervescence). Therefore the laxative was incorporated into Krebs-phosphate buffer (Krebs & Henseleit, 1932) which does not require gassing to maintain luminal pH. The final danthron concentration in the perfusate was 5mM.

When 'Dorbanex' was perfused through the *in situ* jejunum net fluid absorption (Figure 3.5) was reversed ($P<0.001$) to a net secretion of $2.90\pm2.71(8)$ ul/min/100mg dry weight which was not significantly different from zero net fluid absorption. Net hydrogen ion secretion (Figure 3.6) was also significantly ($P<0.001$) reduced to values of $0.06\pm0.01(8)$ uequiv/min/100mg dry weight. This reduction in acidification rate, although small, was highly significant ($P<0.001$) and may have been due to the reduced buffering capacity of phosphate buffer compared to bicarbonate

Figure 3.5 Effect of "Dorbanex" on fluid transport in perfused <u>in vivo</u> loops of rat proximal jejunum. Details as for Figure 3.1.



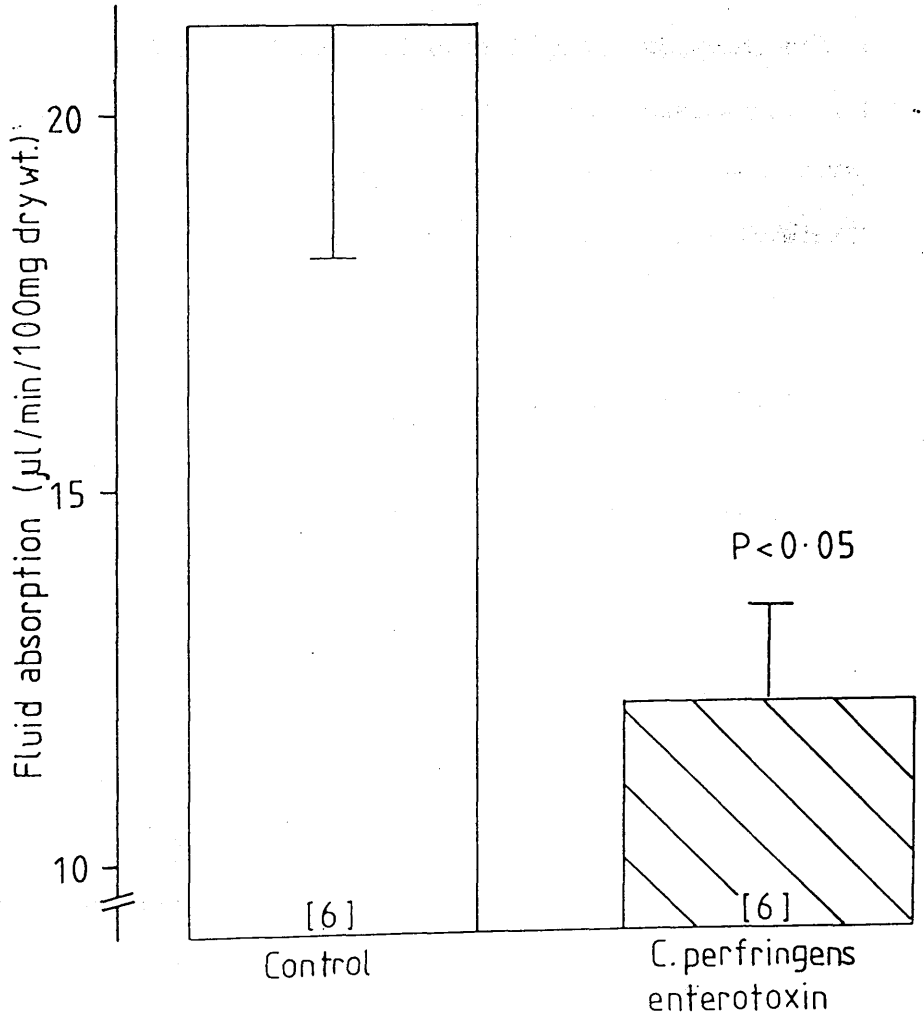
buffer.

Transverse histological sections were cut from the 'Dorbanex'-perfused loops for light microscopy. Comparing these 'Dorbanex' sections to sections cut from control loops it was noted that, although there was no cellular damage and the brush border appeared fully intact the 'Dorbanex'-treated tissue had become extremely flaccid i.e. the smooth muscle appeared to have lost all of its tone. The significance of this finding is unclear but the observation was a very striking one. Therefore, although 'Dorbanex' altered both fluid transport and acid-base balance, its usefulness as a damaging agent was limited by its tendency to effervesce. There was also the possibility that the alterations to intestinal structure induced by the suspension might complicate interpretation of any results. For these reasons no further work was done with 'Dorbanex'.

(d) Effect of Clostridium perfringens enterotoxin on fluid transport and acidification in the jejunum

Clostridium perfringens produces a variety of protein toxins one of which is an enterotoxin (McDonel, 1986). The effect of C.perfringens on jejunal fluid transport was investigated. Whether the toxin produced a similar inhibition of luminal acidification to that observed with E.coli STa was also determined. When Krebs-bicarbonate buffer containing 50ug/ml of purified C.perfringens enterotoxin was perfused through in vivo loops of proximal jejunum (Figure 3.7), net fluid absorption

Figure 3.7 Effect of Clostridium perfringens enterotoxin on fluid transport in perfused in vivo loops of rat proximal jejunum. Details as for Figure 3.1.



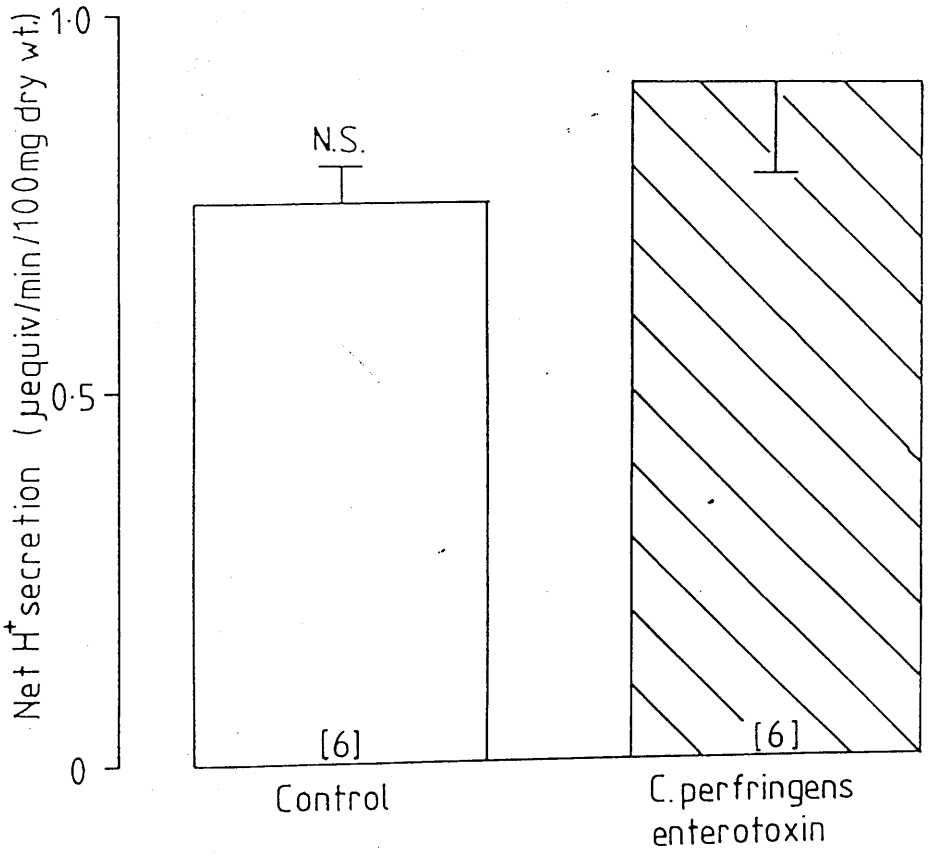
significantly ($P<0.05$) fell from $21.17\pm3.06(6)$ ul/min/100mg dry weight to values of $12.12\pm1.30(6)$ ul/min/100mg dry weight. This 43% reduction in net fluid absorption was the smallest observed with any of the substances tested. This was surprising since the toxin is a well-known secretagogue (Duncan & Strong, 1969). There is therefore a possibility that the toxin had become denatured in some way, rendering it inactive.

There was no significant difference in the apparent net hydrogen ion secretion (Figure 3.8) after toxin challenge. In fact the mean acidification rate was actually enhanced from $0.75\pm0.05(6)$ ul/min/100mg dry weight to $0.91\pm0.12(6)$ uequiv/min/100mg dry weight.

(e) Effect of E.coli STa enterotoxin on luminal acid-base changes in the ileum.

Having established that E.coli STa enterotoxin abolishes luminal acidification in the rat jejunum in vivo, a further series of experiments was undertaken to determine the effect of STa on luminal acid-base balance in the ileum. Unlike the jejunum, rat ileum has been shown to alkalinise its luminal perfusates in vivo (Hubel, 1967; Hubel, 1969). Therefore there were two possible outcomes of toxin challenge - (1) that STa would cause luminal acidification or (2) that STa would elevate basal alkalinisation.

Figure 3.8 Effect of Clostridium perfringens enterotoxin on luminal acid-base balance in perfused in vivo loops of rat proximal jejunum. Details as for Figure 3.1.



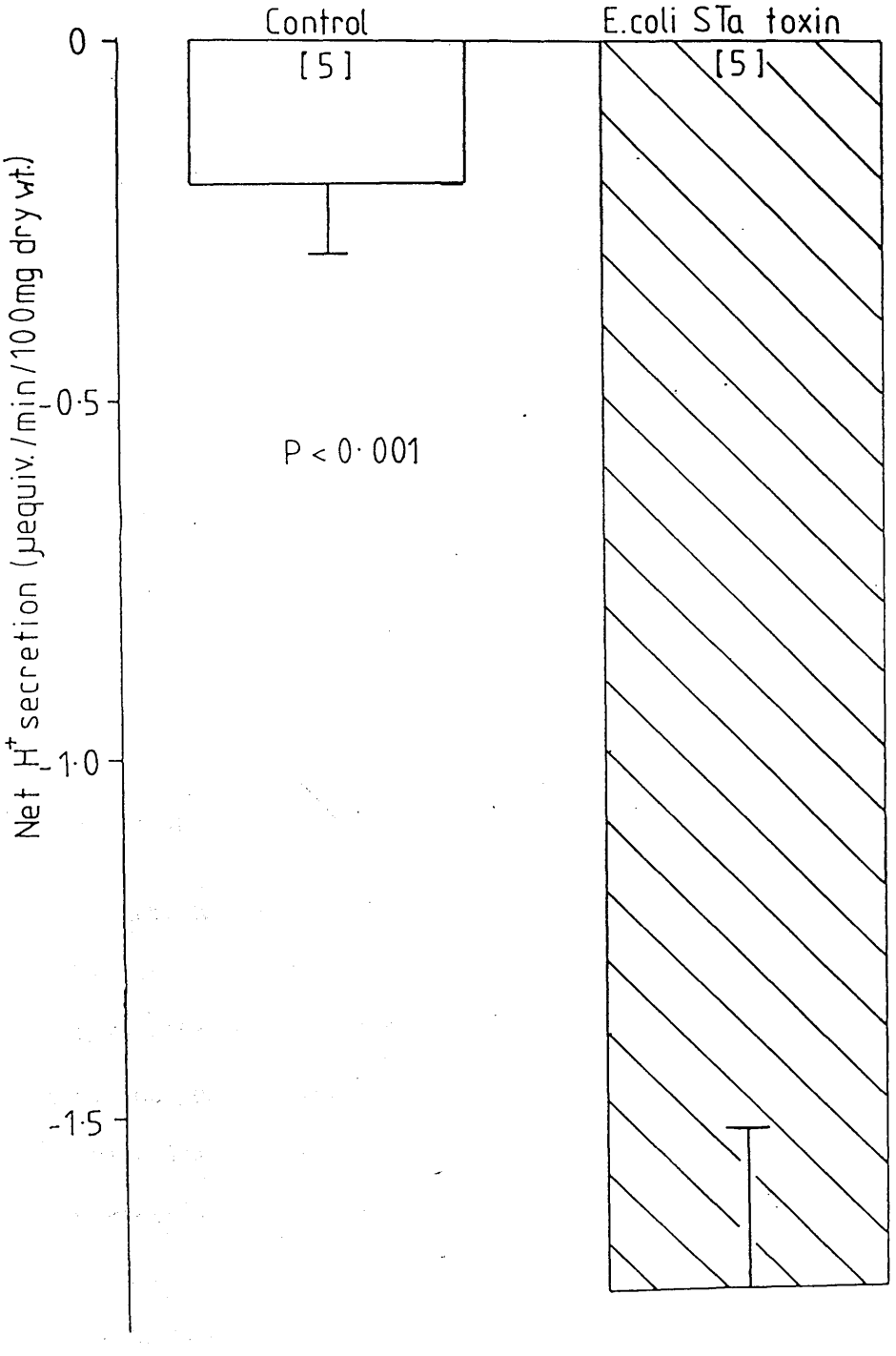
When ileal loops were perfused with Krebs-bicarbonate buffer there was an apparent net secretion of bicarbonate anion into the lumen (Figure 3.9), amounting to 0.20±0.10(5) uequiv/min/100mg dry weight. This apparent bicarbonate secretion (or its converse, hydrogen ion absorption) was very small and did not differ significantly from zero net bicarbonate secretion. There was also no significant difference between this unstimulated bicarbonate secretion in the ileum and the STa driven luminal alkalinisation observed in the jejunum.

In contrast, after E.coli STa enterotoxin (56ug/ml), net bicarbonate secretion was elevated massively ($P<0.001$) to values of 1.77±0.23(5) uequiv/min/100mg dry weight. This induction of bicarbonate secretion is similar to that observed in the ileum after exposure to cholera toxin (Leitch & Burrows, 1968; Carpenter, Sack, Feeley & Steenberg, 1968; Norris, Curran & Schultz, 1969; Moore, Morowaski, Finkelstein & Fordtran, 1971; Hubel, 1974). Therefore it would appear that, while the ileum appears to be a rather quiescent bicarbonate secretor under normal conditions, if it is stimulated it has a very large capacity to elevate its output.

Summary of perfused loop experiments

Exposing in vivo loops of rat jejunum to E.coli STa enterotoxin resulted in a complete inhibition of both net fluid absorption and luminal acidification. In the ileum, STa significantly elevated a normally low luminal alkalinisation. Forskolin and

Figure 3.9 Effect of E.coli STa toxin on luminal acid-base balance in perfused in vivo loops of rat distal ileum. Details as for Figure 3.1.



'Dorbanex' both abolished net fluid absorption in the jejunum, while reducing luminal acidification. C.perfringens enterotoxin had no effect on either net fluid transport or luminal acidification.

3.2 MUCOSAL SURFACE pH EXPERIMENTS

Rat jejunum acidifies its luminal perfusates *in vivo*. It has been proposed that this acidification occurs as a consequence of maintaining a low surface pH at the intestinal mucosal surface (Blair et al., 1975). This low surface pH, or 'acid microclimate' region in the rat jejunum was first demonstrated by Lucas et al (1975) using pH microelectrodes *in vitro*. A low jejunal surface pH has subsequently been demonstrated in several studies both *in vitro* (Lucas & Blair, 1978; Rechkemmer et al., 1979; Lucas et al., 1980; Daniel et al., 1985; Shiau et al., 1985; Daniel & Rehner, 1986; Shimada, 1986) and *in vivo* (Lucas, 1983; Hogerle & Winne, 1983; Iwatsubo et al., 1986).

The finding of the present study, that exposing rat jejunum to E.coli STa enterotoxin results in the cessation of luminal acidification, confirmed a previous report (Lynch, 1986) and raised the possibility that STa might bring about an elevation in the microclimate pH. To investigate this further a series of experiments were undertaken in which the mucosal surface pH of rat small intestine was measured in the presence of E.coli STa enterotoxin. To explore further the likely mechanism of STa action, the effects of a variety of compounds on jejunal mucosal

surface pH were tested.

(a) Measurement of mucosal surface pH of rat jejunum in vivo

When a pH electrode of sufficiently small size was placed onto the mucosal surface of in vivo proximal jejunum (Figure 3.10), an immediate and rapid change in pH was observed with approximately 90% of the final value being detected within fifteen seconds. The rapidity of the response was unchanged over the sixty minute perfusion period.

When the unchallenged proximal jejunum was perfused with Krebs-phosphate buffer the mean mucosal surface pH (Figure 3.11) was $6.34 \pm 0.05(10)$ which was significantly ($P<0.001$) more acid than the perfusing buffer pH ($7.11 \pm 0.01(10)$). The mean surface pH did not alter significantly from this initial value during thirty and sixty minutes of perfusion and remained at values consistent with previously reported measurements (Lucas, 1983)

It has been claimed (Flemstrom et al., 1982) that any mucosal surface acidity measured is an artefact due to tissue anoxia. However as can be seen (Figure 2.5) from a photograph of the tissue preparation adopted for these experiments, the tissue was obviously receiving a full, uninterrupted blood supply. Anoxia is therefore unlikely to be the explanation for the observed phenomenon. When anoxia was induced by occlusion of all afferent mesenteric blood vessels supplying the tissue (Figure 3.11), the mean mucosal surface pH rose significantly ($P<0.001$) to $6.71 \pm 0.05(10)$ within fifteen minutes. This alkalinisation

Figure 3.10 Typical trace showing change in pH when an electrode is placed onto the mucosal surface of rat proximal jejunum <u>in vivo</u> ("On") and returned to the bulk solution ("Off") before (t_0) and after sixty minutes (t_{60}) of perfusion with Krebs phosphate buffer at 1.0ml/min.

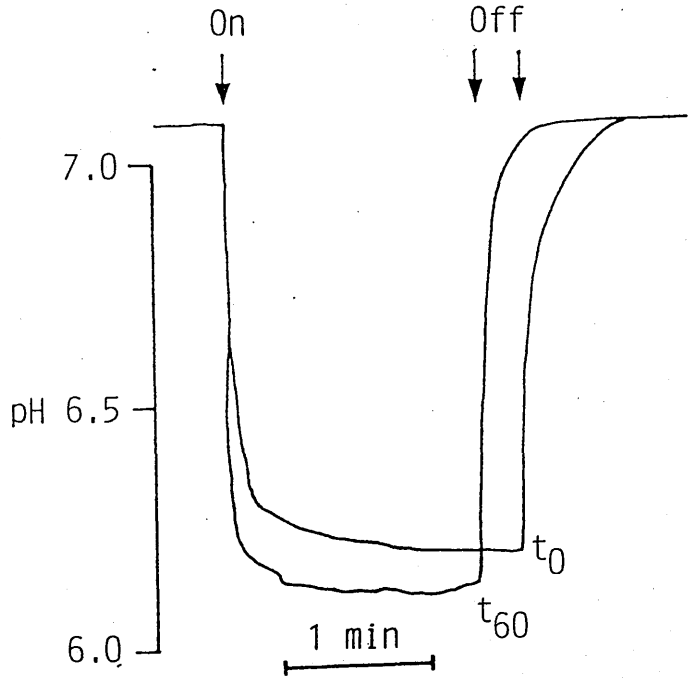
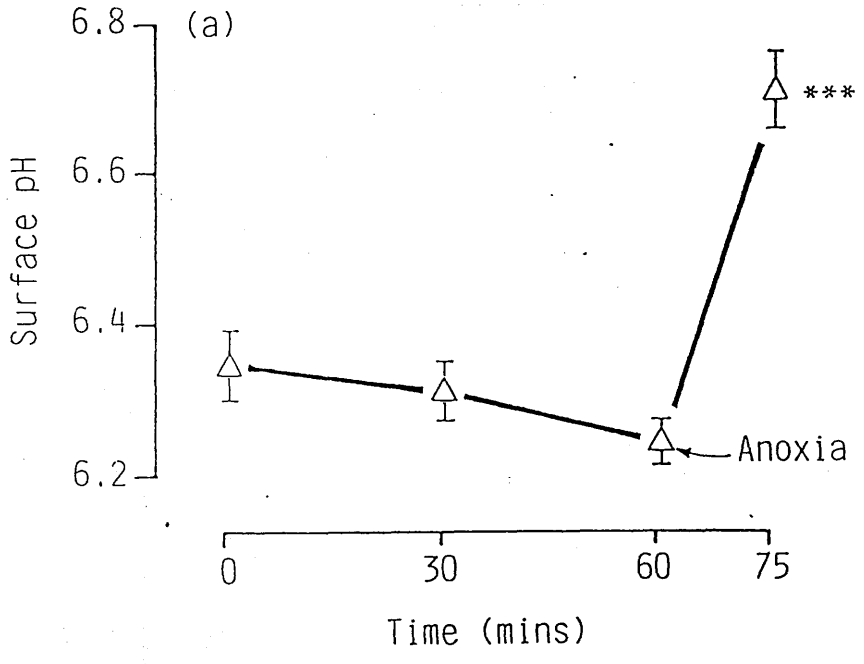


Figure 3.11 Mucosal surface pH of rat proximal jejunum in vivo during sixty minutes of perfusion with Krebs phosphate buffer (pH 7.1). After sixty minutes blood flow was occluded. Values are given as mean S.E.M. for 10 experiments, one observation per animal at the specified times. (*** = $P < 0.001$ compared with value at t_{60}).



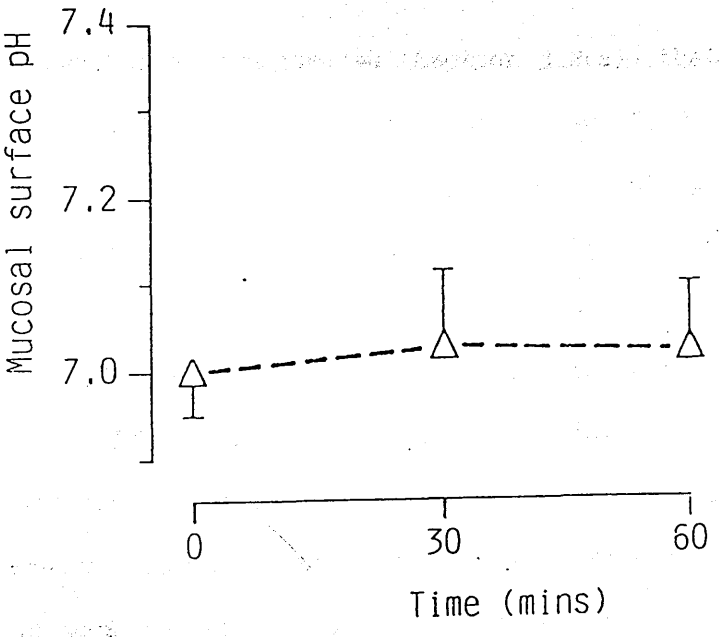
response to anoxia confirmed previous findings (Lucas, 1983). It should also be recalled that in experiments on acidification, using dead animals (i.e. completely anoxic) there was no acidification of the perfusate. Thus in the light of this evidence anoxic artefact can be completely ruled out as the cause of acidification.

(b) Measurement of mucosal surface pH of rat ileum in vivo

The rat ileum has long been considered as capable of active bicarbonate secretion (Parsons, 1956; Hubel, 1967; Hubel, 1969). However the results obtained from the perfused loop experiments presented in Section 3.1(e) suggested that the ileum secretes very little, if any, bicarbonate under unstimulated control conditions. To date, there has only been one measurement of *in vivo* rat ileal mucosal surface pH (Lucas, 1983) where a value of 7.23 was recorded.

In the present study, when rat ileum was perfused with Krebs-phosphate buffer (Figure 3.12) the mean ileal mucosal surface pH was $7.00 \pm 0.05(5)$. As in the jejunum, this value did not change significantly over the sixty minute perfusion period. This neutral surface pH measurement provides further evidence that the ileum is not secreting appreciable amounts of bicarbonate under these control conditions. One might expect a bicarbonate secreting tissue to have a considerably more alkaline surface.

Figure 3.12 Mucosal surface pH of rat distal ileum *in vivo* during sixty minutes of perfusion with Krebs phosphate buffer (pH 7.1). Values are given as mean ± S.E.M. for five experiments, one observation per animal at the specified times.

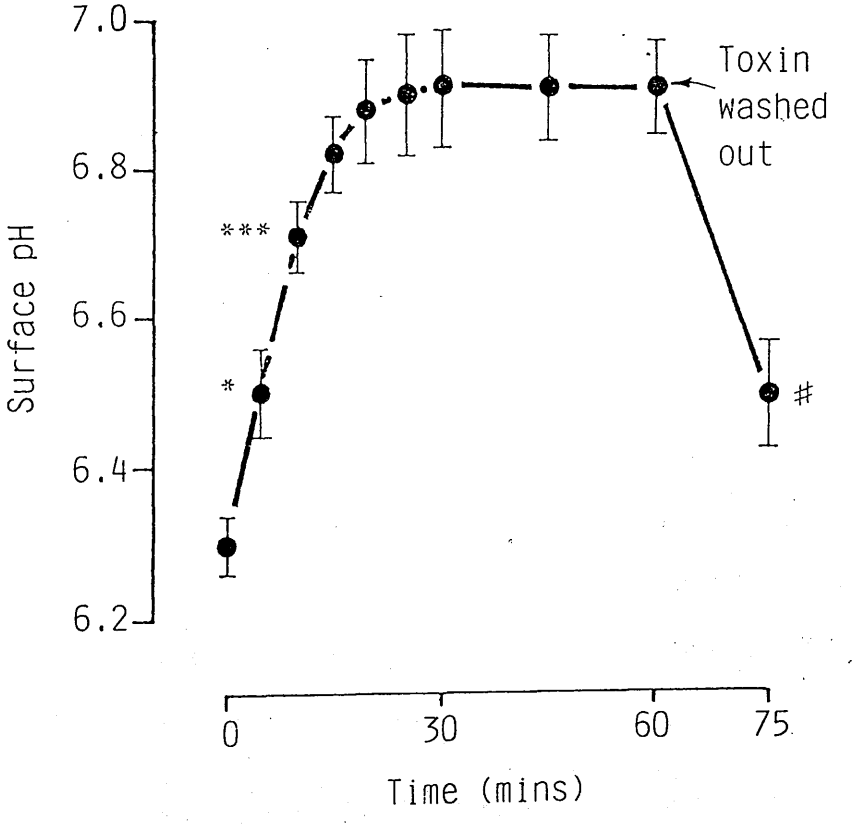


(c) Effect of E.coli STa enterotoxin on jejunal surface pH

From experiments on *in vivo* perfused loops of rat jejunum it was demonstrated that exposing the jejunal mucosa to *E.coli* STa toxin resulted in a cessation of the luminal acidification observed in the unchallenged jejunum. The following experiments were undertaken to ascertain whether this effect might bring about changes in mucosal surface pH in the jejunum.

It was previously demonstrated (Section 3.2(a)) that the mucosal surface pH of *in vivo* rat jejunum was $6.34 \pm 0.05(10)$ and that this remained unchanged during sixty minutes of perfusion with Krebs-phosphate buffer (pH 7.1). When *E.coli* STa enterotoxin was incorporated into this perfusate in a concentration of 14ug/ml (Figure 3.13) the mean mucosal surface pH rose significantly ($P<0.02$) within five minutes of toxin exposure. This alkalinisation effect, the acid mucosal surface becoming effectively neutral, was complete at thirty minutes, by which time the mean surface pH was $6.91 \pm 0.08(10)$. The mucosal surface remained at this elevated level for as long as the enterotoxin was present in the perfusate. When the toxin was washed out of the chamber and replaced with fresh, toxin-free buffer, the mean mucosal surface pH fell significantly ($P<0.001$) to values of $6.50 \pm 0.07(10)$ within fifteen minutes of toxin removal. The alkalinising effect of STa is therefore both rapid and readily reversible as is consistent with STa effects on net fluid transport in the same tissue (Mullen et al., 1978).

Figure 3.13 Effect of E.coli STa toxin on mucosal surface pH of rat proximan jejunum in vivo. After sixty minutes, toxin was washed out and toxin-free buffer was perfused. Values are given as mean $\pm$ S.E.M. for ten experiments, one observation per animal at the specified times (* = $P < 0.02$; ** = $P < 0.001$ compared with value at t_0; # = $P < 0.001$ compared with value at t_{60}).



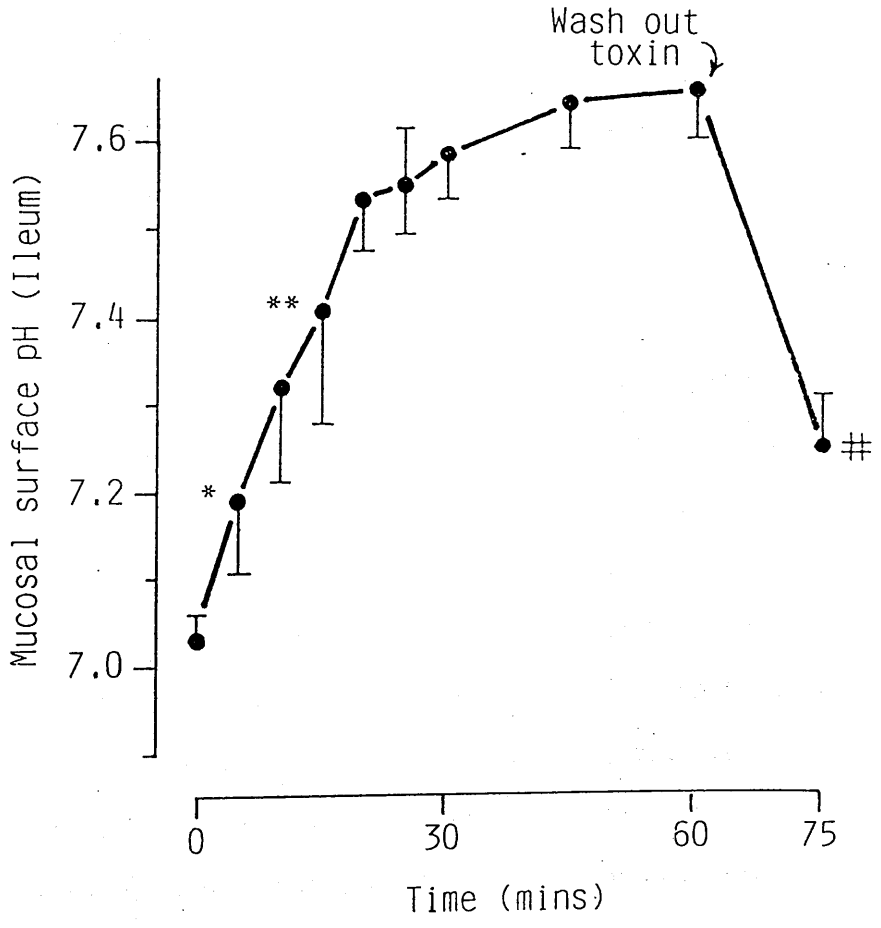
(d) Effect of E.coli STa enterotoxin on ileal surface pH

Exposing the ileal mucosa to E. coli STa toxin resulted in near negligible net bicarbonate secretion in normal ileum being elevated almost nine fold. Since STa elevated the mucosal surface pH in the jejunum there was a strong possibility that this increased ileal luminal alkalinisation after STa challenge would cause an elevation of the mucosal surface pH. To test this, similar experiments to those described in the previous section were undertaken in the distal ileum.

As predicted, when the ileal mucosa was perfused with Krebs-phosphate buffer containing 14ug/ml of E.coli STa enterotoxin (Figure 3.14) mucosal surface pH rose significantly ($P<0.05$) within five minutes. The mucosal surface continued to alkalinise, considerably above bulk solution pH (7.10), reaching values of $7.67\pm0.06(5)$. As in the jejunum, when the toxin was removed from the chamber and the tissue was perfused with toxin-free Krebs-phosphate buffer, the surface pH returned towards control values, falling significantly ($P<0.001$) to $7.26\pm0.03(5)$ after fifteen minutes.

Exposure to E.coli STa enterotoxin resulted in a significant elevation of surface pH in both jejunum and ileum. In both tissues the effect was rapid and readily reversible. The currently accepted view is that STa induces its effects on fluid transport by stimulating particulate guanylate cyclase (Field, Graf, Laird & Smith, 1978) and thereby elevating intracellular

Figure 3.14 Effect of E.coli STa toxin on mucosal surface pH of rat distal ileum in vivo. After sixty minutes, toxin was washed out and toxin-free buffer was perfused. Values are given as mean $\pm$ S.E.M. for five experiments, one observation per animal at the specified times (* = $P < 0.05$; ** = $P < 0.001$ compared with value at t_0; # = $P < 0.001$ compared with value at t_{60}).



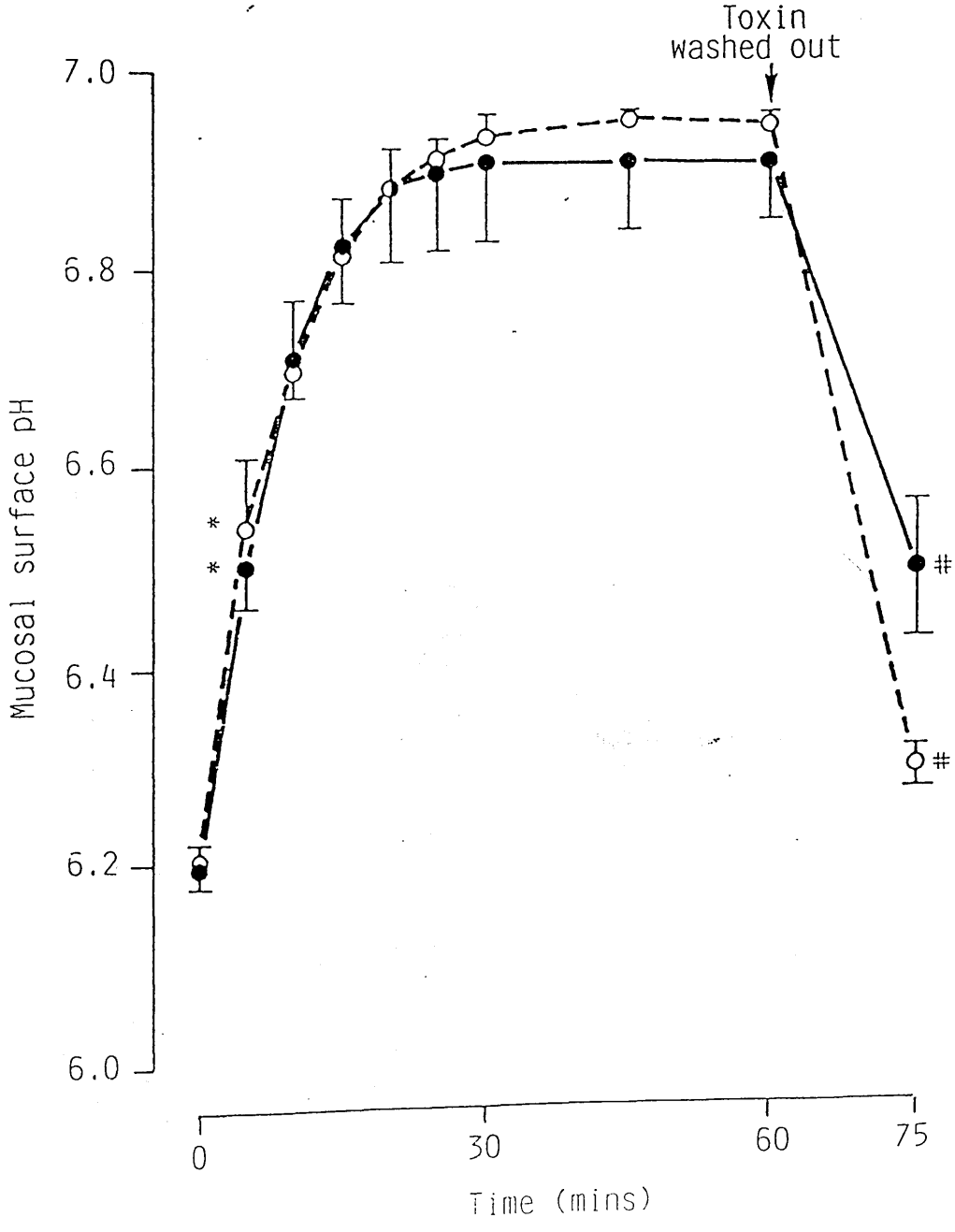
cGMP levels (Hughes, Murad, Chang & Guerrant, 1978; Field et al., 1978). It is thought that elevated cGMP levels have a direct role in the secretory process induced by STa. There is therefore a possibility that STa-induced mucosal alkalinisation is also mediated through cGMP. Experiments were undertaken to test this view. In order to simplify interpretation and exclude location as a factor causing variability, all subsequent surface pH experiments were restricted to the jejunum.

(e) Effect of STh(6-19) peptide on jejunal surface pH

To ensure that the alkalinisation response observed after jejunal exposure to STa was actually induced by the toxin rather than by some indigenous product in the toxin preparation, the effect of a synthetically produced STa analogue on jejunal surface pH was investigated. This toxin analogue was a shorter form of a heat stable enterotoxin produced by a human strain of enterotoxigenic E.coli (strain SK-1) consisting of fourteen amino acid residues (Aimoto, Ikemura, Shimonishi, Takeda, Takeda & Miwatani, 1983). This synthetic peptide has previously been shown to be 2-5 times more potent than native STa but exhibits the same biological properties. The amino acid sequence of this peptide, referred to as STh(6-19) peptide, constitutes the thermodynamically stable and biologically active site of ST toxins (Aimoto et al., 1983).

When the mucosal surface of in vivo rat jejunum was exposed to STh(6-19) peptide (250ng/ml) (Figure 3.15) there was a rapid elevation of surface pH, a significant ($P<0.001$) increase being

Figure 3.15 Comparative effects of E.coli STa toxin (—●—) and STh(6-19) peptide (- -○- -) on mucosal surface pH of rat proximal jejunum in vivo. Values are given as mean ± S.E.M. for five (STh[6-19]) and ten (STa) experiments, one observation per animal at specified times (* = $P < 0.001$ compared with value at t_0; # = $P < 0.001$ compared with value at t_{60}).



detected within five minutes. After sixty minutes exposure the mean surface pH was measured as $6.95 \pm 0.01(5)$. The response to STh(6-19) was statistically indistinguishable (Figure 3.15) in both time course and magnitude from the response to native STa. As with the native toxin, when the peptide was removed from the chamber and replaced with fresh buffer, the surface pH returned rapidly to acid values, falling significantly ($P<0.001$) to $6.30 \pm 0.02(5)$ after fifteen minutes.

This result had two implications. Firstly, since STh(6-19) is a synthetically produced, highly purified chemical, it seems very unlikely that the effect of native STa on intestinal mucosal surface pH is due to anything other than the enterotoxigenic activity of STa alone. Secondly, if this is a valid assumption, then it is not unreasonable to predict that the presented phenomenon may be due to an STa-induced elevation of intracellular cGMP. The following three sets of experiments investigated this possibility further.

(f) Effect of E.coli STa enterotoxin in combination with theophylline on jejunal surface pH

Theophylline, a phosphodiesterase inhibitor which inhibits both guanylate and adenylate cyclase activities in rat small intestine (Quill & Weiser, 1975), has been shown (Hamilton, Forsyth, Roe & Nielsen, 1978) to enhance intestinal secretory activity when combined with cholera toxin, E.coli LT toxin, and E.coli STa toxin. A series of experiments was undertaken to determine

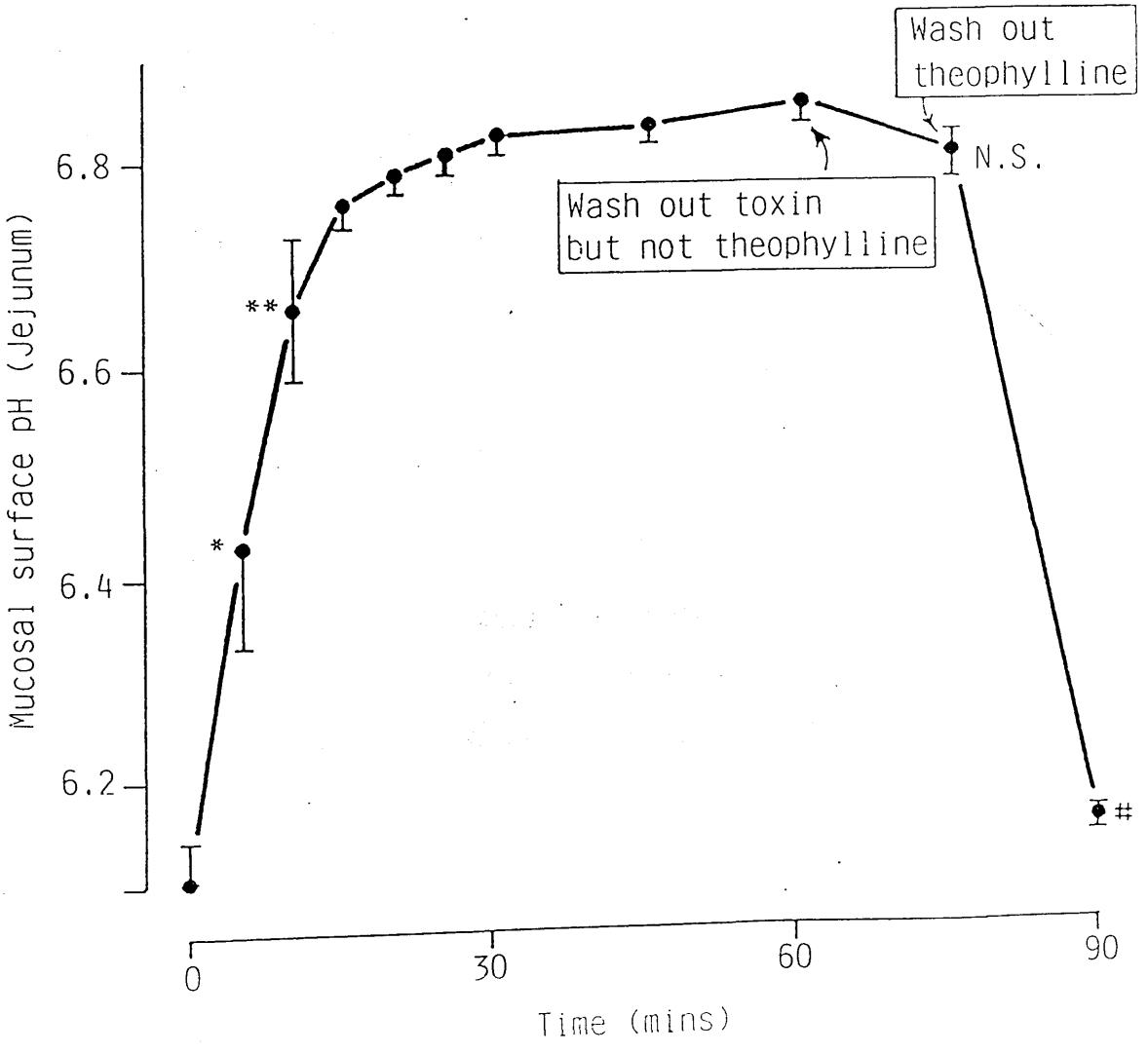
whether theophylline would augment the alkalinisation of jejunal surface pH induced by STa.

Exposing rat jejunum to a combination of E.coli STa toxin (14ug/ml) and theophylline (20mmol/l) (Figure 3.16) resulted in no significant change to either the time course or the magnitude of the normal alkalinisation response to STa (Figure 3.13), and therefore theophylline did not add to the surface pH effects of STa. After sixty minutes, the mean mucosal surface pH of $6.86\pm0.02(6)$ was not significantly different from the value of $6.91\pm0.08(10)$ observed after exposure to STa alone. However, when STa was removed from the chamber and replaced by fresh buffer, containing theophylline (20mmol/l) alone, surface pH did not return to control values as before. After fifteen minutes of exposure to theophylline the mean surface pH had not changed significantly, remaining at an elevated level of $6.81\pm0.04(6)$. Removing theophylline from the chamber at this time resulted in a very rapid return to acid values ($P<0.001$), the mean surface pH after fifteen minutes being $6.15\pm0.01(6)$. This value was not significantly different from the mean surface pH of $6.10\pm0.04(6)$ measured at the onset of these experiments.

(g) Effect of theophylline on jejunal surface pH

To investigate the possibility that the result presented in the previous section might be solely due to the actions of theophylline, in the following experiments the jejunal mucosa was exposed to theophylline alone. Theophylline (20mmol/l) had no

Figure 3.16 Effect of E.coli STa toxin in combination with theophylline (20mM) on mucosal surface pH of rat proximal jejunum in vivo. STa and theophylline were replaced after sixty minutes by toxin-free Krebs buffer containing theophylline (20mM) followed by Krebs buffer alone after seventy five minutes. Values are given as mean $\pm$ S.E.M. for six experiments, one observation per animal at the specified times (* = $P < 0.02$; ** = $P < 0.001$ compared with value at t_0; # = $P < 0.001$ compared with value at t_{60}).



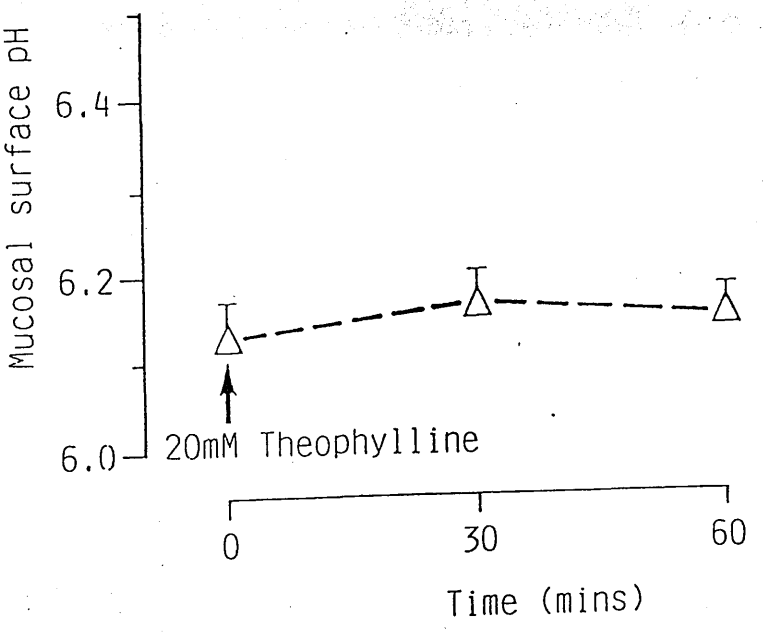
effect (Figure 3.17) on jejunal surface pH. The pH of 6.16±0.03(5) measured after sixty minutes did not differ significantly from the value of 6.13±0.04(5) measured at the onset of these experiments.

Theophylline seems incapable of altering surface pH when applied alone. This may be because the basal cGMP phosphodiesterase activity in the rat small intestine is low and hence inhibiting this does not change the intracellular cGMP levels to any great extent. Alternatively, since theophylline is a weak base with a pKa of 8.75, there is a possibility that, at the surface pH prevailing in the jejunum, there is insufficient unionised theophylline presented to the mucosal surface to permit enough drug to traverse the brush border membrane. In the presence of STa toxin, however, the surface pH is elevated and, therefore more of the unionised species would be present to enter the cell. This latter argument seems the more plausible since the more permeable form of theophylline, aminophylline (an ethylenediamine/theophylline complex) had profound effects on rat jejunal surface pH *in vitro* (Lucas & Blair, 1978).

(h) Effect of 8-bromo cGMP on jejunal surface pH

Since the results of the preceeding experiments all pointed to cGMP as the likely mediator of toxin induced alkalinisation an attempt was made to induce an elevation of surface pH using the 8-bromo analogue of cGMP (8-bromo cGMP). This species is more permeable than the native cGMP molecule. Exogenously applied

Figure 3.17 Effect of theophylline (20mM) on mucosal surface pH of rat proximal jejunum *in vivo*. Values are given as mean ± S.E.M. for five experiments, one observation per animal at the specified times.

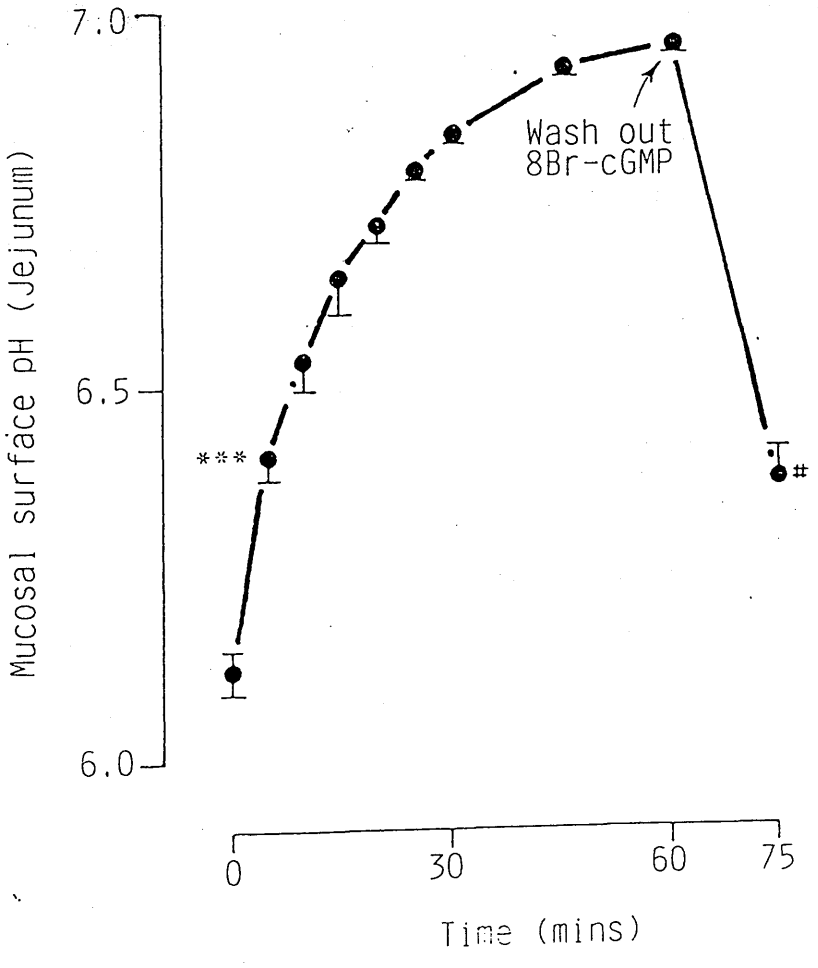


8-bromo cGMP resembles STa effects on intestinal fluid secretion (Hughes et al., 1978) and therefore, if cGMP is involved 8-bromo cGMP should elevate the mucosal surface pH.

In preliminary experiments 8-bromo cGMP was added exogenously to the mucosal surface of rat jejunum in a concentration of 1mmol/l. At this concentration there was no effect of 8-bromo cGMP on surface pH. The mean pH after sixty minutes exposure was $6.24\pm0.03(5)$, not significantly different from the value of $6.20\pm0.04(5)$ measured at the onset of these experiments.

Five mM 8-bromo cGMP does not always alter fluid transport in rat jejunum *in vivo* (Eklund, Jodal & Lundgren, 1986), therefore it is not surprising that 1mM 8-bromo cGMP had no effect on the same tissue; 20mM induces a maximal secretion (Eklund et al., 1986). Therefore, these higher concentrations of 8-bromo cGMP were used. Within five minutes of exposure to 20mM 8-bromo cGMP (Figure 3.18) the jejunal mucosal surface had alkalinised significantly ($P<0.001$) above control values.The mucosa continued to alkalinise for the remainder of the sixty minute perfusion, finally reaching a mean pH of $6.96\pm0.01(5)$. As with STh(6-19) peptide and with the combination of native *E.coli* STa toxin and theophylline, this final value was not significantly different from mean surface pH measured after sixty minutes of perfusion with *E.coli* STa toxin alone. The time course of the response to 20mM 8-bromo cGMP was also not significantly different from the response obtained with native toxin. As with STa, when 8-bromo cGMP was removed from the

Figure 3.18 Effect of 8-bromo cGMP (20mM) on mucosal surface pH of rat proximal jejunum *in vivo*. After sixty minutes 8-bromo cGMP was removed and replaced by fresh buffer. Values are given as mean $\pm$ S.E.M. for five experiments, one observation per animal at the specified times (*** = $P < 0.001$ compared with value at t_0; # = $P < 0.001$ compared with value at t_{60}).



chamber the surface pH returned to acid values, falling significantly ($P<0.001$) to $6.37\pm0.04(5)$ within fifteen minutes. This is perhaps more convincing evidence that cGMP is the mediator of STa induced alkalinisation of *in vivo* rat jejunal mucosa.

To summarise, *E.coli* STa enterotoxin abolished luminal acidification in rat jejunum and increased luminal alkalinisation in rat ileum, thereby causing the elevation of the intestinal mucosal surface pH in both jejunum and ileum after STa challenge. This alkalinisation is both rapid and reversible as is consistent with STa activation of guanylate cyclase and STa inhibition of net fluid absorption. A synthetic, highly purified ST analogue (the fourteen amino acid peptide, STh(6-19)) had the same action on jejunal surface pH as native STa toxin and hence, the effect is probably due to the enterotoxigenic activity of STa, rather than to some contaminant in the STa preparation used in these experiments. Since the known action of STa is to stimulate guanylate cyclase, this would suggest that cGMP is the mediator of the observed surface alkalinisation. Theophylline, a phosphodiesterase inhibitor, prevented the normal reversal of STa effects after toxin removal, presumably by maintaining STa-elevated intracellular cGMP levels. Theophylline alone had no effect on surface pH. Finally the 8-bromo analogue of cGMP reproduced STa action on jejunal surface pH. If cGMP is the mediator of STa action on intestinal surface pH, the question of whether this phenomenon is a specific consequence of cGMP

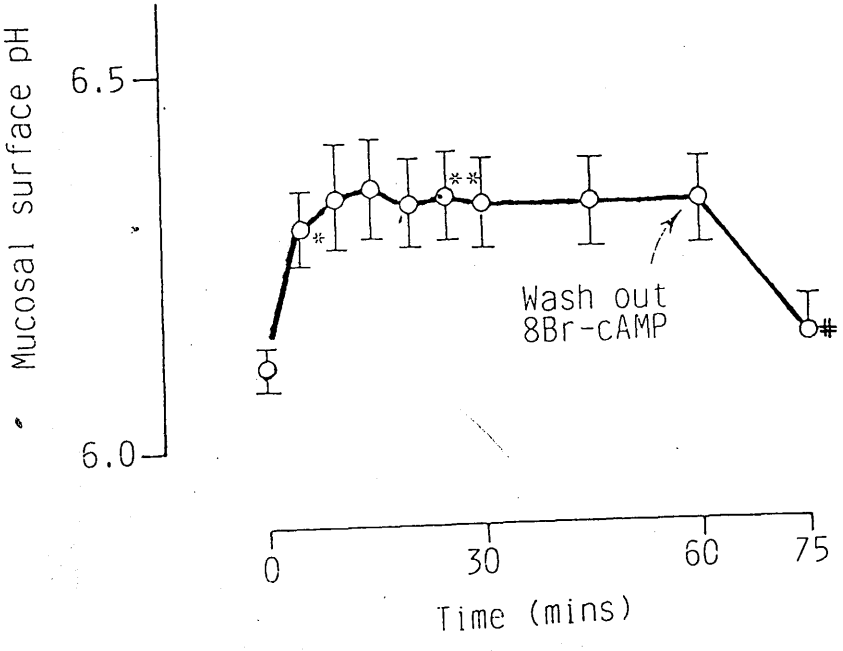
dependent secretory processes or merely a general response to any secretory stimulus arises. In an attempt to answer this question, experiments investigated the effects of several cAMP dependent secretory agents on jejunal surface pH.

(i) Effect of 8-bromo cAMP on jejunal surface pH

As a direct comparison between cGMP and cAMP dependent secretory processes, the effect of the 8-bromo analogue of cAMP on jejunal surface pH was investigated (Figure 3.19). As with 8-bromo cGMP when 8-bromo cAMP was applied to the mucosal surface in a concentration of 1mmol/l there was no change in surface pH, the mean pH after sixty minutes exposure was $6.16\pm0.05(5)$. This was not significantly different from the value of $6.12\pm0.04(5)$ measured at the commencement of these experiments.

Increasing the concentration of 8-bromo cAMP to 20mmol/l elevated the surface pH significantly ($P<0.02$) within five minutes, however the effect was significantly ($P<0.001$) lower than that induced by 8-bromo cGMP at the same concentration. The maximum pH attained was $6.35\pm0.07(5)$, after fifteen minutes of exposure. The surface pH remained approximately at this level until the end of the sixty minute perfusion when the mean pH was $6.33\pm0.06(5)$. Removing 8-bromo cAMP from the chamber resulted in the surface pH returning to control values, falling significantly ($P<0.05$) to values of $6.15\pm0.05(5)$, not significantly different from the value of $6.11\pm0.03(5)$ measured at the onset of the experiments.

Figure 3.19 Effect of 8-bromo cAMP (20mM) on mucosal surface pH of rat proximal jejunum *in vivo*. After sixty minutes 8-bromo cAMP was removed and replaced by fresh buffer. Values are given as mean $\pm$ S.E.M. for five experiments, one observation per animal at the specified times (* = $P < 0.02$; ** = $P < 0.01$ compared with value at t_0; # = $P < 0.05$ compared with value at t_{60}).



Although 8-bromo cAMP causes an elevation of mucosal surface pH, the effect is very small compared to that of 8-bromo cGMP, E.coli STa toxin and STh(6-19) peptide. This would suggest that cAMP is a less potent effector of surface pH changes than cGMP.

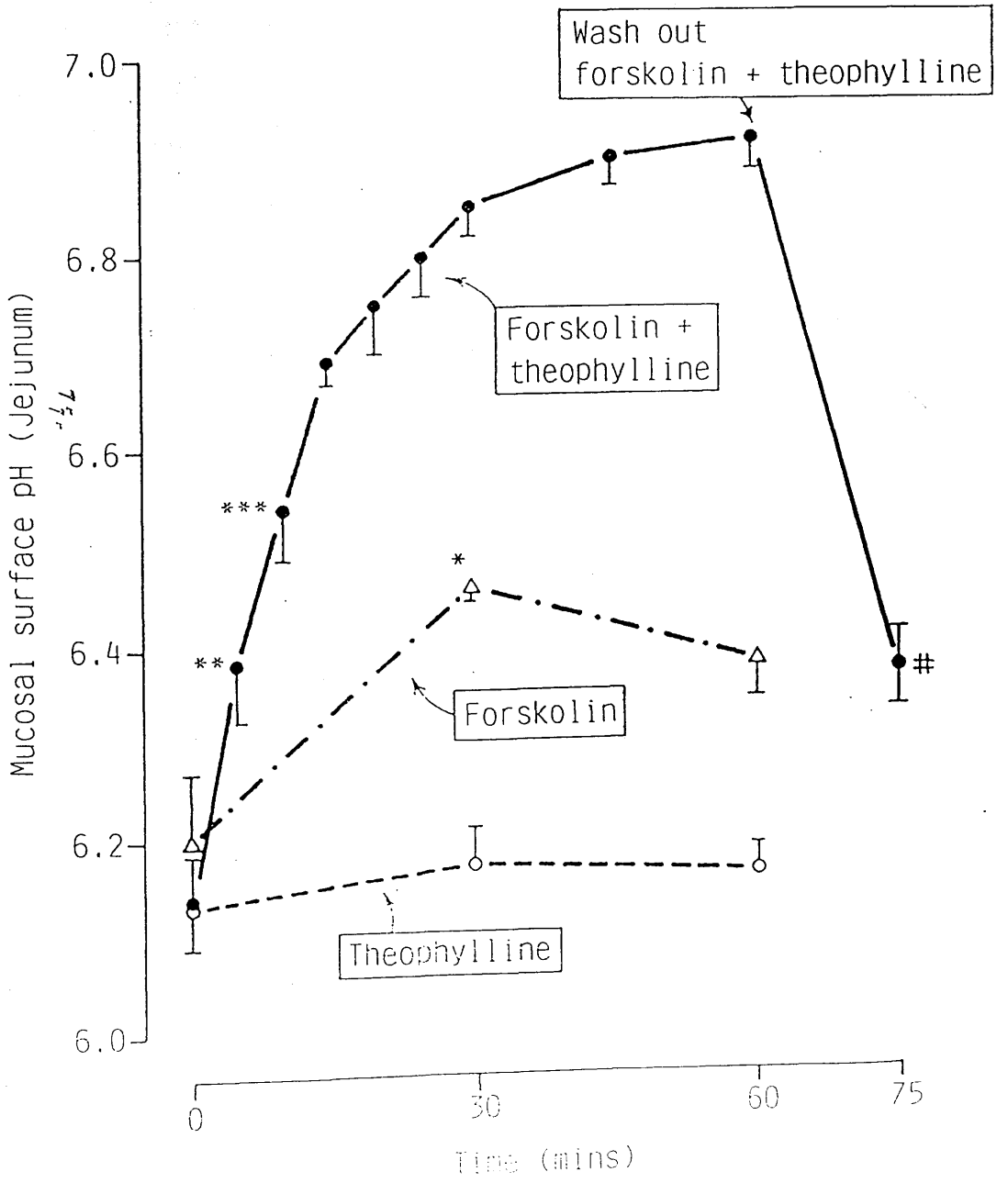
(j) Effect of forskolin on jejunal surface pH

To investigate whether the effect of 8-bromo cAMP could be reproduced by elevating intracellular cAMP levels through adenylate cyclase ,the effect of forskolin on surface pH was studied. Forskolin, a diterpene of the labdane family, is a potent adenylate cyclase activator in a variety of tissues (Seamon & Daly, 1981), including the intestine (Boige et al., 1984).

Because in previous experiments 0.1mM forskolin reduced luminal acidification in the jejunum but failed to abolish it completely the concentration of forskolin in the perfusate was increased to 1mmol/l. This is unusually high for in vitro experiments but seems to be necessary to demonstrate similar effects in vivo (B. Bridges, personal communication), perhaps because of the rapid absorption and hence clearance away from the site of action of forskolin.

When the mucosal surface was exposed to forskolin (1mM), there was a significant ($P<0.05$) elevation (Figure 3.20) of surface pH after thirty minutes, the mean pH being $6.46\pm0.01(3)$. However, the surface pH fell again over the next thirty minutes to $6.38\pm0.04(3)$ which was not significantly different from the mean

Figure 3.20 Effects of forskolin (1mM) (-·-△-·-), theophylline (20mM) (--O--) and forskolin (1mM) plus theophylline (20mM) (—●—) on mucosal surface pH of rat proximal jejunum <u>in vivo</u>. Values are given as the mean ± S.E.M. for five (theophylline and forskolin + theophylline) and three (forskolin) experiments, one observation per animal at specified times (* = $P < 0.05$; ** = $P < 0.02$; $P < 0.001$ compared with value at t_0; # = $P < 0.001$ compared with value at t_{60}).



starting pH, 6.20±0.07(3). This effect of forskolin is consistent with the reduced, but not abolished, luminal acidification observed in perfused loop experiments when forskolin was incorporated in the perfusate.

In separate experiments, a combination of theophylline (20mM) and forskolin (1mM) (Figure 3.20) caused an elevation of surface pH which, like the response to STh(6-19), was statistically indistinguishable in both time course and magnitude from that induced by native STa. A significant ($P<0.02$) increase in surface pH was detected within five minutes and this continued to rise for the remainder of the sixty minute perfusion period when values of 6.92±0.03(5) were recorded. As with STa, removing forskolin and theophylline from the tissue resulted in a reversal of the response, surface pH falling significantly ($P<0.001$) to values of 6.37±0.04(5).

Therefore, the responses to forskolin and to 8-bromo cAMP were very similar, demonstrating that cAMP is less effective than cGMP at inducing mucosal surface alkalinisation. However, when theophylline was combined with forskolin the alkalinisation response was virtually identical to those obtained with the cGMP elevating agents.

Since theophylline was ineffectual when applied alone, the presence of forskolin must have permitted more theophylline to enter the enterocytes than was possible under control conditions. This is possible since the elevation of surface pH observed with

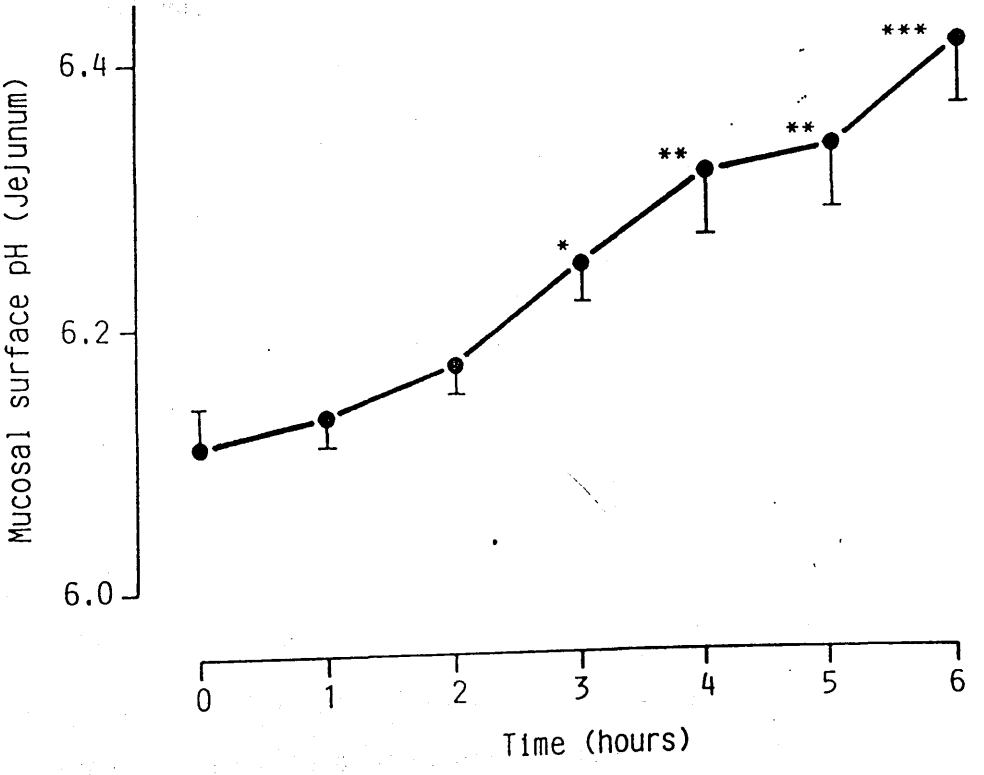
forskolin might allow sufficient theophylline to enter the cell by non-ionic diffusion than would be possible under the normally acid surface conditions. Since theophylline inhibits cAMP and cGMP phosphodiesterase (Quill & Weiser, 1975), breakdown of both these compounds would be prevented. However, since 8-bromo cAMP was relatively ineffective in elevating surface pH it seems more likely that the increased alkalinisation with theophylline is due to a build up of cGMP.

(k) Effect of cholera toxin on jejunal surface pH

To investigate further the effects of cAMP-dependent secretory processes on surface pH, the jejunal mucosa was subjected to prolonged exposure to cholera toxin. Cholera toxin, like forskolin, stimulates adenylate cyclase and thereby raises intracellular cAMP levels (Field, Fromm, Al-Awqati & Greenough, 1972). This effect, following a characteristic lag period, is essentially irreversible for the duration of the lifetime of the cell (Lonnroth, 1983).

Cholera toxin (20ug/ml) had no significant effect on jejunal surface pH (Figure 3.21) until 150 minutes had elapsed. After six hours the mucosal surface had alkalinised to values of $6.42\pm0.05(5)$, significantly ($P<0.001$) higher than the value measured at the onset of these experiments, $6.11\pm0.03(5)$. This was a very similar, although much slower, elevation of surface pH to that observed with both 8-bromo cAMP and forskolin. This must be regarded as evidence that cAMP-dependent secretory processes

Figure 3.21 Effect of cholera toxin on mucosal surface pH of rat proximal jejunum *in vivo*. Values are given as mean ± S.E.M. for five experiments, one observation per animal at the specified times (* = $P < 0.02$; ** = $P < 0.01$; *** = $P < 0.001$ compared with value at t_0).



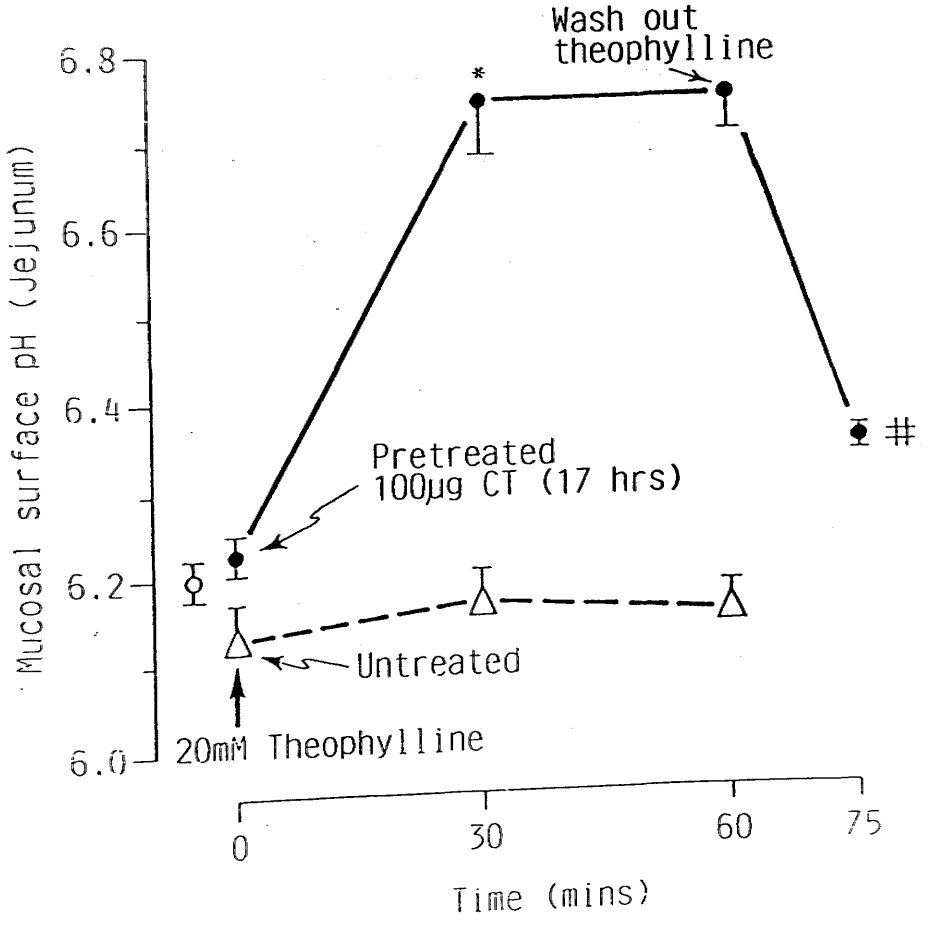
are much less effective inducers of surface alkalinisation than cGMP-dependent processes.

Additional experiments were undertaken in which rat jejunum was pretreated with 100ug of cholera toxin seventeen hours prior to surface pH measurement. In these animals, when the intestine was prepared for surface pH measurement, the marked and localised oedematous state of the tissue was evidence that the toxin had been fully active.

When the jejunal mucosal surface pH was measured in these cholera toxin treated animals (Figure 3.22) the mean pH was $6.23\pm0.04(4)$. This value is significantly higher than the mean jejunal surface pH obtained from all the surface pH experiments performed in this study ($6.18\pm0.01(84)$). This difference is only significant because of the large number of control observations (n=84) and probably does not reflect a true population difference.

However, when the cholera toxin treated intestine was perfused with buffer containing 20mM theophylline (Figure 3.22) the surface pH rose significantly ($P<0.001$) to $6.75\pm0.06(4)$ after thirty minutes and $6.76\pm0.04(4)$ after sixty minutes. Removal of theophylline from the perfusate resulted in the surface pH falling significantly ($P<0.001$) within fifteen minutes to $6.36\pm0.01(4)$. Therefore, as with forskolin, adding theophylline to cholera toxin-treated tissue caused a larger elevation of surface pH than was evident when either compound was applied alone.

Figure 3.22 Effect of theophylline (20mM) on mucosal surface pH of *in vivo* rat proximal jejunum pretreated (17 hours) with cholera toxin. Pretreatment with cholera toxin is presented at t_0 (●) with control value (○) for comparison. Cholera toxin plus theophylline (20mM) is shown (—●—) with theophylline replaced by Krebs buffer after sixty minutes and compared with untreated jejunum (- - △- -) exposed to theophylline (20mM) alone. Values are given as mean ± S.E.M. for 67 (control), 4 (cholera toxin pretreatment) and 5 (theophylline alone) experiments, one observation per animal at specified times (* = $P < 0.001$ compared with CT at t_0; # = $P < 0.001$ on comparison with CT plus theophylline at 60 minutes).



(1) Effect of Staphylococcus aureus d toxin on jejunal surface pH

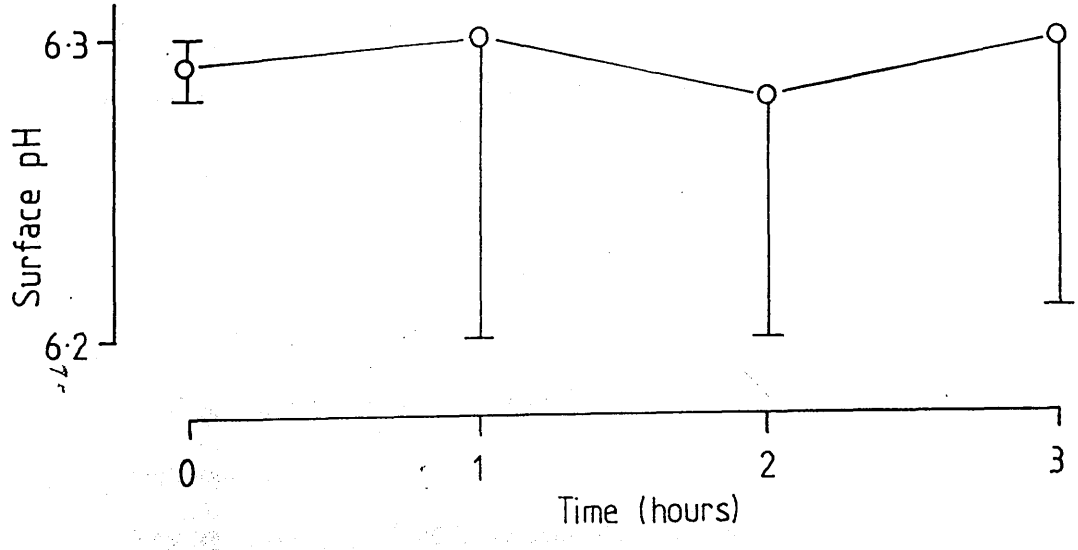
Staphylococcus aureus d toxin has been shown to exhibit enterotoxigenic properties, inhibiting net fluid absorption in guinea pig and rabbit small intestine (Kapral, O'Brien & Drugen, 1976). The toxin induces this fluid secretion by elevating intracellular cAMP levels. However the mechanism whereby cAMP production is increased is not fully understood.

When rat jejunal mucosa was exposed to S.aureus d toxin (1mg/ml) (Figure 3.23), there was no significant change in the mucosal surface pH over the three hour perfusion period. The mean pH after three hours was 6.30±0.09(3) compared to 6.29±0.01(3) at the onset of the experiments. It must be concluded that S.aureus d toxin had no effect on rat jejunal surface pH in vivo.

Summary of surface pH experiments

The mucosal surface pH of rat small intestine was measured in vivo. With a buffer pH of 7.1, surface pH in the jejunum was 6.18±0.01(84) while a value of 7.00±0.05(5) was measured in the ileum. E.coli STa enterotoxin induced a rapid and reversible alkalinisation of both jejunal and ileal mucosae to values of 6.91±0.08(10) and 7.67±0.06(5) respectively. The synthetic ST analogue, STh(6-19), and 8-bromo cGMP had similar effects to STa on jejunal mucosal surface pH while theophylline maintained the STa-elevated jejunal surface pH after toxin removal. These findings suggest that cGMP is the likely mediator of STa-induced

Figure 3.23 Effect of Staphylococcus aureus d toxin on mucosal surface pH of rat proximal jejunum in vivo. Values are given as mean ± S.E.M. for three experiments, one observation per animal at the specified times.



surface alkalinisation. 8-bromo cAMP, forskolin and cholera toxin had considerably lesser effects on jejunal surface pH, although combining forskolin and cholera toxin with theophylline resulted in significant alkalinisation of the jejunal mucosa. Theophylline alone and S.aureus d toxin had no effect on jejunal surface pH. These results indicate that cAMP-dependent secretory processes are less capable of inducing surface pH changes than those dependent on cGMP.

3.3 MEASUREMENT OF pH PROFILE OF INTESTINAL VILLI

The existence of a pH gradient along the villus-crypt axis of rat jejunal villi *in vitro* has been demonstrated using antimony microelectrodes (Daniel et al., 1985). Detection of gradients presents the possibility of localising the mucosal surface alkalinisation measured after STa challenge to a specific villus region. Unlike the previous study of Daniel and co-workers, the present investigation used pH-sensitive liquid ion exchange (LIX) microelectrodes.

(a) Effect of E.coli STa enterotoxin on jejunal villus pH profile

When the tip of a pH microelectrode was placed at the base of a jejunal villus and then withdrawn in 50um steps to the villus tip, pH could be determined at discrete points along the villus. From traces from electrode experiments (Figure 3.24), surface pH could be calculated to provide a distribution profile (Figure 3.25) for a particular villus. Variations in individual villus lengths made it difficult to construct a complete mean pH profile for an average villus. Because of this the values presented are

Figure 3.24 Typical trace obtained by placing the tip of a pH sensitive LIX microelectrode at a jejunal villus base and then removing it in 50um steps to the villus tip (electrode details: slope 55mV/pH unit; tip diameter 10um; response time < 12 seconds).

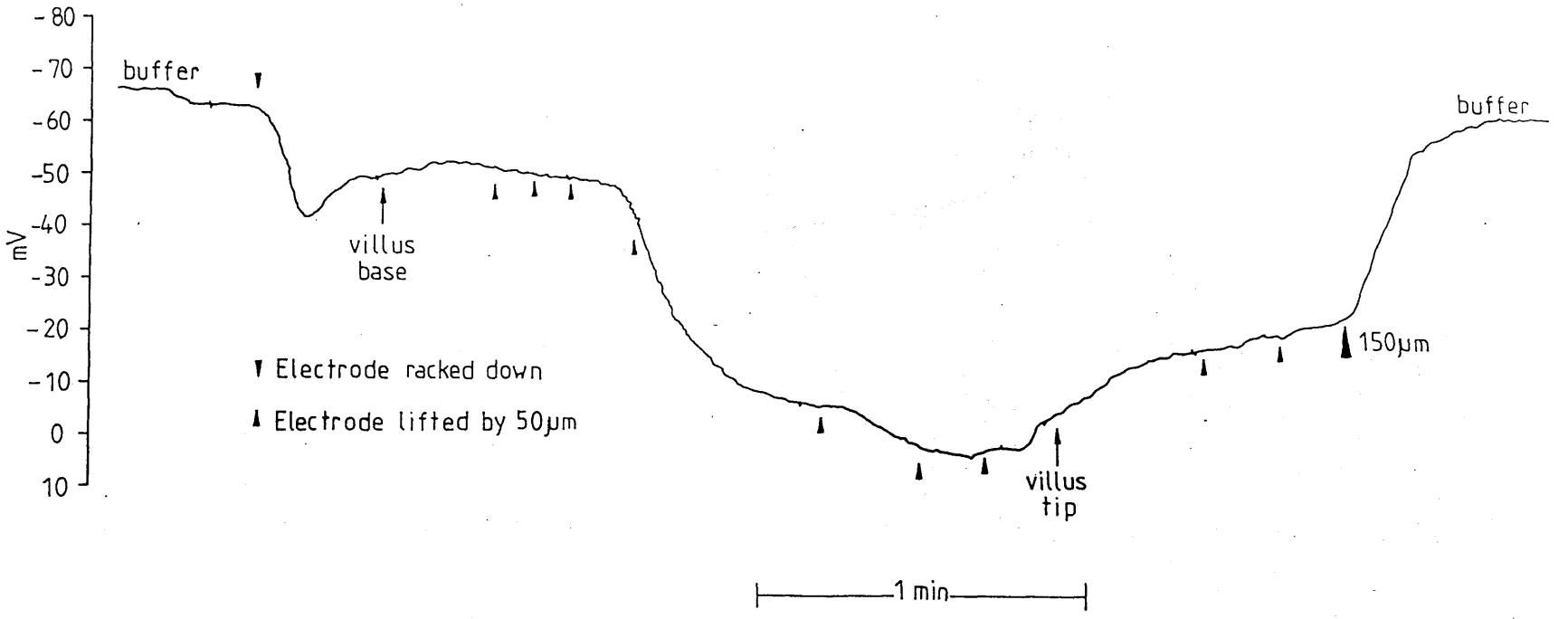
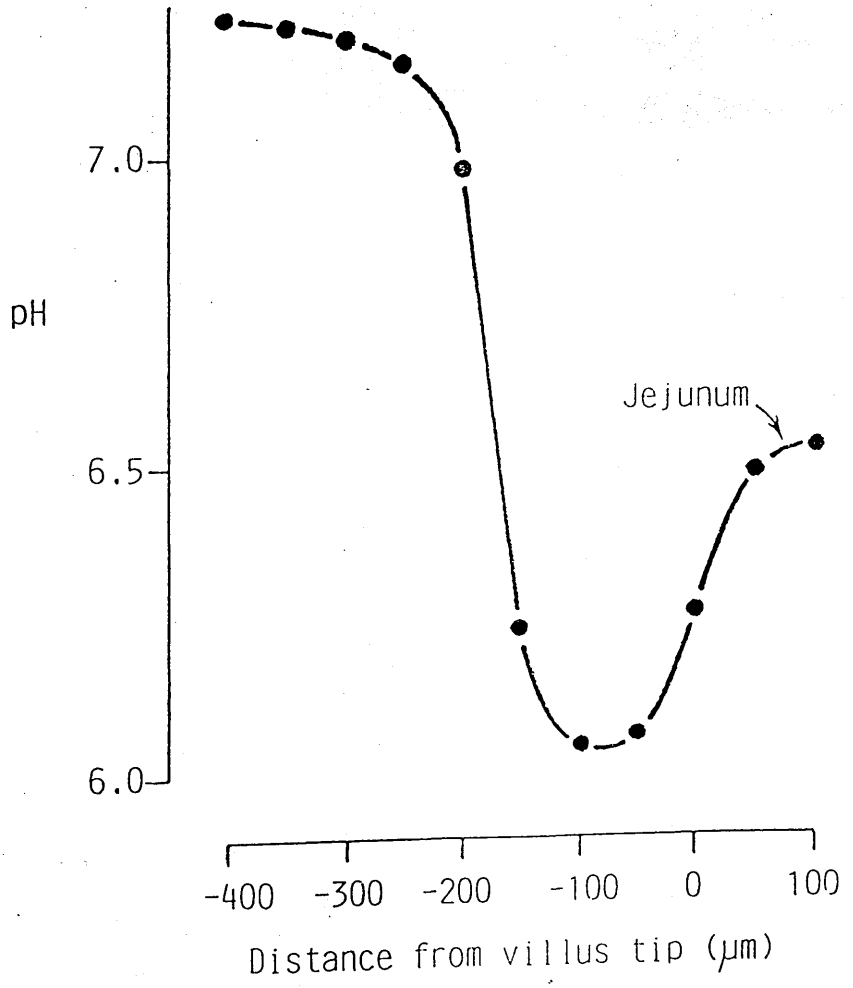


Figure 3.25 Representative pH profile of jejunal villus measured by LIX pH microelectrode in rat intestine <u>in vitro</u> (see text for details).



the mean pH at the villus tip, villus base and the maximum and minimum pH values expressed at their respective positions relative to the villus tip.

In the jejunum (Figure 3.26), with a buffer pH of 7.40 the mean pH at the villus base was 6.86±0.05(10) at a mean villus length of 448±16(10)um. As the electrode was withdrawn along the villus-crypt axis, the local pH rose slightly to a maximum value of 6.93±0.05(10) at a distance of 274±52(10)um below the villus tip. Beyond this there was a sharp fall in pH, reaching a minimum value of 6.50±0.07(10) at 213±27(10)um below the villus tip. The pH then increased up to the villus tip where values of 6.75±0.08(10) were recorded.

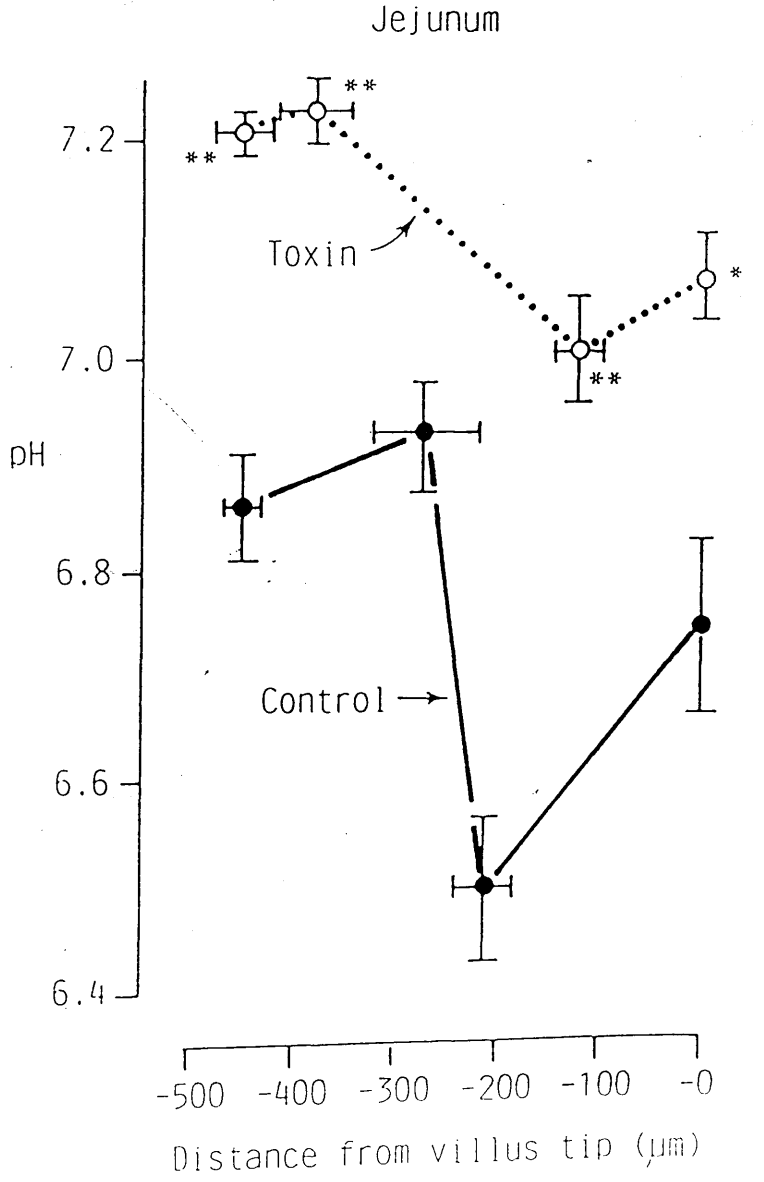
These observations confirm the previous finding with antimony microelectrodes (Daniel et al., 1985) that a pH gradient does exist along the villi of the jejunum but differs in one important respect. In this study, using LIX microelectrodes, the mean pH measured at the villus base was 6.86±0.05(10), much lower than the value of 8.15±0.25(13) measured using antimony electrodes. A similar discrepancy between LIX and antimony electrodes has recently been observed in the duodenum (Daniel, unpublished observations). It has been demonstrated (Tourky & Mousa, 1948; El Wakkad, 1950; Daniel et al., 1985) that at low PO_2 values the pH response of antimony microelectrodes deviates from the normal response in solutions with high PO_2 values. Therefore, there is a distinct possibility that, because of the inaccessibility of the

crypts, there will be a low oxygen tension in this region (despite a high oxygen saturation in the bulk solution). This low PO_2 at the the base of the villi would account for the comparatively high pH values measured by the antimony electrodes in this region. Since the LIX electrodes are not affected by the PO_2 of the solution, the values presented here are probably closer to the true pH values in the lower villus regions.

The lowest pH values measured *in vitro* were higher than the *in vivo* mucosal surface pH values measured using the larger surface electrode. However, it should be noted that the *in vitro* incubation medium did not contain glucose. It has been shown (Lucas & Blair, 1978; Daniel & Rehner, 1986) that a low jejunal surface pH *in vitro* is highly dependent on the presence of metabolisable sugars. It appears that this is not important *in vivo* (Lucas, 1983), presumably because of glucose availability from the blood. Therefore, in the present study, had glucose been present in the incubation medium the pH values attained would probably have been more comparable with the presented *in vivo* data.

After exposure to *E.coli* STa enterotoxin (14ug/ml) (Figure 3.26), the local pH values measured along the jejunal villus-crypt axis were all higher than those measured in unchallenged tissue. The region which had previously demonstrated the lowest pH (100-200um below the villus tip) underwent the most prominent alkalinisation, rising significantly ($P<0.001$) to $7.01\pm0.05(9)$.

Figure 3.26 Effect of E.coli STa toxin on rat jejunal villus pH profile in vitro. pH at villus tip, villus base, maximum and minimum pH values are presented at respective positions below the villus tip in control (—●—) and STa treated (·····○·····) tissue. Values represent mean ± S.E.M. for 10 (control) and 9 (toxin) experiments, one observation per jejunum (* = $P < 0.01$; ** = $P < 0.001$ compared with respective control value).



The maximum pH value attained was 7.23±0.03(9) in the region just above the villus base where the pH was 7.21±0.02(9). Both of these values were significantly ($P<0.001$) increased over there respective control values. Additionally, the regions of maximum and minimum acidity were shown to be more ($P<0.05$) spatially separated after STa challenge, 257±40(9)um compared to 60±79um in control tissue.

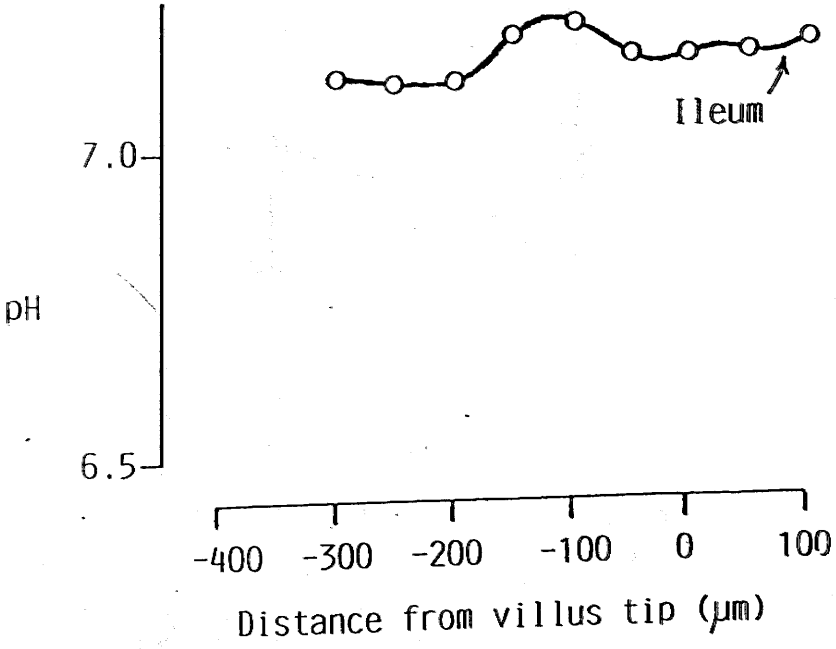
Thus, it would appear that STa induced surface alkalinisation in the jejunum can be localised to the region 100-200um below the villus tip - the region demonstrating the the greatest acidity in unchallenged tissue. This would suggest that STa acts by inhibiting the normal acidification process in this region, which could be either hydrogen ion secretion or bicarbonate anion absorption. It is unlikely that the effect can be attributed to an elevated OH^- and/or HCO_3^- secretion emanating from the crypts, since one would expect to find a considerably higher pH in this region if this were the case.

(b) Effect of E.coli STa enterotoxin on ileal pH profile

Similar experiments to those described in the previous section were executed on rat distal ileum giving similar profiles (Figure 3.27) for ileal villi *in vitro*.

Using the same form of data presentation as that described for the jejunum, the mean pH profile for an average ileal villus was calculated (Figure 3.28). The mean pH at the villus base was 7.16±0.03(6) at a mean villus length of 316±46um. Withdrawing the

Figure 3.27 Representative pH profile of ileal villus measured by LIX pH microelectrode in rat intestine *in vitro* (see text for details).

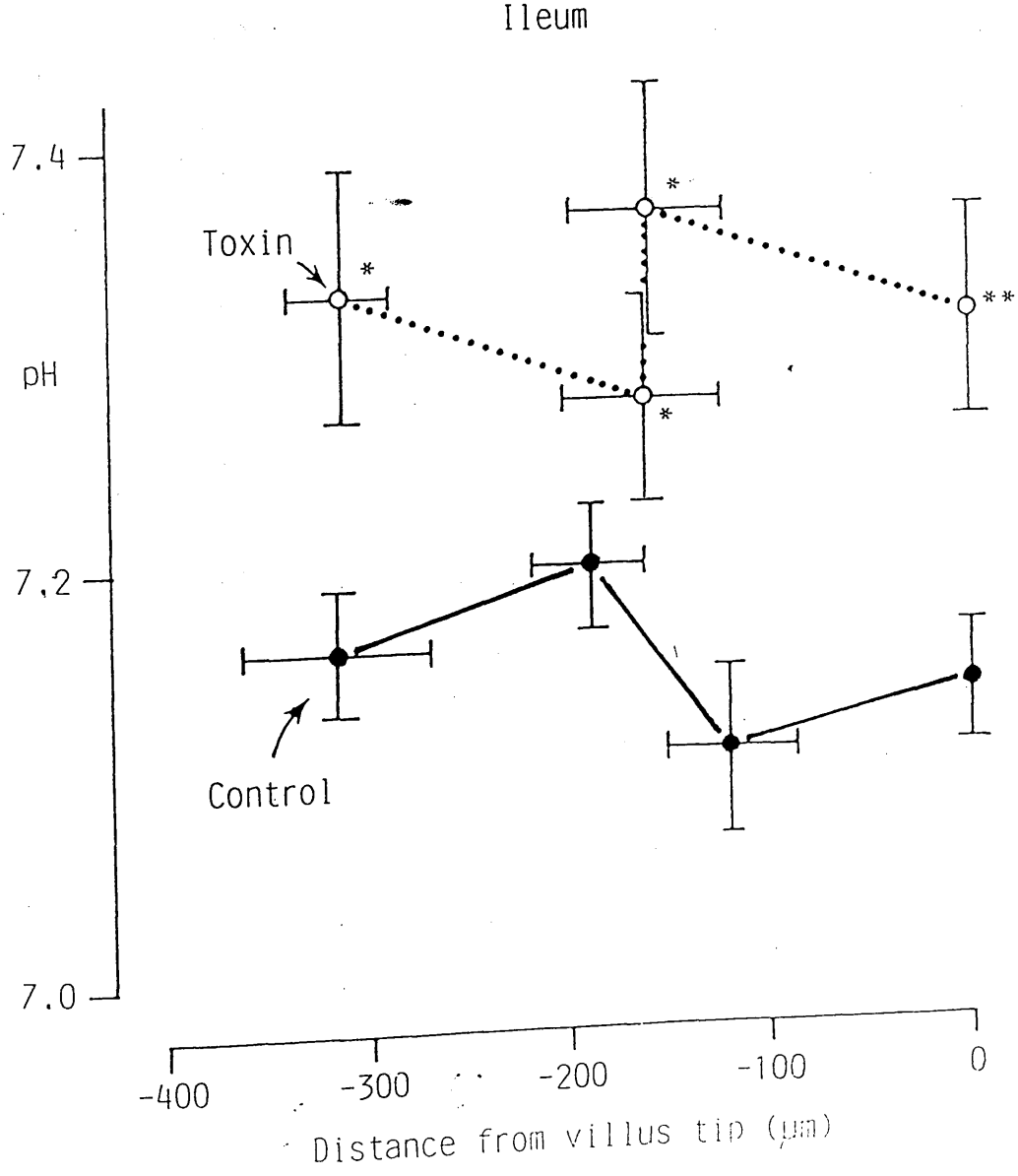


electrode, the pH rose slightly to a maximum value of 7.20±0.03(6), 188±29(6) um below the villus tip, before falling to 7.11±0.04(6) (118±33(6) um below the villus tip). The mean villus tip pH was 7.14±0.03(6).

Therefore, unlike the jejunum, there was no striking pH gradient observed along ileal villi, the pH remaining relatively constant between the villus base and the tip. The values measured were only slightly higher than those measured in the ileum *in vivo*, again possibly due to the lack of glucose in the incubation medium.

Similar to the jejunum, *E.coli* STa enterotoxin (Figure 3.28), elevated pH at all regions along the ileal villus-crypt axis. As in the unchallenged ileum, no striking villus-crypt pH gradient was observed, the pH ranging from 7.28±0.05(7) to 7.37±0.06(7), both of which were significantly ($P<0.05$) higher than the respective minimum and maximum pH values measured in the control ileum. The pH at the villus tip and base (7.32±0.05(7) and 7.33±0.06(7)) were also significantly ($P<0.02$ and $P<0.05$) higher than those measured in unchallenged tissue. The values attained after toxin challenge were lower than those measured *in vivo* but this was possibly due to the rapid wash-out of toxin when the tissue was added to the perfusion chamber.

Figure 3.28 Effect of E.coli STa toxin on rat ileal villus pH profile in vitro. Details as for Figure 3.26. Values represent mean ± S.E.M. for 6 (control [—●—]) and 7 (toxin [····O····]) experiments, one observation per ileum (* = $P < 0.05$: ** = $P < 0.02$ compared with respective control values).



Because of the lack of any truly discernible pH gradient along the ileal villi, it is not really possible to make any justifiable statements about the mechanism of STa-induced alkalinisation in this tissue. However the relatively low pH measured at the villus base would make it unlikely that crypt bicarbonate secretion has been stimulated. Perhaps a more likely explanation would be that STa specifically inhibits an acidification process in the ileum and, in so doing, merely unmasks an ongoing bicarbonate secretion which continues unaffected by STa. This is a similar mechanism to that proposed for the jejunum in the previous section.

Summary of villus pH profile experiments

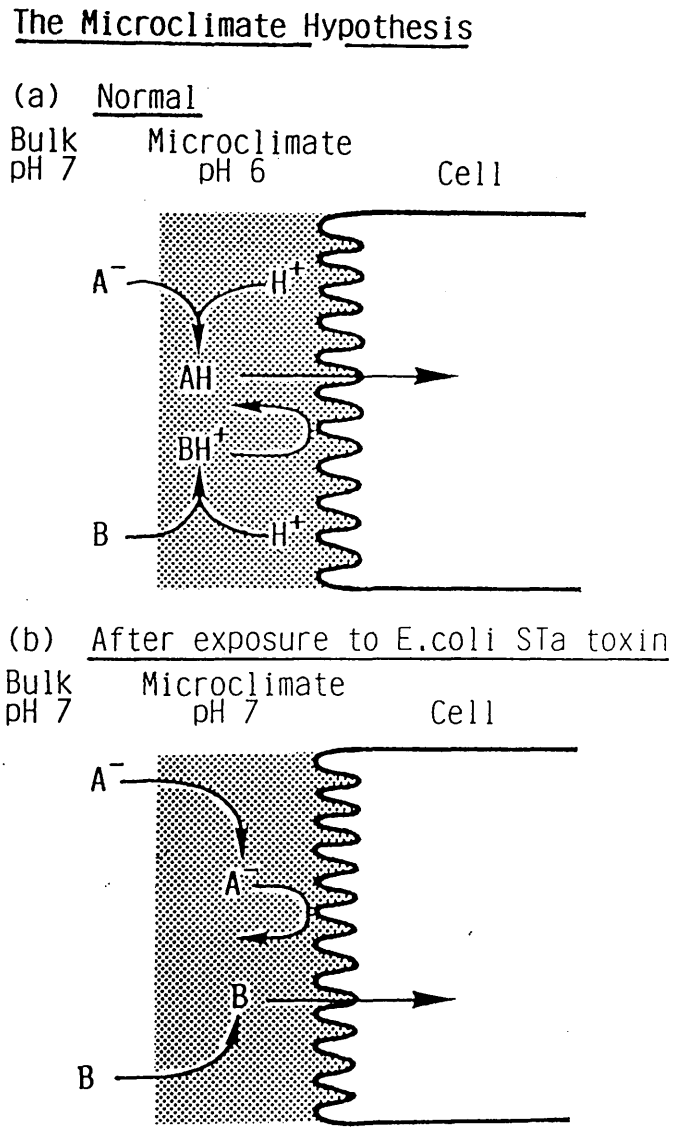
A pH gradient was detected along jejunal but not ileal villi. In the jejunum the lowest pH values attained were located in the region 100-200um below the villus tip. Exposure to E.coli STa enterotoxin resulted in significant elevation of all local pH values measured along the villus-crypt axis of both jejunal and ileal villi. In the jejunum, the region most affected was the region which had previously demonstrated the most acid values. The results suggest that STa-induced alkalinisation in both jejunum and ileum may be due to STa-inhibition of a normally occurring acidification process, unmasking an ongoing bicarbonate secretion in the ileum.

3.4 DRUG ABSORPTION EXPERIMENTS

Regardless of mechanism, exposure to *E.coli* STa enterotoxin results in a considerable alkalinisation of the mucosal surface of both jejunum and ileum *in vivo* and *in vitro*. In the jejunum, this pH shift amounts to almost one pH unit. This property of STa make it a potentially powerful tool with which to investigate the microclimate hypothesis (Figure 3.29) for weak electrolyte absorption (Hogben et al., 1959). This implies that the rate at which weakly dissociable compounds are absorbed is determined primarily by the pH prevailing at the mucosal surface. Under normal conditions in the jejunum the mucosal surface pH is acidic; therefore, weakly acidic compounds will have their absorption promoted since, at this low pH, these compounds will be presented largely in the unionised form (the form in which dissociable compounds traverse lipid membranes). Conversely, weak base absorption will be impeded.

If the microclimate hypothesis does apply, then altering the mucosal surface pH should also alter the rate of weak electrolyte absorption. Since the present results indicate that *E.coli* STa toxin causes an elevation of mucosal surface pH, one would expect, from the microclimate hypothesis (Figure 3.29), that weak acids would be malabsorbed while weak bases should have their absorption enhanced. This prediction, for weak acids, may be deemed not very remarkable, since, in the presence of enterotoxin, the intestine is in a secretory state. Therefore, weak acid malabsorption could be explained in terms of solvent

Figure 3.29 The microclimate hypothesis



drag effects. However, it would be difficult to explain increased weak base absorption under these conditions, without recourse to the mucosal surface pH.

Consequently, in an attempt to add experimental support to the microclimate hypothesis, the following series of experiments investigated the effects of E.coli STa toxin on the absorption of a variety of weakly dissociable drugs from the rat jejunum *in vivo*. All experiments were confined to the jejunum to simplify interpretation of the results.

(a) Effect of E.coli STa toxin on salicylic acid absorption

Salicylic acid was dissolved in 15ml of Krebs-bicarbonate buffer to give a drug concentration of 1mmol/litre. When this solution was recirculated through *in vivo* loops of proximal jejunum, 8.65±0.92(8) umoles of drug were absorbed over the three hour perfusion period. This amounted to approximately 58% of the total administered dose. The line of best fit for perfusate drug amounts against time was obtained and, standardising for an intestinal loop of 100mg dry weight, the mean rate of absorption (rate of luminal disappearance) (Figure 3.30) was 33.02±3.41(8)nmol/min/100mg dry weight.

Incorporating E.coli STa enterotoxin into the perfusate significantly ($P<0.02$) reduced salicylic acid absorption (Figure 3.30) by approximately 39% to values of 20.17±3.40(7) nmol/min/100mg dry weight.

This reduced drug absorption in the the presence of STa was confirmed by a significant ($P<0.01$) reduction in the mean peripheral blood drug concentration from $99.8\pm9.0(8)$ nmol/l under control conditions to $58.0\pm7.0(7)$ nmol/l after enterotoxin challenge. This reduced appearance of drug in the peripheral blood became significant ($P<0.02$) after 105 minutes of perfusion (Figure 3.31) and remained significantly lower for the remainder of the three hour perfusion.

In the same experiments, exposure to E.coli STa toxin also significantly ($P<0.02$) reduced a net fluid absorption (Figure 3.32) in control loops of $10.99\pm1.93(8)$ ul/min/100mg dry weight to values of $1.41\pm2.68(7)$ ul/min/100mg dry weight. The latter figure was not significantly different from zero net fluid movement.

STa challenge also significantly ($P<0.001$) reversed an apparent net secretion of hydrogen ions into the intestinal lumen (Figure 3.33) of $0.54\pm0.08(8)$ uequiv./min/100mg dry weight to a significant ($P<0.001$) apparent hydrogen ion absorption (bicarbonate anion secretion) of 0.54 ± 0.12 uequiv./min/100mg dry weight.

Thus, whilst inducing the expected inhibition of fluid absorption and reversing luminal acidification to alkalinisation, E.coli STa toxin caused a reduced jejunal absorption of salicylic acid. This reduced absorption was confirmed by a similarly reduced appearance of drug in the peripheral blood, implying that total

Figure 3.30 Effect of E.coli STa toxin on salicylic acid absorption from perfused loops of rat jejunum. Values are given as mean ± S.E.M. for number of experiments shown, one observation per animal.

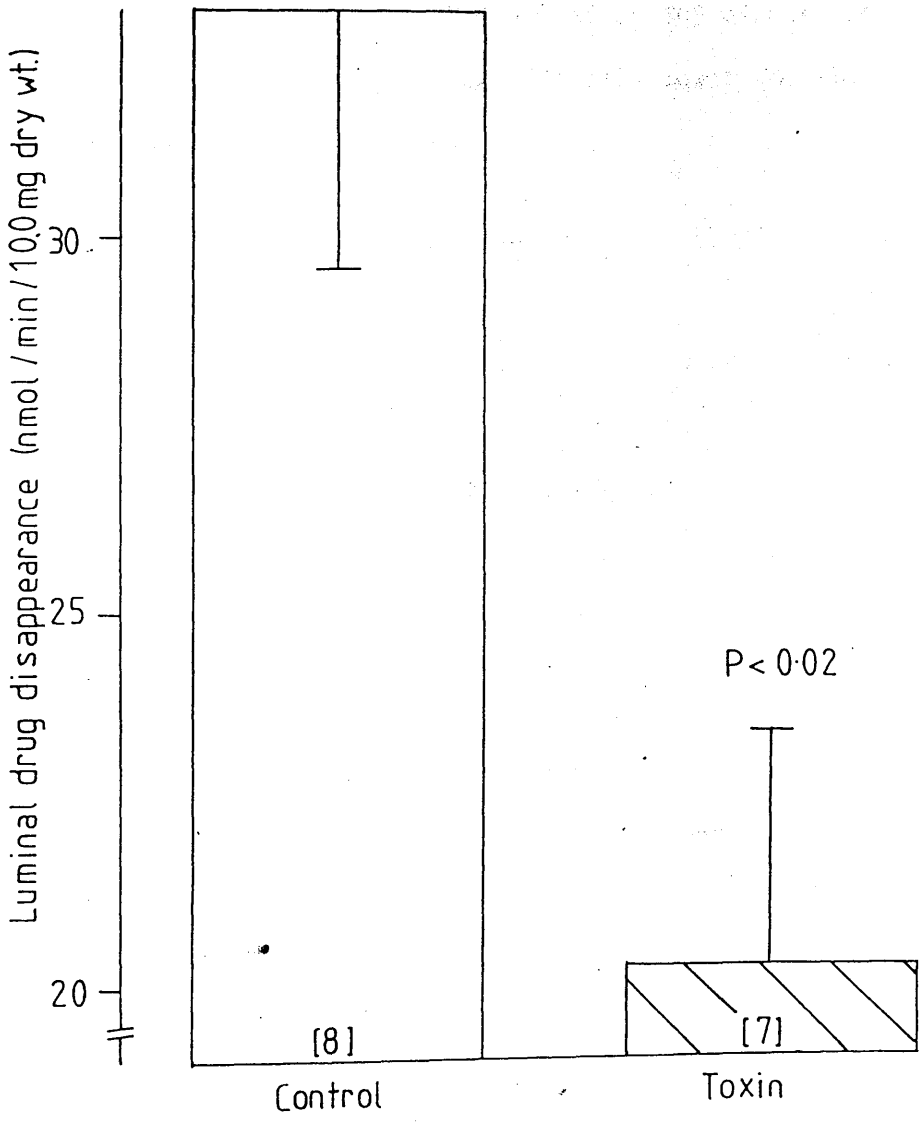


Figure 3.31 Salicylic acid concentration in peripheral blood after jejunal exposure to E.coli STa toxin. Values are given as mean $\pm$ S.E.M. for number of experiments in parentheses, one observation per animal at specified times. (* = P< 0.02; ** = P< 0.01 compared with respective control value).

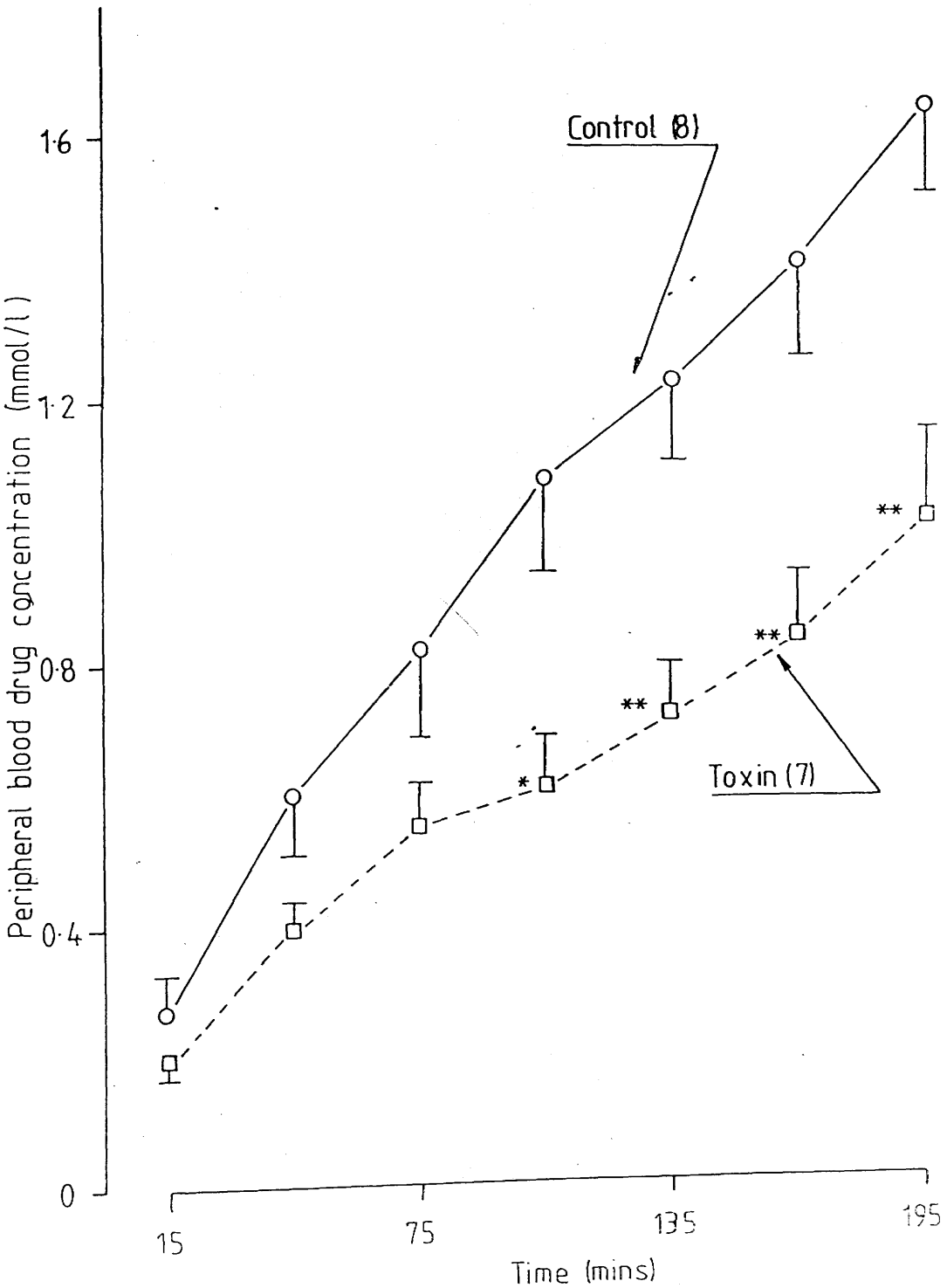


Figure 3.32 Effect of E.coli STa toxin on fluid transport in perfused loops of rat proximal jejunum during salicylic acid absorption. Details as for Figure 3.1.

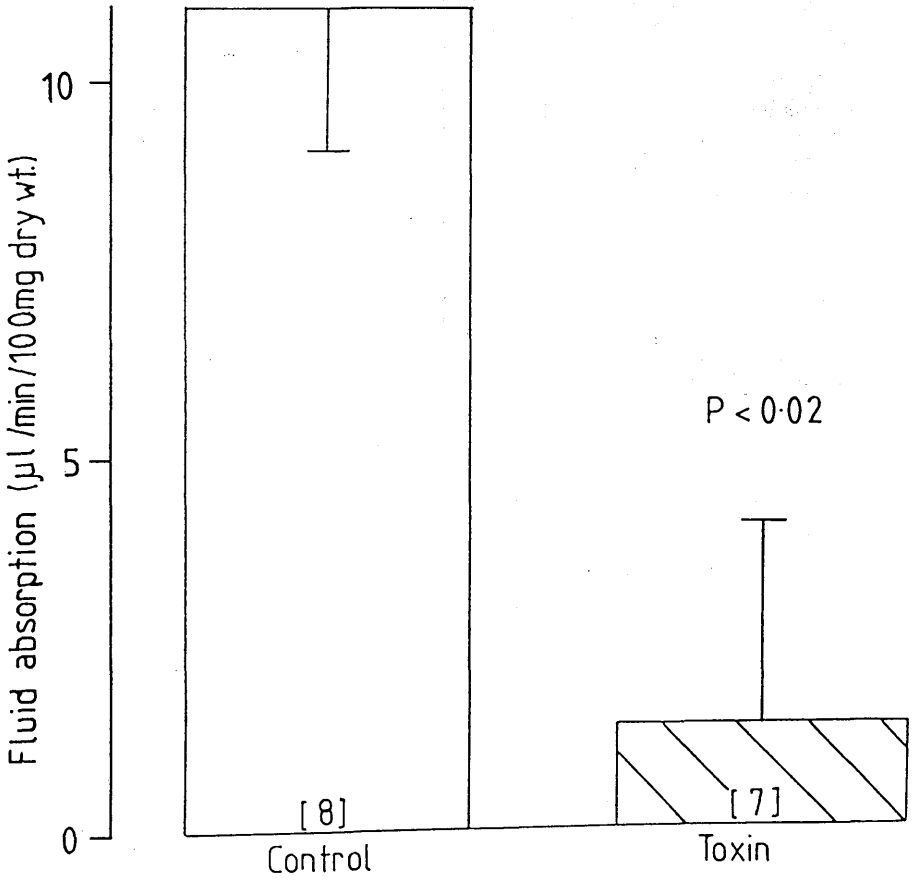
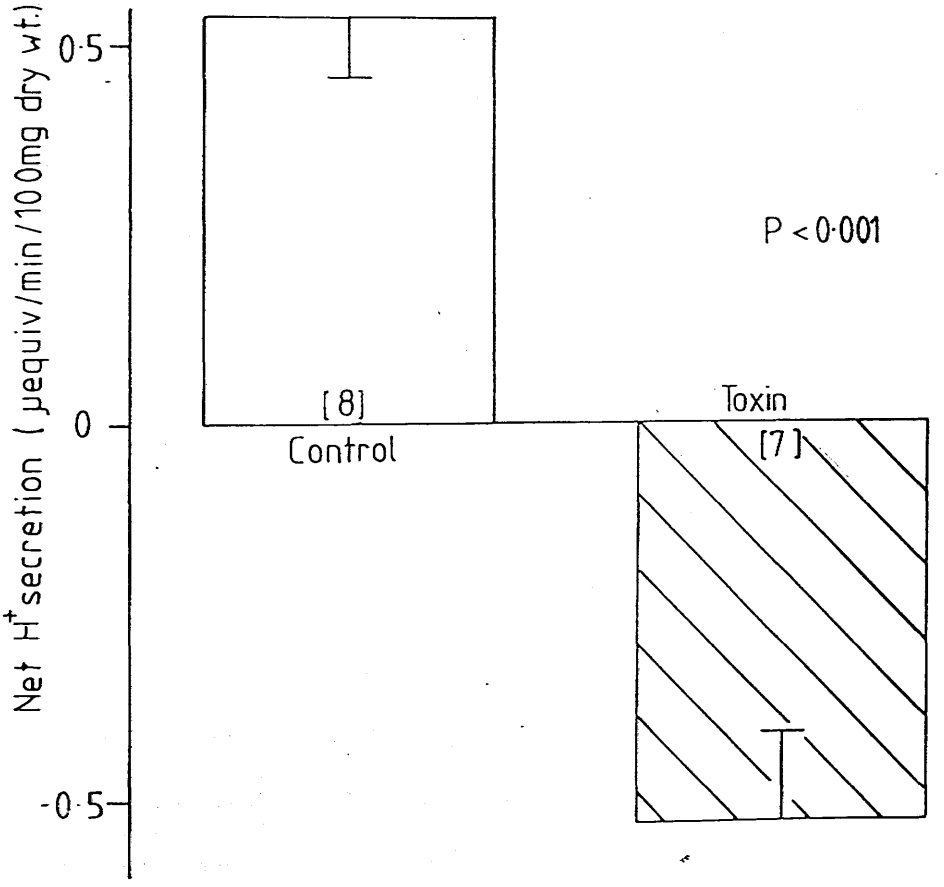


Figure 3.33 Effect of E.coli STa toxin on luminal acid-base balance in perfused loops of rat proximal jejunum during salicylic acid absorption. Details as for Figure 3.1.



transfer and not only cellular uptake of the drug is affected by enterotoxin challenge.

In addition to the empirical analysis of data presented above, the mean luminal drug concentration data were analysed in terms of the absorption models previously described. Rate constants and proportionality constants were estimated for a first order (two parameter) model and a second order (four parameter) model. In addition correlation coefficients were calculated as an index of how well each model described the data. This treatment of the data was compared with more empirical treatment of the data by linear regression analysis.

The absorption rate constants obtained for the first order model (Table 3.1) confirmed the finding that STa reduced salicylic acid absorption. The absorption rate constant for STa treated loops, 2.88×10^{-3} min^{-1}, was significantly ($P<0.001$) lower than that estimated for control experiments, 4.08×10^{-3} min^{-1}. The predicted first order exponential curve for both control and toxin experiments is shown in Figure 3.34.

Comparing the correlation coefficients calculated for the first and second order exponential models (Table 3.1) it is evident that the second order model provided a better fit for the data from both control and toxin experiments (the predicted best fit second order exponential curve for both control and toxin experiments is shown in Figure 3.35). This implies that salicylic acid absorption from the jejunum does not follow the expected

Figure 3.34 Least squares best fit first order exponential curves for change in luminal salicylic acid concentration with time in control (—●—) and toxin (– –○– –) treated loops. Values are given as mean ± S.E.M. for 8 (control) and 7 (toxin) experiments, one observation per animal at specified times.

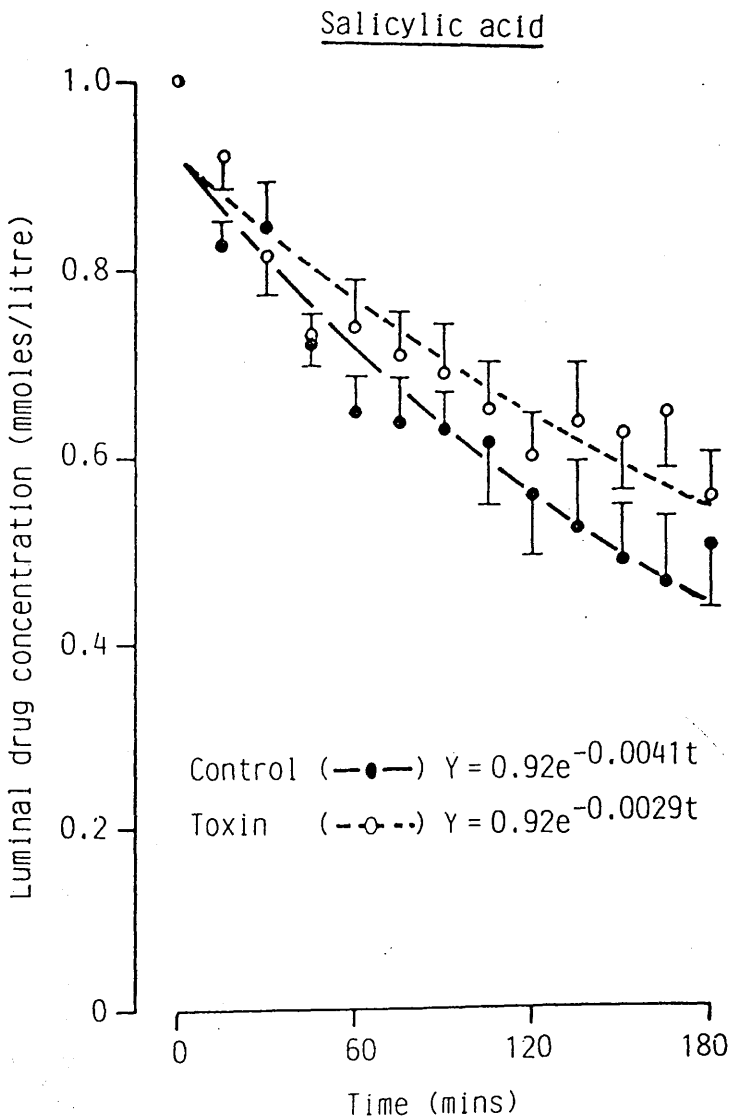
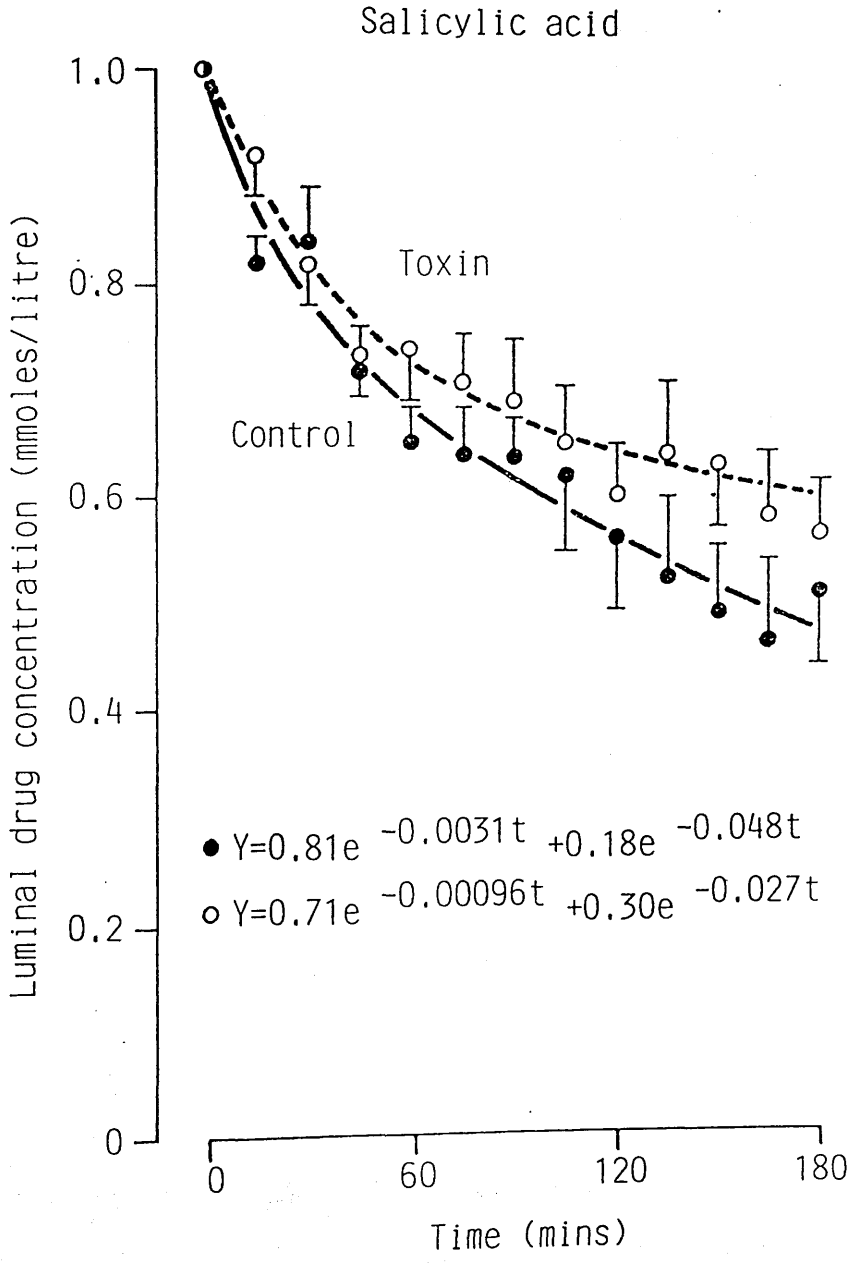


Fig 3.35 Least squares best fit second order exponential curves for change in luminal salicylic acid concentration with time in control (—●—) and toxin (- - O - -) treated loops. Details as for Figure 3.34.

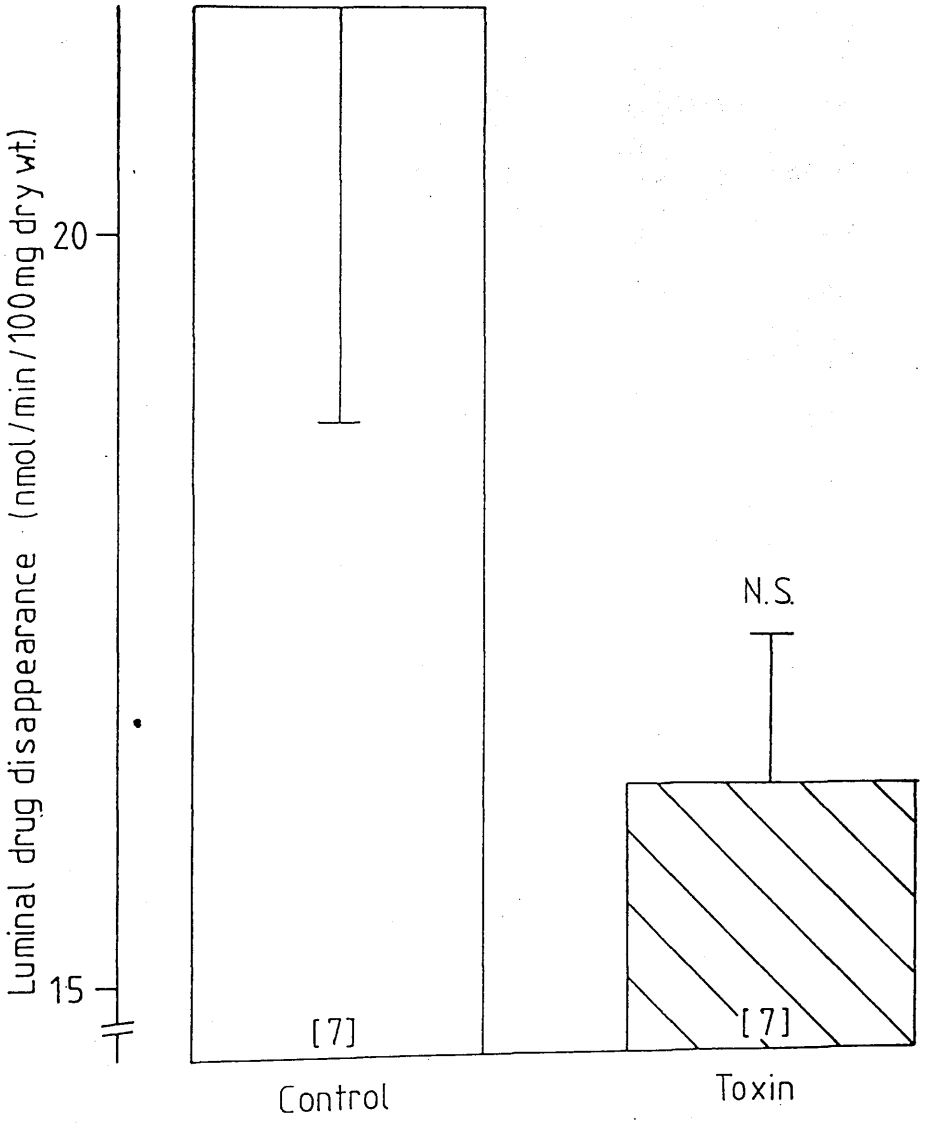


first order exponential pattern consistent with diffusion across a single barrier. The preferred second order fit is more consistent with the exchange compartment model. When this model is solved it is not possible to extract the three rate constants on their own, without additional information on the concentration changes in either the tissue or the intermediate compartmment. However, the ratio k_{23}/k_{21} can be calculated. This ratio, as can be seen from the diagramatic representation of this model (Figure 2.13), compares the rate constant for drug transfer across the cell membrane (K_{23}) with diffusion out of the exchange compartment (K_{21}). This ratio was reduced from 0.471 in control experiments to 0.184 after STa challenge. This is again consistent with STa reducing salicylic acid absorption.

(b) Effect of E.coli STa toxin on phenytoin absorption

When 15ml of Krebs-bicarbonate buffer containing 1mmol/l of phenytoin was perfused through in vivo loops of proximal jejunum there was considerable absorption of drug from the intestinal lumen. The rate of luminal disappearance of drug (Figure 3.36) in control loops was 21.48±2.74(7) nmol/min/100mg dry weight. Thus approximately 26% of the initial 15umol dose was absorbed over the three hour perfusion period. Challenge of the jejunal mucosa with E.coli STa toxin resulted in a reduced phenytoin absorption (Figure 3.36) to values of 16.27±1.03(7) nmol/min/100mg dry weight, which was not significantly different from the control absorption rate. There was a significant ($P<0.05$) reduction in the mean peripheral blood drug concentration observed after STa

Figure 3.36 Effect of E.coli STa toxin on phenytoin absorption from perfused loops of rat jejunum. Details as for Figure 3.30.



challenge. The value of 16.14±1.79(7) nmol/l measured in control experiments was reduced to 11.60±0.89(7) nmol/l in the presence of STa. An inspection of the mean blood drug concentrations at each sample time (Figure 3.37) shows that there was a significant ($P<0.05$) difference between control and toxin treatment after 45 minutes of perfusion.

E.coli toxin significantly ($P<0.001$) enhanced a net fluid secretion (Figure 3.38) of 9.24±0.87(7) ul/min/100mg dry weight in control loops to values of 22.01±3.81(7) ul/min/100mg dry weight. It is unclear why phenytoin should have induced intestinal secretion in these experiments. However, whatever mechanism of drug action was responsible also produced a massive ($P<0.001$) increase in fluid secretion in the presence of STa compared to STa treatment in the absence of phenytoin. STa also reversed ($P<0.001$) acidification into the intestinal lumen (Figure 3.39) of 0.46±0.09(7) uequiv./min/100mg dry weight to alkalinisation of 0.15±0.04(7) uequiv./min/100mg dry weight.

Thus, as with salicylic acid, phenytoin absorption from the jejunum was reduced after STa challenge. This reduced absorption was confirmed by significantly lower peripheral blood drug levels, indicating that STa affected the overall transfer of the drug.

Figure 3.37 Phenytoin concentration in peripheral blood after jejunal exposure to E.coli STa toxin. Details as for Figure 3.31 (* = P< 0.05 compared with respective control value).

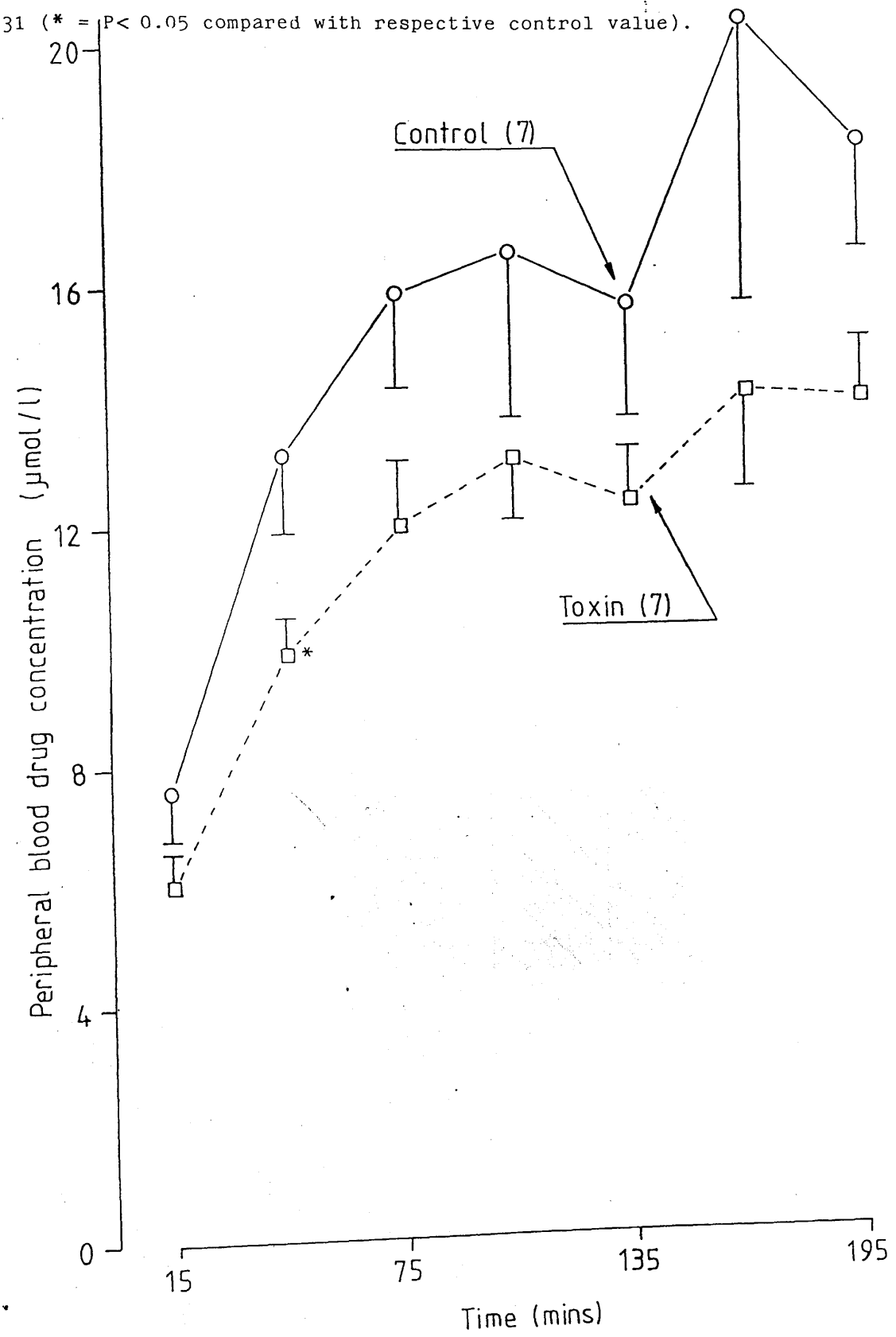
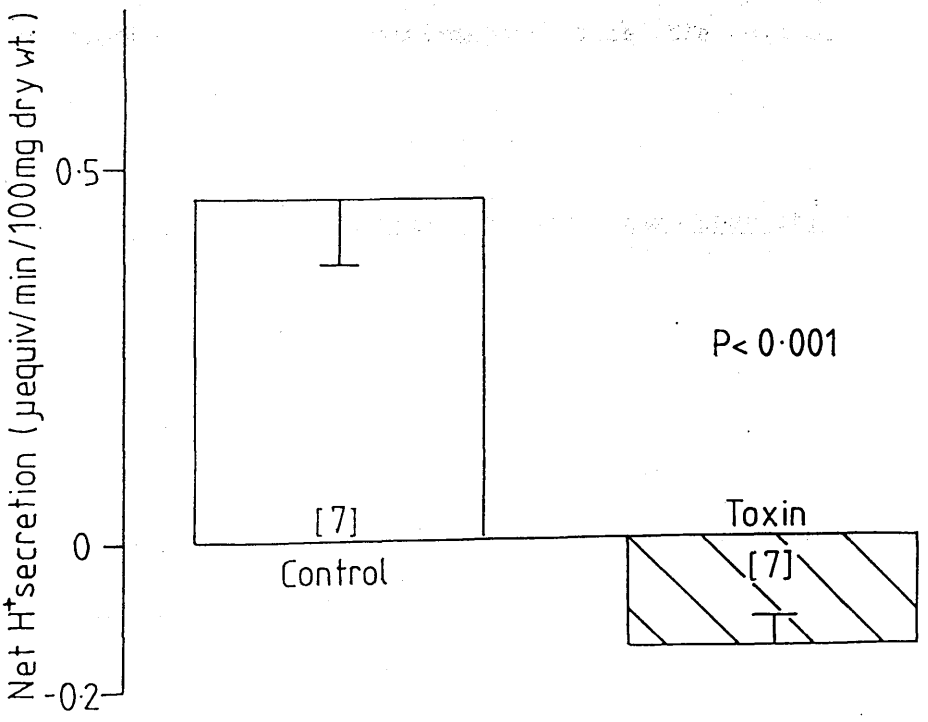


Figure 3.39 Effect of E.coli STa toxin on luminal acid-base balance in perfused loops of rat proximal jejunum during phenytoin absorption. Details as for Figure 3.1.



The mean luminal drug concentration data from control and enterotoxin treated loops were also analysed by non-linear regression. As can be seen from the correlation coefficients (Table 3.2) the first order exponential model provided the best fit for the phenytoin data. Second order model fitting defaults to the simpler first order model coefficients and graphical analysis also failed to show a second component to the curve (Figure 3.40). Unlike the more empirical data analysis, the first order absorption rate constant estimated for STa challenge experiments of 2.98×10^{-3} min^{-1}, was significantly ($P<0.001$) lower than the value of 5.25×10^{-3} min^{-1} calculated for control experiments. This is confirmation that STa reduces phenytoin absorption from the jejunum.

(c) Effect of E.coli STa toxin on amphetamine absorption

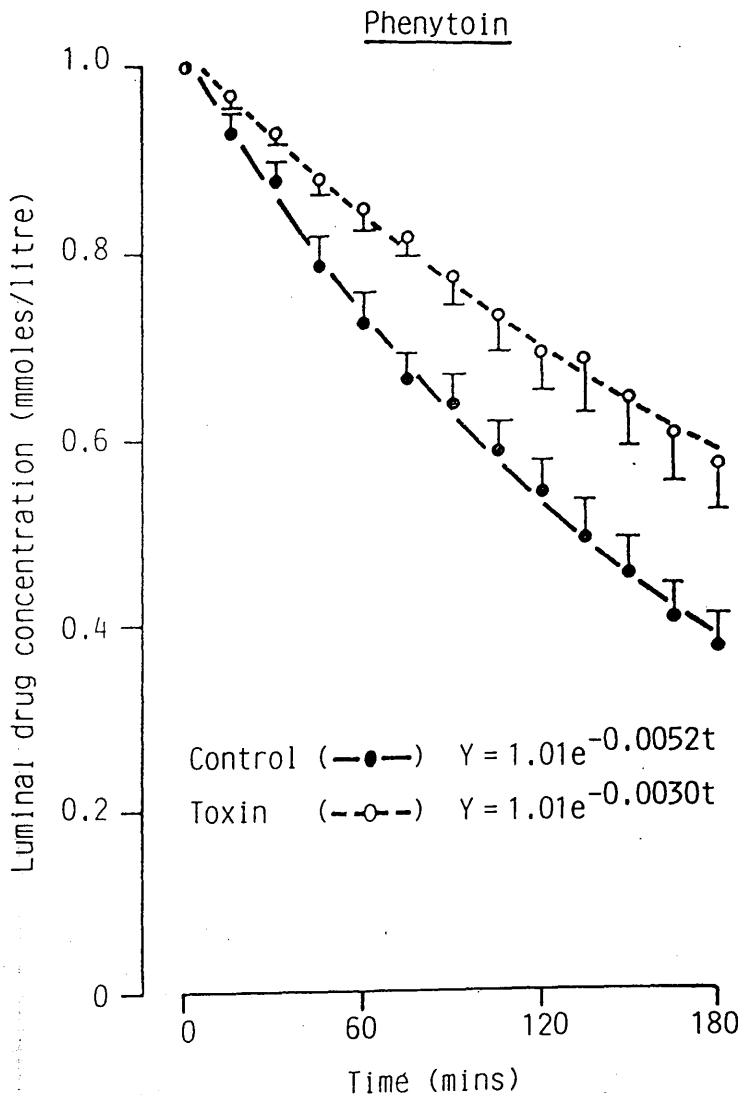
Amphetamine was dissolved in 15ml of Krebs-bicarbonate buffer to give a drug concentration of 1mmol/l. Perfusing this solution through loops of rat proximal jejunum *in vivo* resulted in amphetamine being absorbed from the intestinal lumen. From the luminal drug data the rate of luminal disappearance (Figure 3.41) was calculated to be $4.98 \pm 0.41(6)$ nmol/min/100mg dry weight. This value was considerably lower than those measured for salicylic acid or phenytoin. In separate experiments, when *E.coli* STa toxin was incorporated into the perfusate, amphetamine absorption was significantly ($P<0.01$) enhanced to values of $9.68 \pm 1.02(6)$ nmol/min/100mg dry weight, representing an increase of

Table 3.2

Model	Parameters	Control	Toxin	Sig.
$C(t) = A - Bt$	A (mM)	0.963	0.995	
	B (μM/min)	3.41	2.34	*
	r	0.993	0.998	
$C(t) = Ae^{-kt}$	A (mM)	1.009	1.012	
	k ($x10^{-3}$ min^{-1})	5.25	2.98	*
	r	0.999	0.998	

Estimated parameters for zero and first order pharmacokinetic models for phenytoin luminal concentration data. Correlation coefficients (r) are presented as an index of the degree of fit of each model to the data (* = $P < 0.001$ compared with control).

Figure 3.40 Least squares best fit first order exponential curves for change in luminal phenytoin concentration with time in control (—●—) and toxin (--○--) treated loops. Values are given as mean ± S.E.M. for 7 (control and toxin) experiments, one observation per animal at specified times.



approximately 94% over control values.

This elevated amphetamine absorption was confirmed by a significant ($P<0.02$) increase in the mean peripheral blood drug concentrations after toxin challenge, the levels rising from $4.83\pm0.73(6)$ nmol/l in control experiments to 7.47 ± 0.44 nmol/l after exposure to STa. From the blood drug concentration profile (Figure 3.42) it can be seen that the blood levels became significantly ($P<0.02$) higher in STa experiments after 105 minutes of perfusion.

In the same experiments STa significantly ($P<0.001$) enhanced a modest net fluid secretion (Figure 3.43) of $2.35\pm0.91(6)$ ul/min/100mg dry weight to a secretion of $25.95\pm3.19(6)$ ul/min/100mg dry weight. This was a similar result to that achieved with phenytoin.

STa exposure also significantly ($P<0.001$) reversed an apparent net secretion of hydrogen ions (Figure 3.44) into the jejunal lumen of 0.46 ± 0.05 uequiv./min/100mg dry weight to an insignificant alkalinisation of 0.04 ± 0.03 uequiv./min/100mg dry weight.

Therefore, unlike salicylic acid and phenytoin, amphetamine absorption from the jejunum was enhanced by STa, a finding which was confirmed by similarly elevated peripheral blood drug concentrations after toxin. This increase in absorption was despite unfavourable fluid movement.

Figure 3.42 Amphetamine concentration in peripheral blood after jejunal exposure to E.coli STa toxin. Details as for Figure 3.31 (* = P< 0.02; ** = P< 0.01; *** = P< 0.001 compared with respective control value).

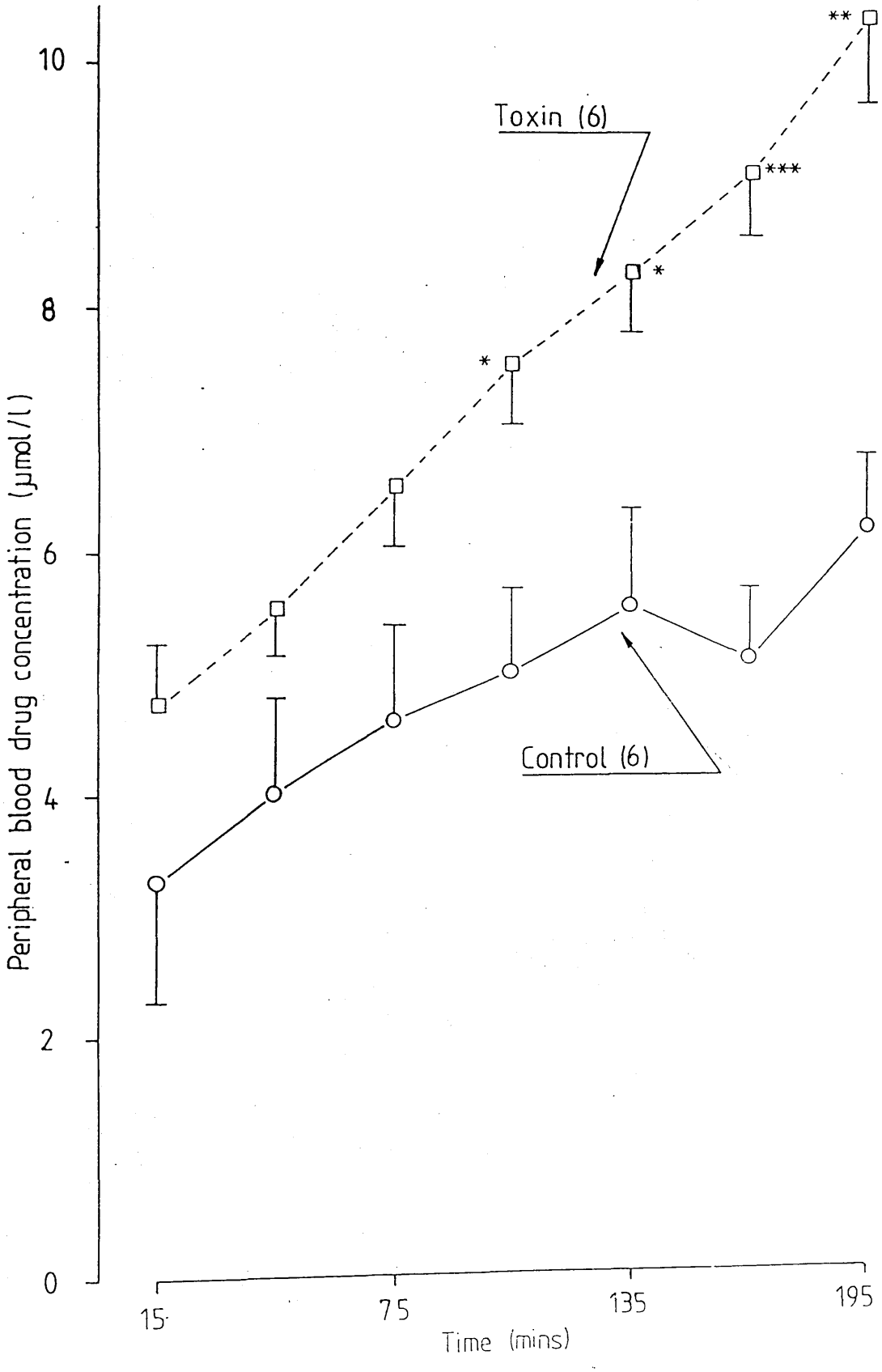


Figure 3.43 Effect of E.coli STa toxin on fluid transport in perfused loops of rat proximal jejunum during amphetamine absorption. Details as for Figure 3.1.

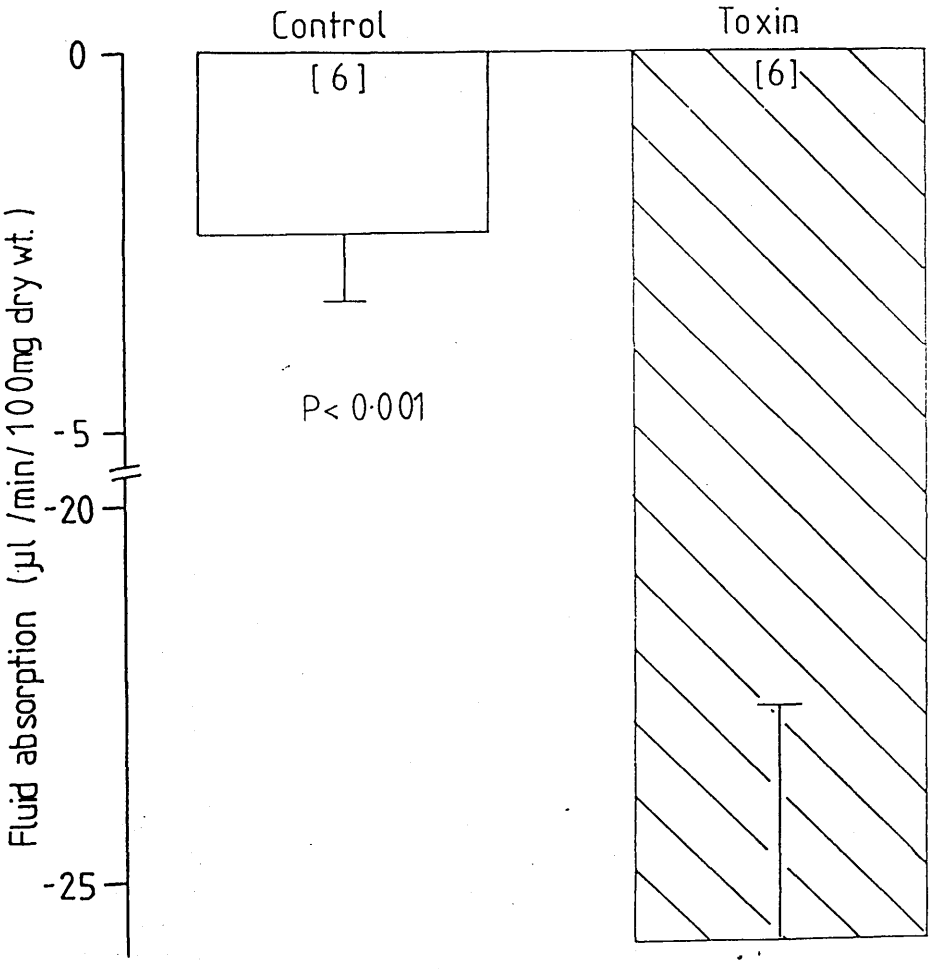
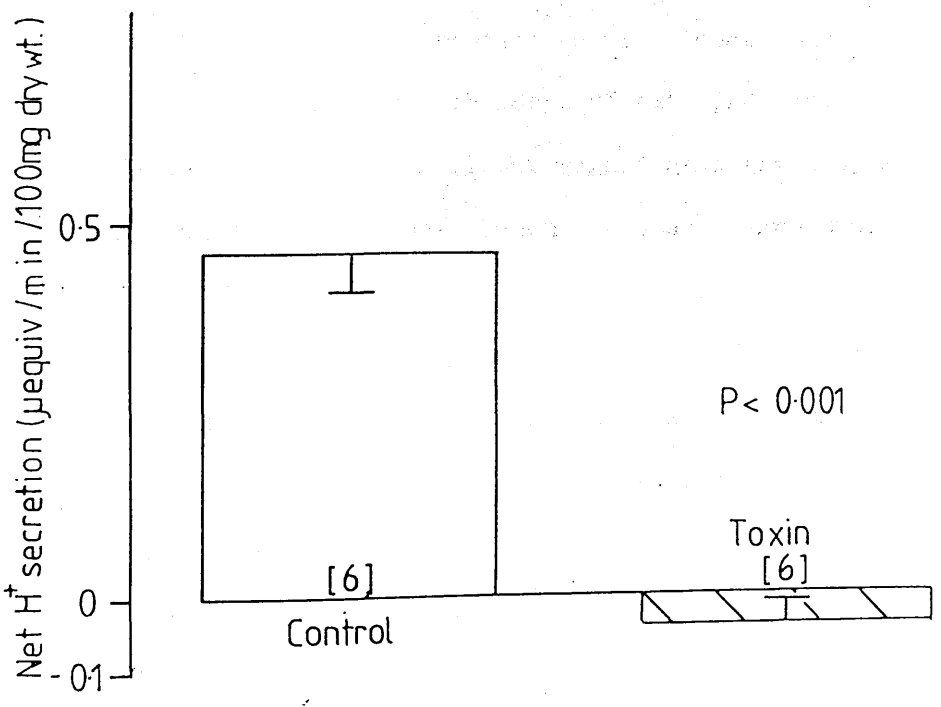


Figure 3.44 Effect of E.coli STa toxin on luminal acid-base balance in perfused loops of rat proximal jejunum during amphetamine absorption. Details as for Figure 3.1.



This finding was further strengthened when the mean luminal drug concentrations were analysed by non-linear regression. As can be seen from the correlation coefficients (Table 3.3), a first order exponential model did not provide a better fit to the data than the more empirical zero order curve. However, the first order model (Figure 3.45) absorption rate constant estimated for the toxin experiments (Table 3.3), 1.49×10^{-3} min^{-1}, was significantly ($P<0.001$) higher than the value of 0.95×10^{-3} min^{-1} estimated for control experiments. As with phenytoin a second order exponential curve could not be fitted to the amphetamine data.

(d) Effect of E.coli STa toxin on morphine absorption

When 15ml of Krebs-bicarbonate buffer containing 1mmol/l of morphine was perfused through loops of rat proximal jejunum *in vivo* there was a very small but significant ($P<0.01$) absorption of drug from the intestinal lumen. Linear regression analysis estimated the rate of luminal disappearance (Figure 3.46) to be $0.39 \pm 0.12(6)$ nmol/min/100mg dry weight. This amounted to approximately 0.47% of the initial administered dose being absorbed over the three hour experimental period. This was, by far, the lowest absorption rate measured for any of the drugs tested in this study. Similar to amphetamine, exposure to *E.coli* STa toxin caused this absorption to significantly ($P<0.01$) increase (Figure 3.46) to values of $2.84 \pm 0.60(6)$ nmol/min/100mg dry weight. This represents more than a seven fold increase in morphine absorption with STa.

Figure 3.41 Effect of E.coli STa toxin on amphetamine absorption from perfused loops of rat jejunum. Details as for Figure 3.30.

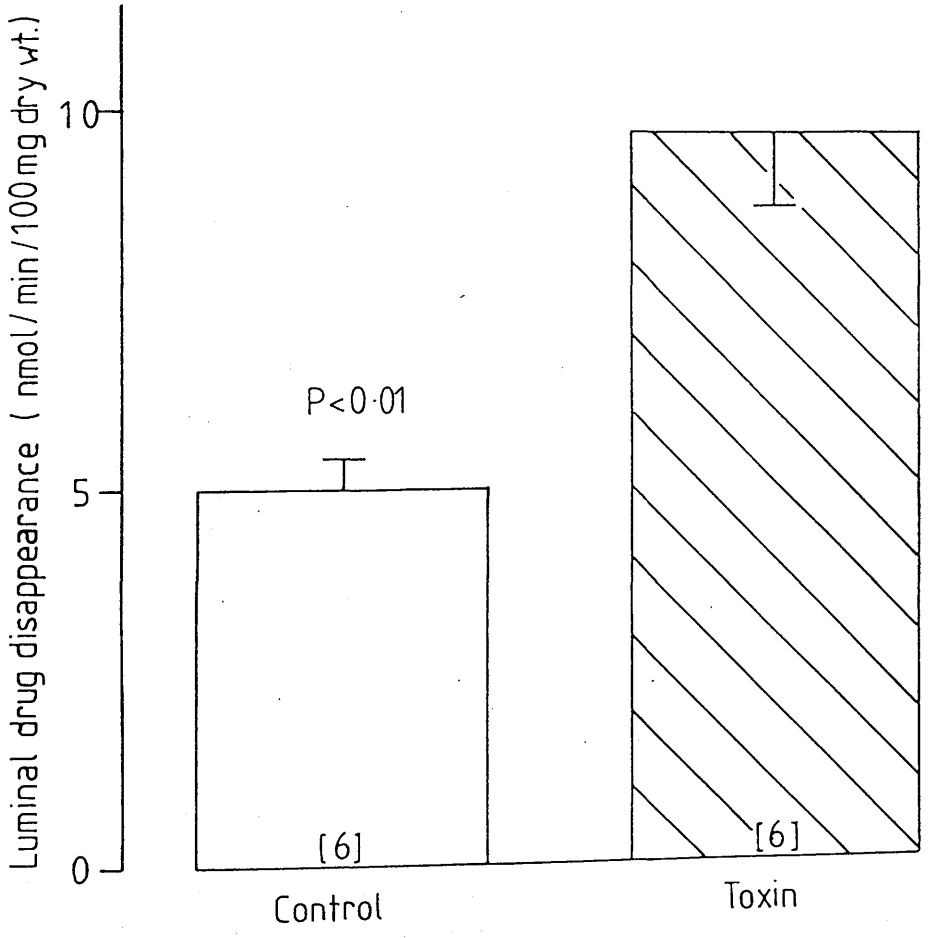


Table 3.3

Model	Parameters	Control	Toxin	Sig.
$C(t) = A - Bt$	A (mM)	1.009	1.004	
	B (μM/min)	0.88	1.32	*
	r	0.984	0.995	
$C(t) = Ae^{-kt}$	A (mM)	1.011	1.008	
	k ($x10^{-3}$ min^{-1})	0.95	1.49	*
	r	0.983	0.995	

Estimated parameters for zero and first order pharmacokinetic models for amphetamine luminal concentration data. Correlation coefficients (r) are presented as an index of the degree of fit of each model to the data (* = $P < 0.001$ compared with control).

Figure 3.45 Least squares best fit first order exponential curves for change in luminal amphetamine concentration with time in control (—●—) and toxin (--○--) treated loops. Values are given as mean ± S.E.M. for 6 (control and toxin) experiments, one observation per animal at specified times.

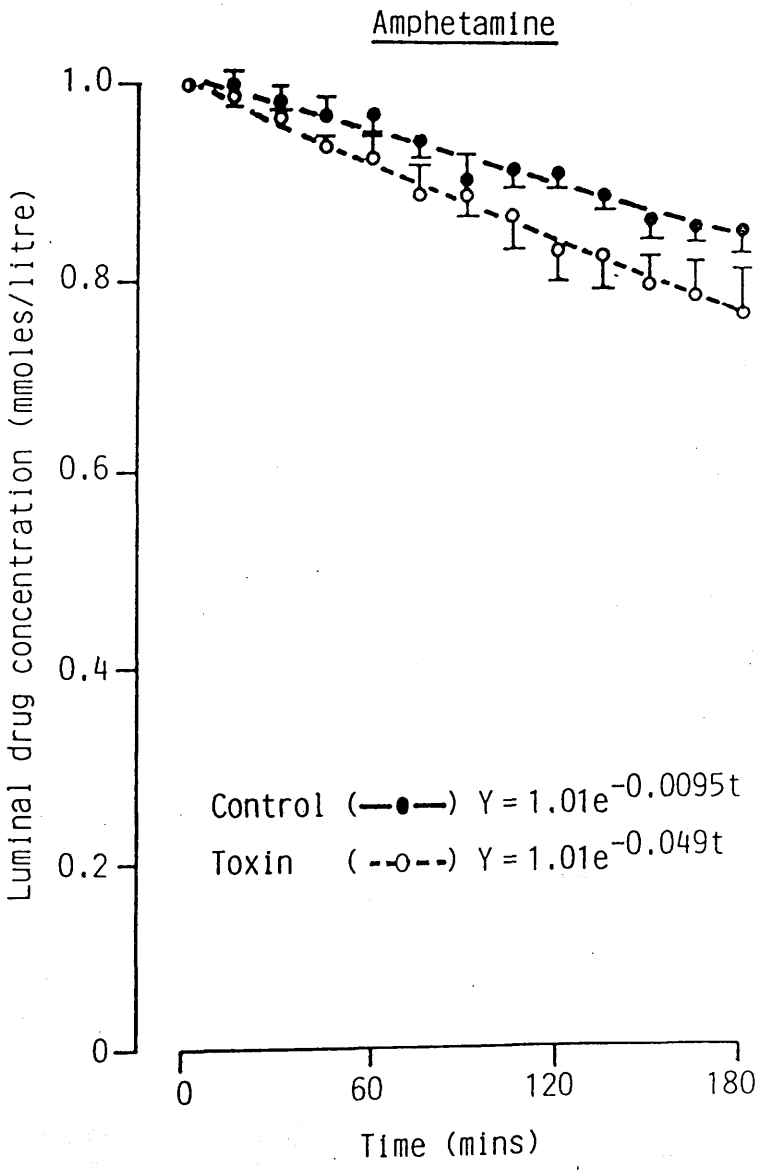
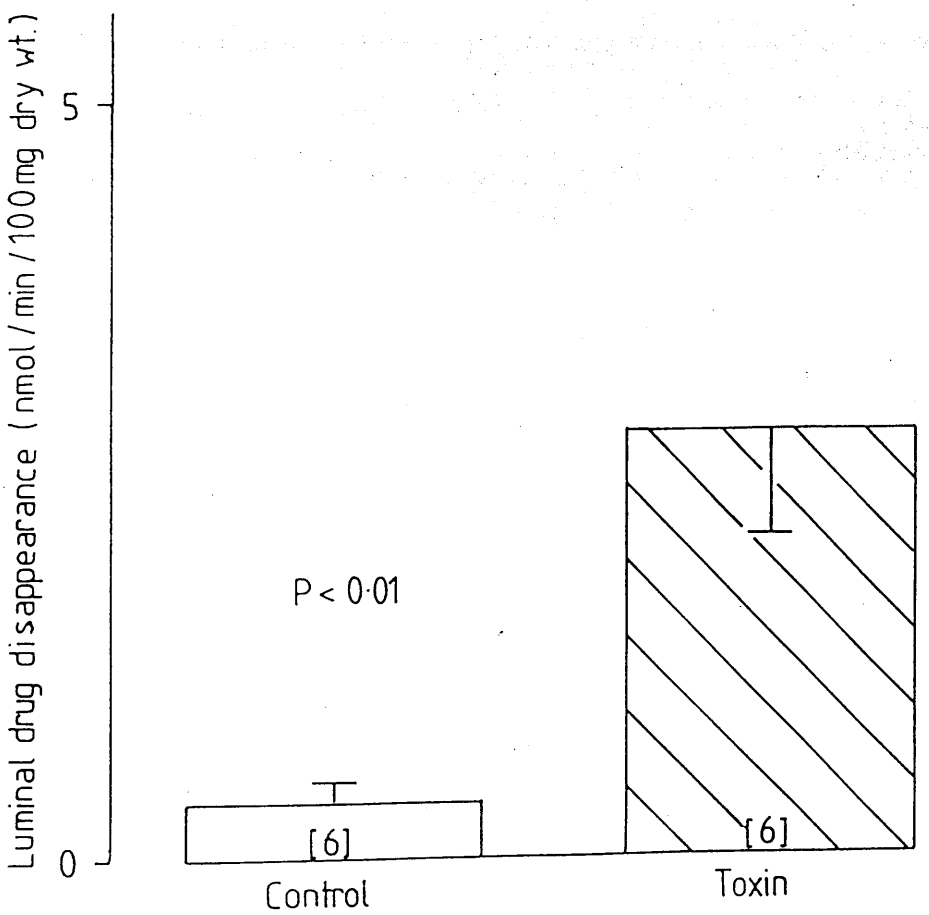


Figure 3.46 Effect of E.coli STa toxin on morphine absorption from perfused loops of rat jejunum. Details as for Figure 3.30.



The mean peripheral blood drug concentrations was also increased after STa challenge, from 5.47±0.29(6) nmol/l in control experiments to values of 6.87±0.67(6) nmol/l. This difference was not significant. However, an inspection of the peripheral blood drug concentration profiles for control and toxin experiments (Figure 3.47) shows that there was a significant ($P<0.05$) increase in blood drug concentration in the presence of toxin after 165 minutes of perfusion.

STa challenge also resulted in the net fluid absorption (Figure 3.48) measured under control conditions of 5.64±0.58(6) ul/min/100mg dry weight being reversed ($P<0.001$) to a net secretion of 2.78±0.69(6) ul/min/100mg dry weight.

As previously, STa caused a cessation of luminal acidification (Figure 3.49), reducing ($P<0.001$) an apparent net hydrogen ion secretion into the lumen of 0.56±0.05(6) uequiv/min/100mg dry weight in control experiments to values of 0.004±0.02(6) uequiv/min/100mg dry weight, the latter figure being not significantly different zero net hydrogen ion secretion.

Therefore, morphine absorption from the proximal jejunum is greatly enhanced in the presence of E.coli STa. This enhanced absorption occured again, despite net fluid secretion.

The mean luminal drug concentration data was analysed by non-linear regression. Because of the very small absorption of morphine in control experiments neither zero order nor first

Figure 3.47 Morphine concentration in peripheral blood after jejunal exposure to E.coli STa toxin. Details as for Figure 3.31 (* = P< 0.05 compared with control value).

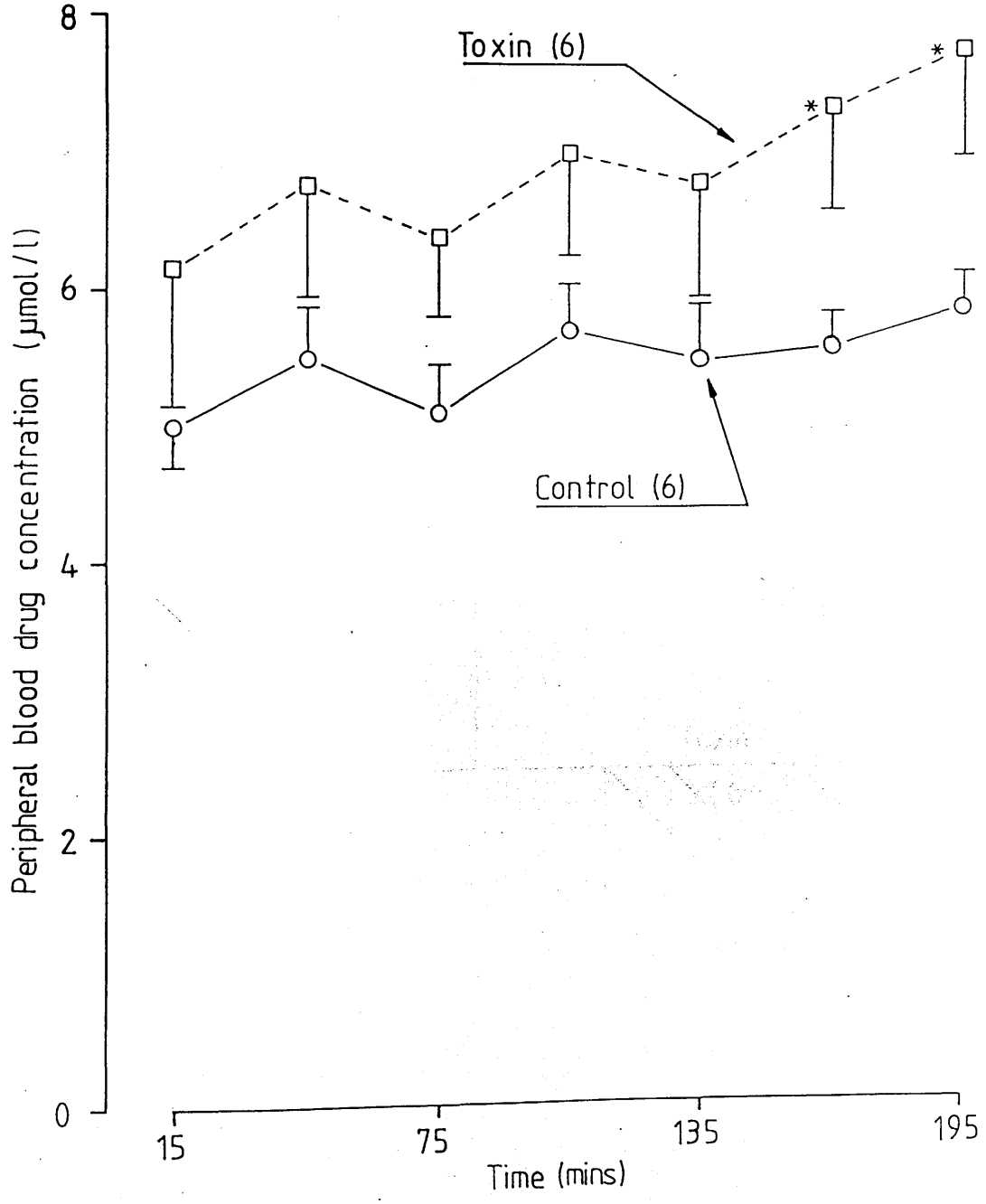


Figure 3.48 Effect of E.coli STa toxin on fluid transport in loops of rat proximal jejunum during morphine absorption. Details as for Figure 3.1.

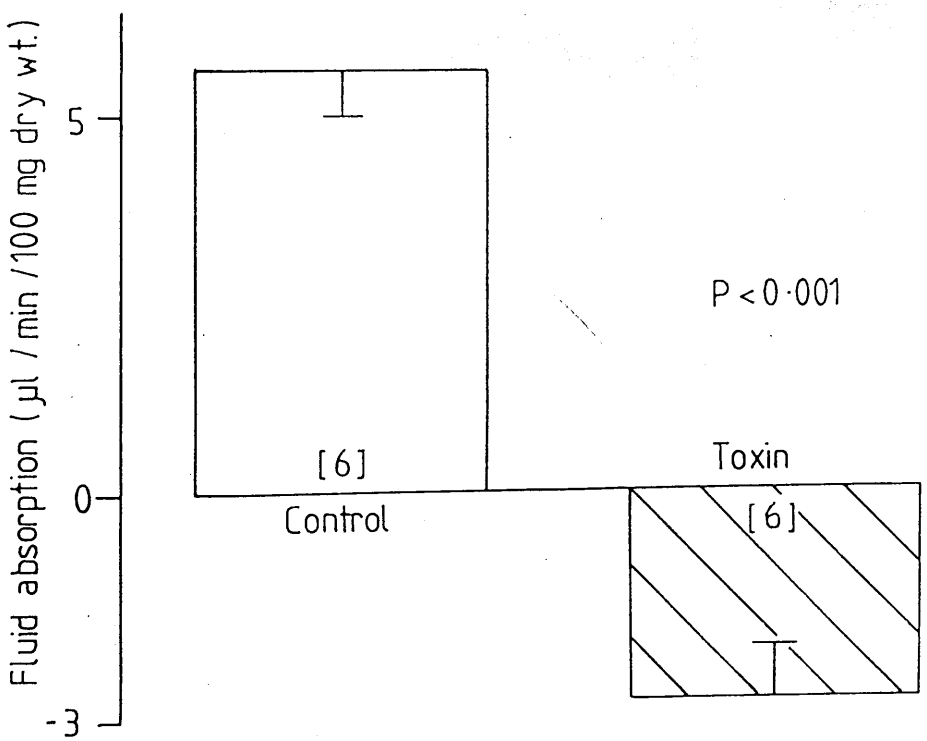
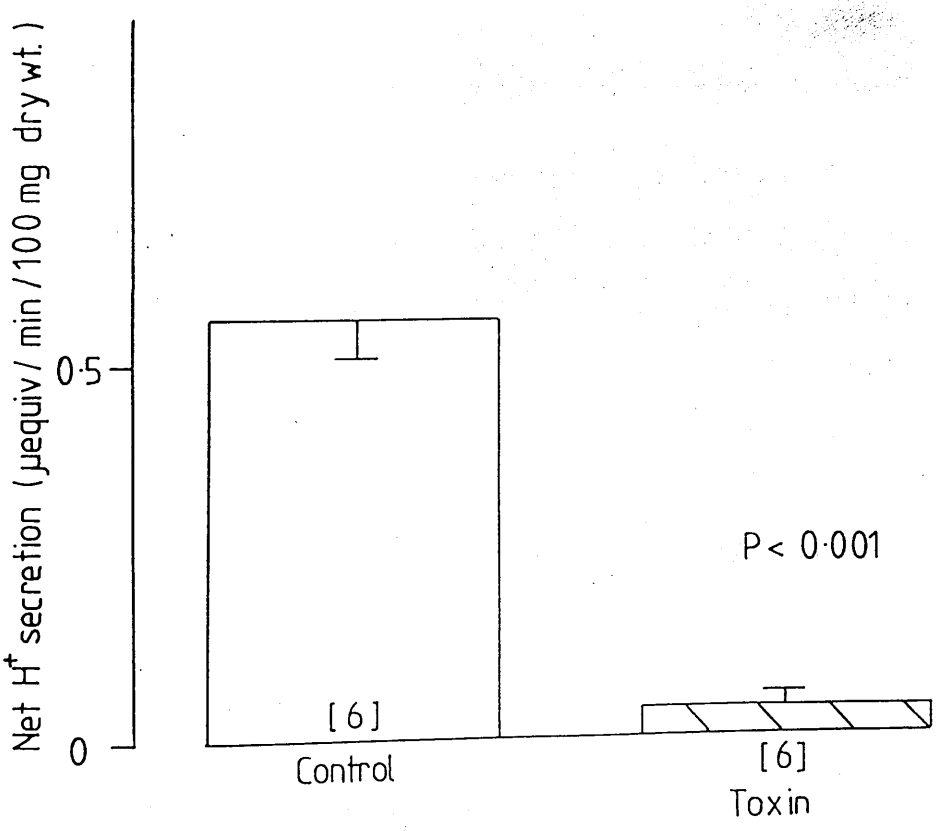


Figure 3.49 Effect of E.coli STa toxin on luminal acid-base balance in perfused loops of rat proximal jejunum during morphine absorption. Details as for Figure 3.1.



order curves provided a particularly good fit for the data, as seen by the very low correlation coefficients (Table 3.4). After STa challenge the degree of fit for both of these models improved considerably (Table 3.4), although as with amphetamine the first order model did not provide an appreciably better fit than the simpler zero order model. However, the estimated first order model (Figure 3.50) absorption rate constants confirmed the finding that STa enhances the jejunal absorption of morphine. The value obtained for toxin experiments, 4.50×10^{-4} min^{-1}, was significantly ($P<0.001$) higher than the value of 4.20×10^{-5} min^{-1} estimated from control experiments. A second order model regression fitted to the data defaulted to a first order model.

(e) Effect of E.coli STa toxin on lignocaine absorption

Lignocaine was dissolved in 15ml of Krebs-bicarbonate buffer in a concentration of 1mmol/l. When this solution was recirculated through loops of rat jejunum *in vivo* there was absorption of lignocaine from the lumen. Linear regression analysis estimated the rate of this absorption (Figure 3.51) to be $16.70 \pm 1.69(9)$ nmol/min/100mg dry weight. This amounted to approximately 20% of the initial dose disappearing out of the lumen over the three hour perfusion period. Exposing the jejunal mucosa to *E.coli* STa toxin significantly ($P<0.01$) increased lignocaine absorption to values of $25.35 \pm 1.97(9)$ nmol/min/100mg dry weight, a 52% increase over control values.

Table 3.4

Model	Parameters	Control	Toxin	Sig.
$C(t) = A - Bt$	A (mM)	1.028	0.998	
	B (μM/min)	0.046	0.43	*
	r	0.206	0.433	
$C(t) = Ae^{-kt}$	A (mM)	1.028	0.988	
	k ($x10^{-3}$ min^{-1})	0.042	0.45	*
	r	0.068	0.960	

Estimated parameters for zero and first order pharmacokinetic models for morphine luminal concentration data. Correlation coefficients (r) are presented as an index of the degree of fit of each model to the data (* = $P < 0.001$ compared with control; NS = not significant).

Figure 3.50 Least squares best fit first order exponential curves for change in luminal morphine concentration with time in control (—●—) and toxin (--O--) treated loops. Values are given as mean ± S.E.M. for 6 (control and toxin) experiments, one observation per animal at the specified times.

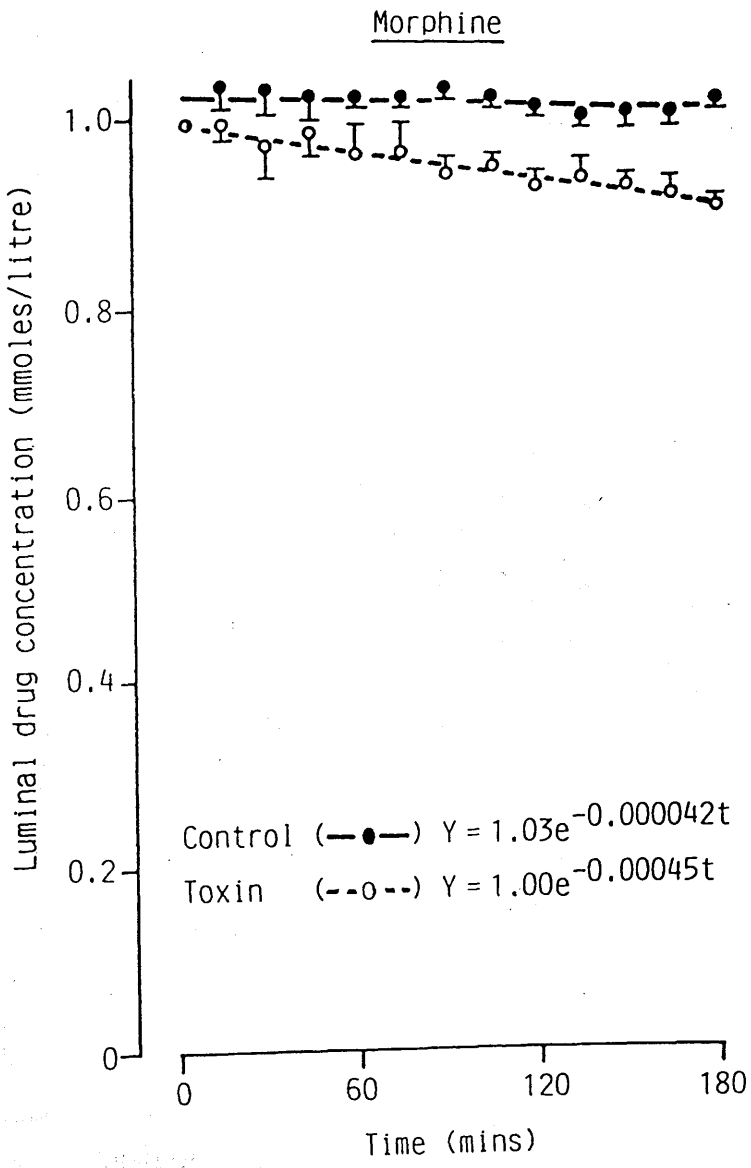
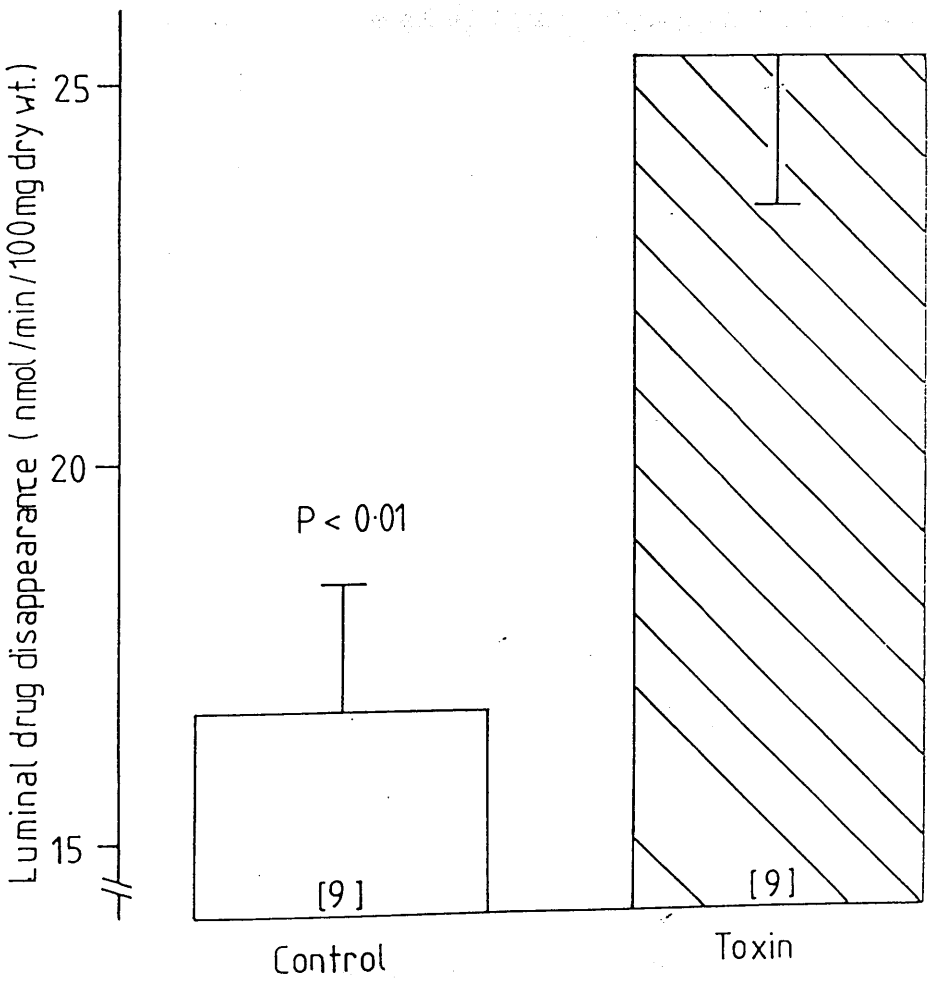


Figure 3.51 Effect of E.coli STa toxin on lignocaine absorption from perfused loops of rat jejunum. Details as for Figure 3.30.



This increased absorption was mirrored by a significant ($P<0.01$) increase in the mean peripheral blood drug concentrations after STa challenge, from values of $7.20\pm0.79(9)$ nmol/l in controls to $12.70\pm1.66(9)$ nmol/l in the presence of STa. From the peripheral blood drug concentration profiles represented in Figure 3.52, it can be seen that the blood drug concentrations in the presence of STa became significantly ($P<0.01$) elevated over control values after 45 minutes and remained higher throughout the remainder of the experiments.

STa reversed ($P<0.001$) a net fluid absorption (Figure 3.53) of $4.37\pm0.88(9)$ ul/min/100mg dry weight to a net secretion of $8.31\pm2.10(9)$ ul/min/100mg dry weight. STa also reversed ($P<0.001$) an apparent net hydrogen ion secretion (Figure 3.54) into the lumen of $0.53\pm0.07(9)$ uequiv./min/100mg dry weight in control experiments to an apparent hydrogen ion absorption (bicarbonate anion secretion) of $0.12\pm0.03(9)$ uequiv./min/100mg dry weight.

Therefore, as with the other weak bases studied, lignocaine absorption was enhanced after STa challenge of <u>in vivo</u> jejunal loops. Mean peripheral blood drug concentrations were also elevated in the presence of enterotoxin indicating that total drug transfer was affected. This enhanced absorption of lignocaine with toxin was despite unfavourable fluid movement.

Figure 3.52 Lignocaine concentration in peripheral blood after jejunal exposure to E.coli STa toxin. Details as for Figure 3.31 (* = P< 0.02; ** = P< 0.01 compared with respective control value).

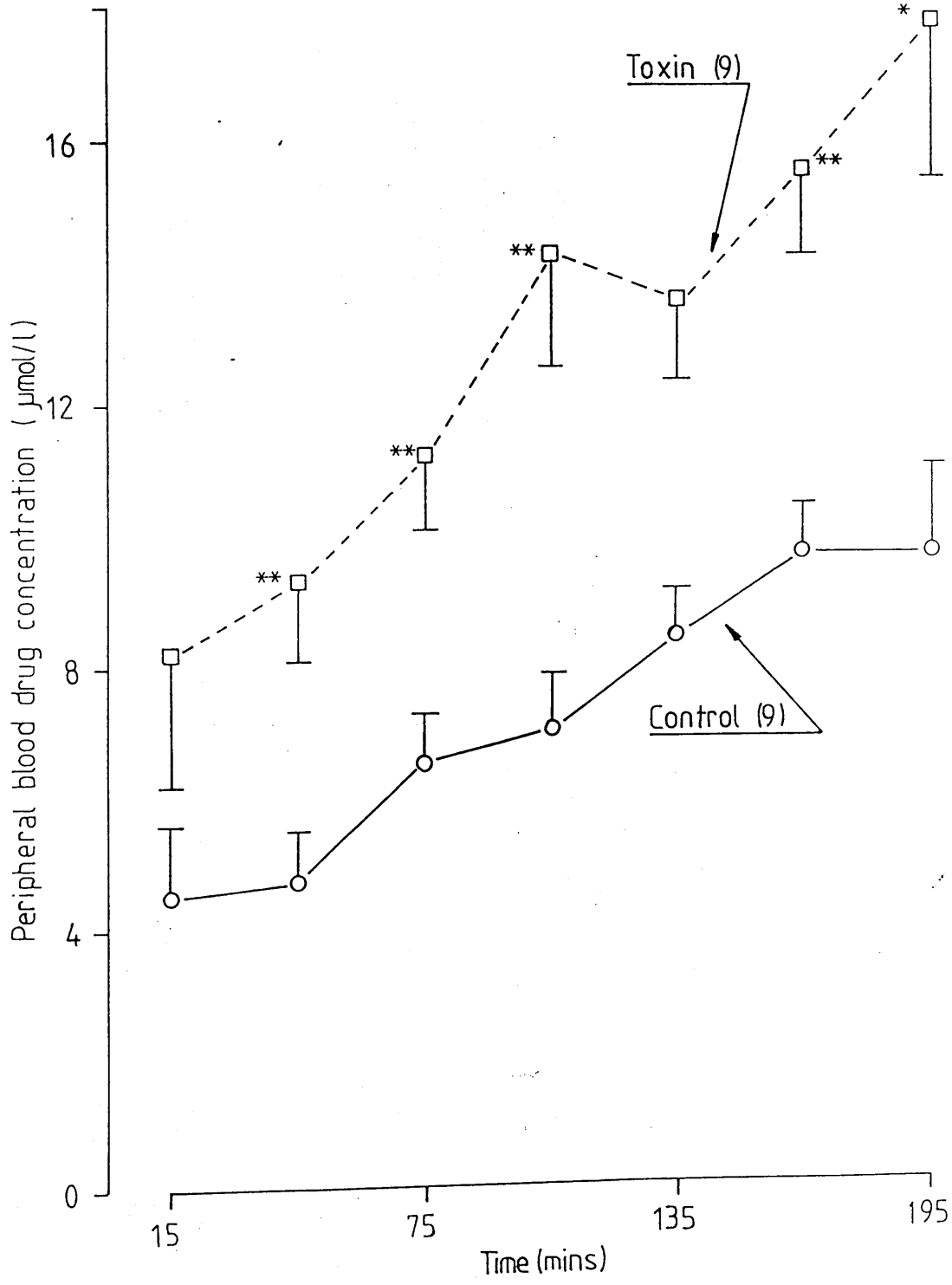


Figure 3.53 Effect of E.coli STa toxin on fluid transport in loops of rat proximal jejunum during lignocaine absorption. Details as for Figure 3.1.

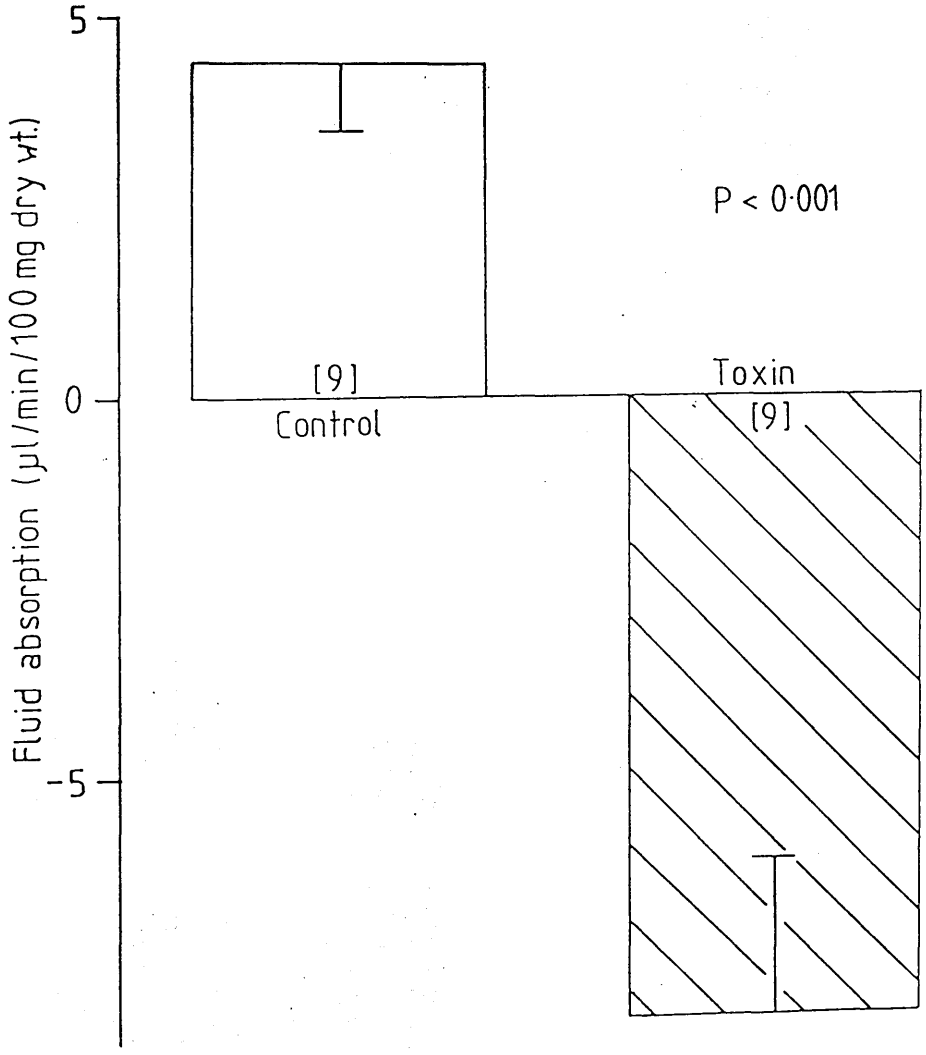
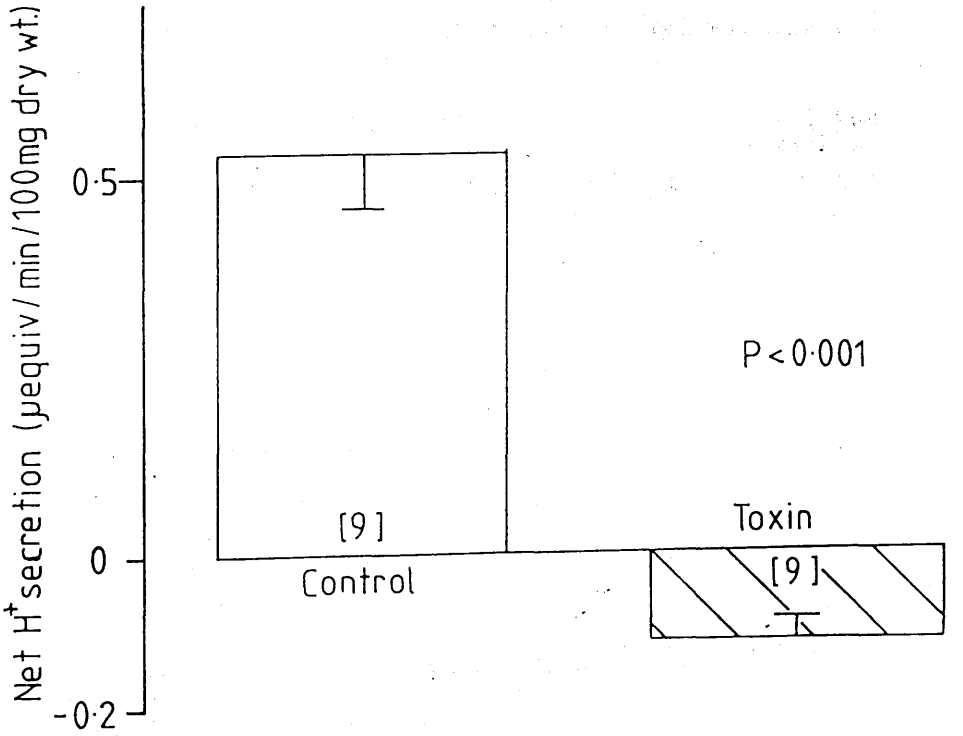


Figure 3.54 Effect of E.coli STa toxin on luminal acid-base balance in perfused loops of rat proximal jejunum during lignocaine absorption. Details as for Figure 3.1.



These findings were confirmed when the mean luminal drug concentration data was analysed by non-linear regression. The correlation coefficients calculated for the different absorption models (Table 3.5) indicate that the best fit for the data was provided by the second order exponential curve (Figure 3.56) rather than a first (Figure 3.55) order model. The first order absorption rate constant estimated for the toxin experiments, 5.78×10^{-3} min^{-1} was significantly ($P<0.001$) greater than the value of 2.86×10^{-3} min^{-1} calculated for control experiments.

As in the salicylic acid experiments (Section 3.4(a)) the ratio between the rate constant for drug transport across the cell membrane (k_{23}) and the rate constant for diffusion of drug out of an exchange compartment (k_{21}) was calculated from the four parameters of the second order exponential equation (Table 3.5). This ratio (k_{23}/k_{21}) of 0.99 was higher for toxin experiments than the 0.62 for control experiments, and is consistent with STa increasing lignocaine absorption in the jejunum.

(f) Effect of forskolin in combination with theophylline on lignocaine absorption

As a further test of the microclimate hypothesis the effect of forskolin combined with theophylline on the jejunal absorption of lignocaine was tested. A combination of forskolin and theophylline elevated the jejunal mucosal surface pH in a manner very similar to that observed after STa challenge. If the noted increase in lignocaine absorption was due to STa-induced changes

Table 3.5

Model	Parameters	Control	Toxin	Sig.
$C(t) = A - Bt$	A (mM)	0.934	0.875	
	B (μM/min)	2.08	3.19	*
	r	0.972	0.961	
$C(t) = Ae^{-kt}$	A (mM)	0.954	0.937	
	k ($x10^{-3}$ min^{-1})	2.86	5.78	*
	r	0.985	0.989	
$C(t) = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t}$	A (mM)	0.852	0.731	
	λ_1 ($x10^{-3}$ min^{-1})	2.02	3.88	*
	B (mM)	0.148	0.264	
	λ_2 ($x10^{-2}$ min^{-1})	2.95	2.74	NS
	r	0.999	0.999	

Estimated parameters for zero, first and second order pharmacokinetic for lignocaine luminal concentration data. Correlation coefficients (r) are presented as an index of the degree of fit of each model to the data (* = $P < 0.001$ compared with control; NS = not significant).

Figure 3.55 Least squares first order exponential curves for change in luminal lignocaine concentration with time in control (—●—) and toxin (--O--) treated loops. Values are given as mean ± S.E.M. for 9 (control and toxin) experiments, one observation per animal at specified times.

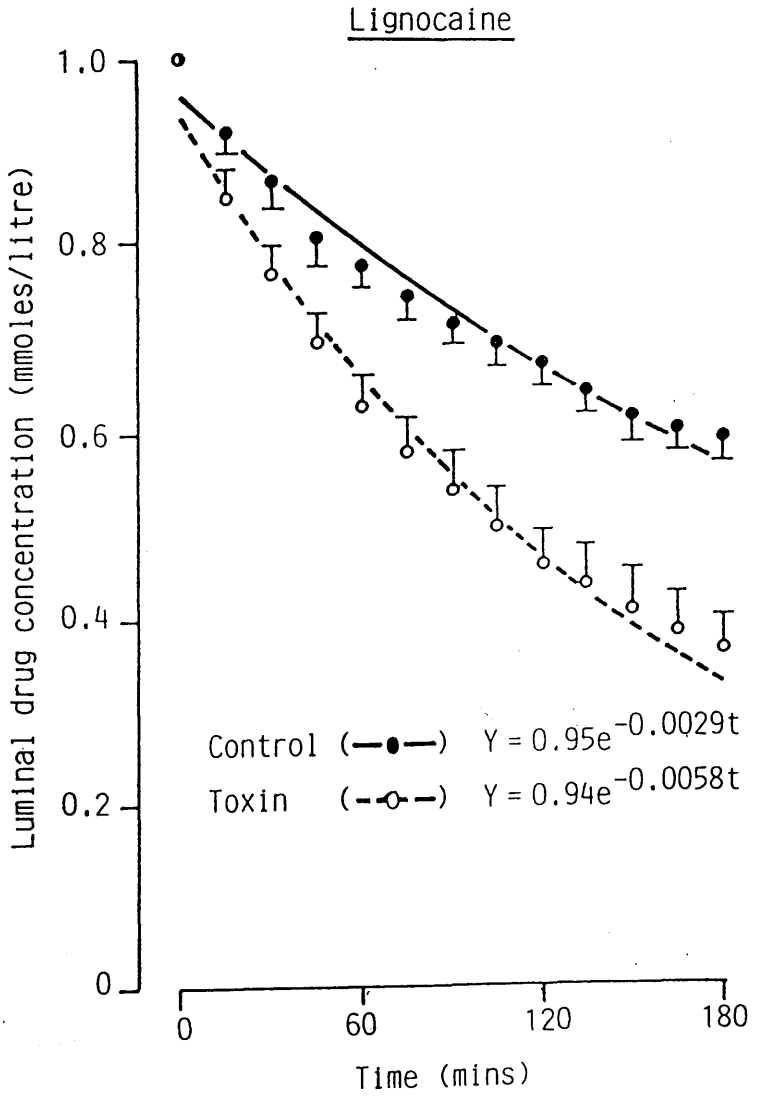
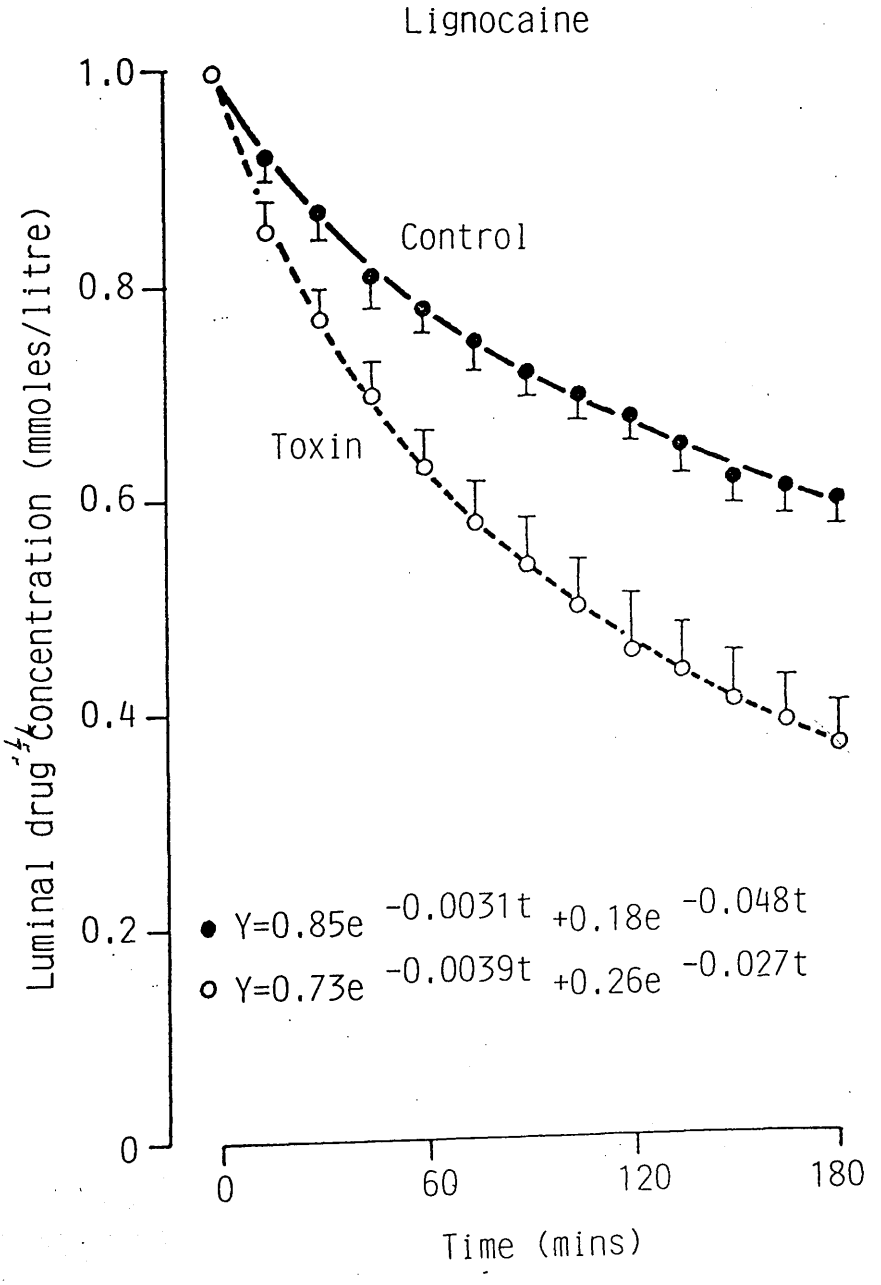


Figure 3.56 Least squares best fit second order exponential curves for change in liminal lignocaine concentration with time in control (—●—) and toxin (--O--) treated loops. Details as for Figure 3.55.



in jejunal mucosal surface pH, forskolin and theophylline should exert similar effects.

Forskolin (1mmol/l) and theophylline (20mmol/l) were incorporated into 15ml of Krebs-bicarbonate buffer containing 1mM lignocaine. This solution was then recirculated for three hours through *in vivo* loops of proximal jejunum. Under these conditions lignocaine absorption (Figure 3.57) was significantly ($P<0.01$) increased from $16.70\pm1.69(9)$ nmol/min/100mg dry weight in control experiments (see previous section) to values of $24.37\pm1.29(5)$ nmol/min/100mg dry weight, which were not significantly different from those obtained after *E.coli* STa toxin exposure.

As with STa, this increased absorption was confirmed by an increased ($P<0.01$) mean peripheral blood concentration in the presence of forskolin and theophylline to values of 11.36 ± 1.06 nmol/l, similar to those found with STa. These elevated blood drug concentrations (Figure 3.58) in the presence of forskolin and theophylline became significant ($P<0.05$) after 75 minutes and remained higher for the remainder of the experiments.

Net fluid absorption (Figure 3.59) in control experiments ($4.37\pm0.88(9)$ ul/min/100mg dry weight) was reversed ($P<0.001$) in the presence of forskolin and theophylline to a net secretion of $23.17\pm2.92(5)$ ul/min/100mg dry weight. This secretion was almost three times greater than that produced by STa, indicating that forskolin and theophylline are operating through different mechanisms (cAMP mediated secretion). Similarly to STa, the

Figure 3.57 Effect of forskolin (1mM) combined with theophylline (20mM) on lignocaine absorption from perfused loops of rat jejunum. Details as for Figure 3.30.

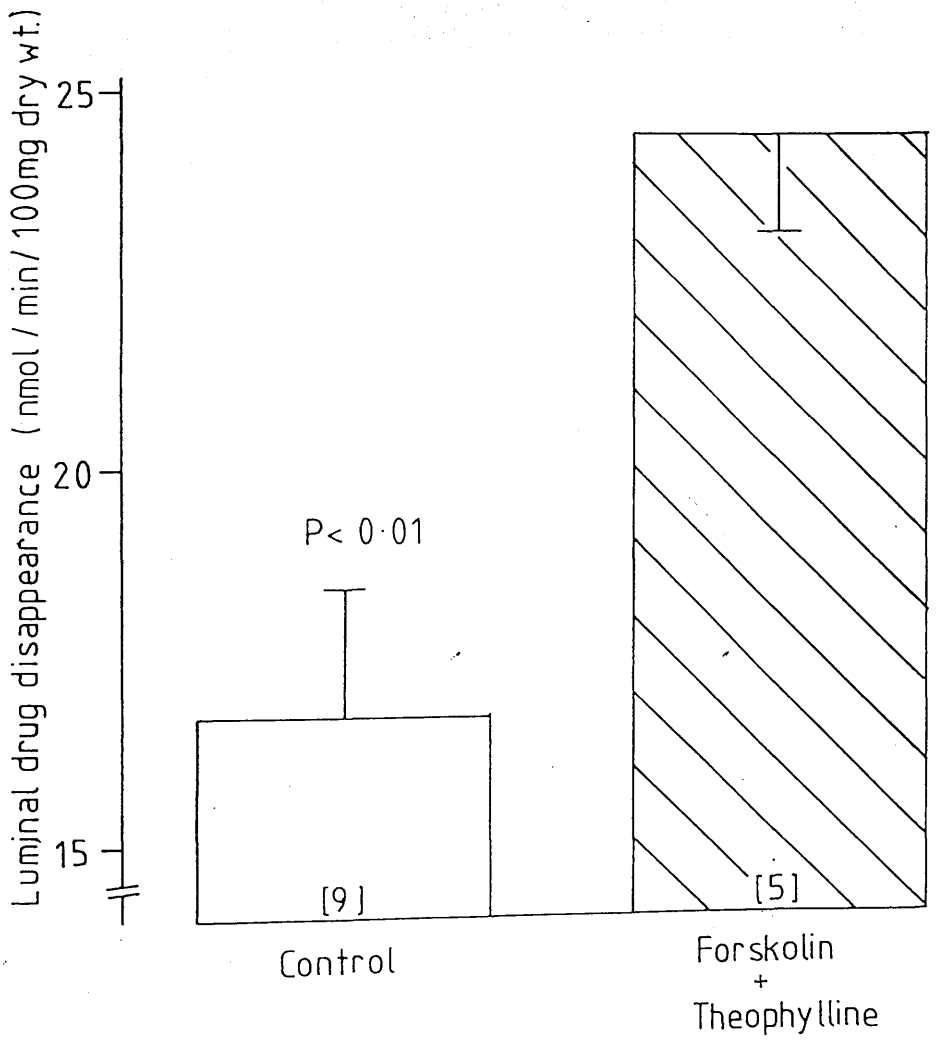


Figure 3.58 Lignocaine concentration in peripheral blood after jejunal exposure to forskolin (1mM) in combination with theophylline (20mM). Details as for Figure 3.31 (* = P< 0.05; ** = P< 0.01; *** = P< 0.001 compared with respective control value).

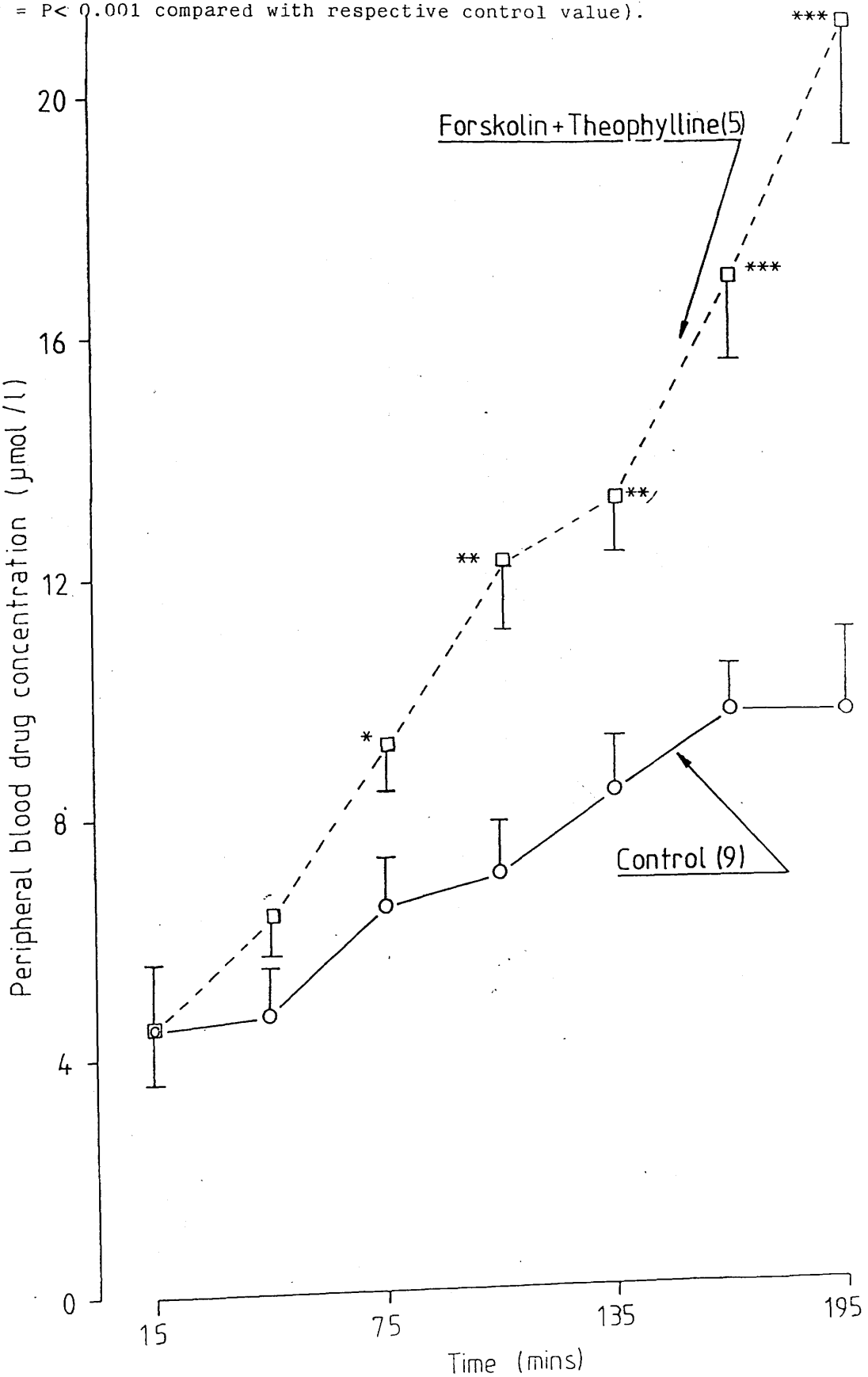
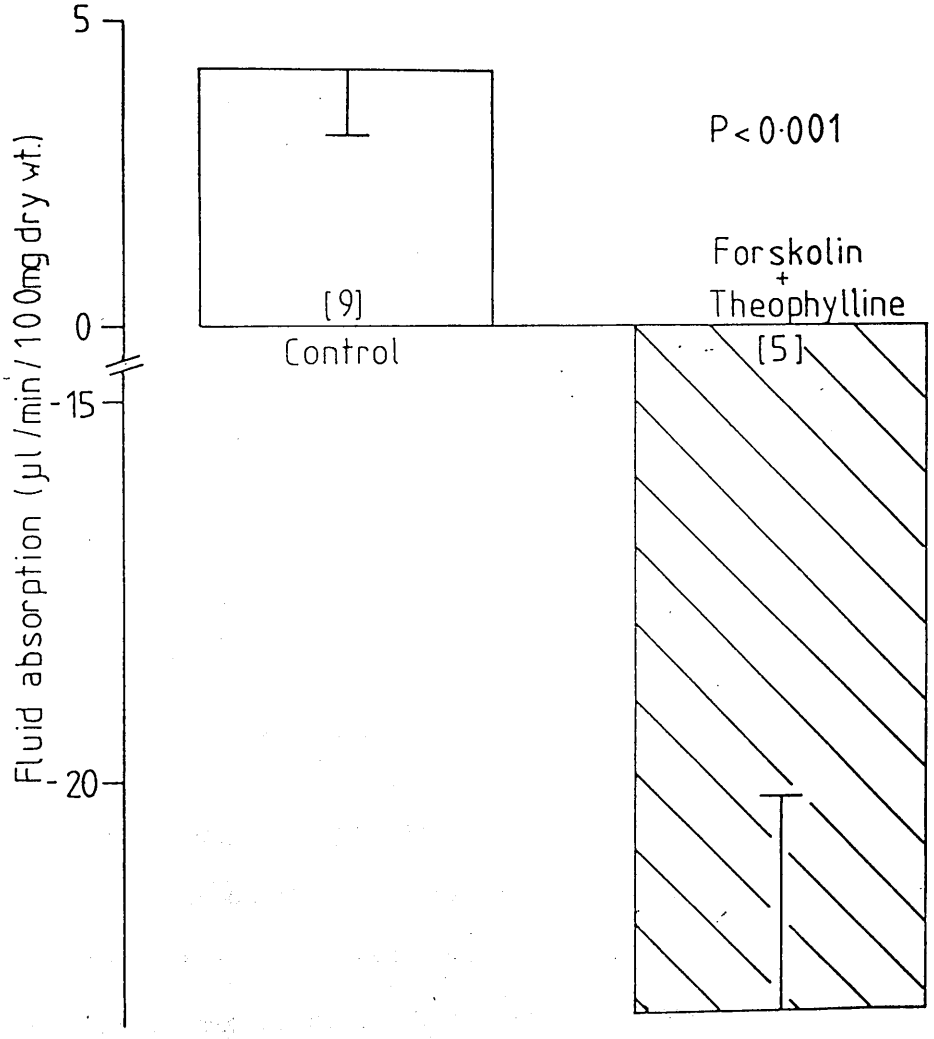


Figure 3.59 Effect of forskolin (1mM) in combination with theophylline (20mM) on fluid transport during lignocaine absorption. Details as for Figure 3.1.



forskolin/theophylline combination reversed net luminal acidification (Figure 3.60) in control experiments of 0.53±0.07(9) uequiv/min/100mg dry weight to alkalinisation of 0.08±0.02(5) uequiv/min/100mg dry weight.

Unlike the lignocaine control and STa experiments, non-linear regression analysis (Figure 3.61) of the mean luminal drug concentration data, indicated that a first order model only slightly enhanced the fit when the calculated correlation coefficient (Table 3.6) was compared to that for the zero order model. Confirming empirical data, the first order model absorption rate constant of 3.71×10^{-3} min^{-1} from the forskolin/theophylline experiments, was significantly ($P<0.001$) higher than the control value of 2.86×10^{-3} min^{-1}. A second order model for the forskolin/theophylline data defaulted to the simpler first order model.

As predicted, the combined effects of forskolin and theophylline are similar to those of STa on lignocaine absorption. Since both of these treatments alter mucosal surface pH, possibly through different mechanisms, this result is strong evidence that the presented alterations in weak electrolyte absorption are due to changes in the microclimate pH.

Summary of drug absorption experiments

The jejunal absorption characteristics of two weakly acidic and three weakly basic drugs were investigated *in vivo*. As predicted by the microclimate hypothesis, the weak acids, salicylic acid

Figure 3.60 Effect of forskolin (1mM) in combination with theophylline (20mM) on luminal acid-base balance in perfused loops of rat proximal jejunum during lignocaine absorption. Details as for Figure 3.1.

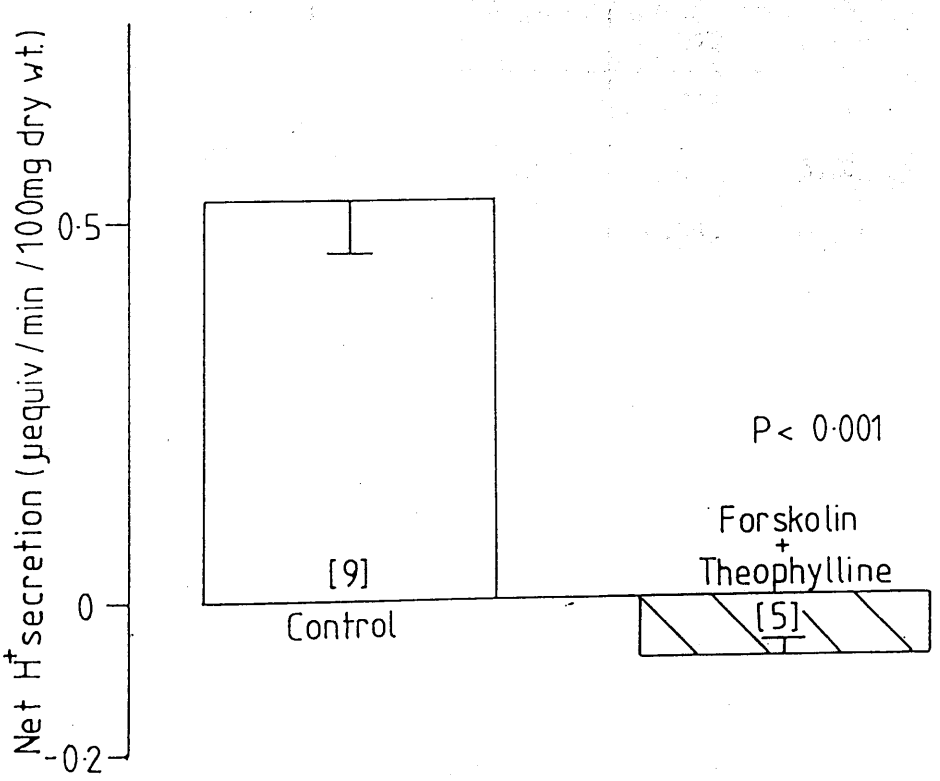
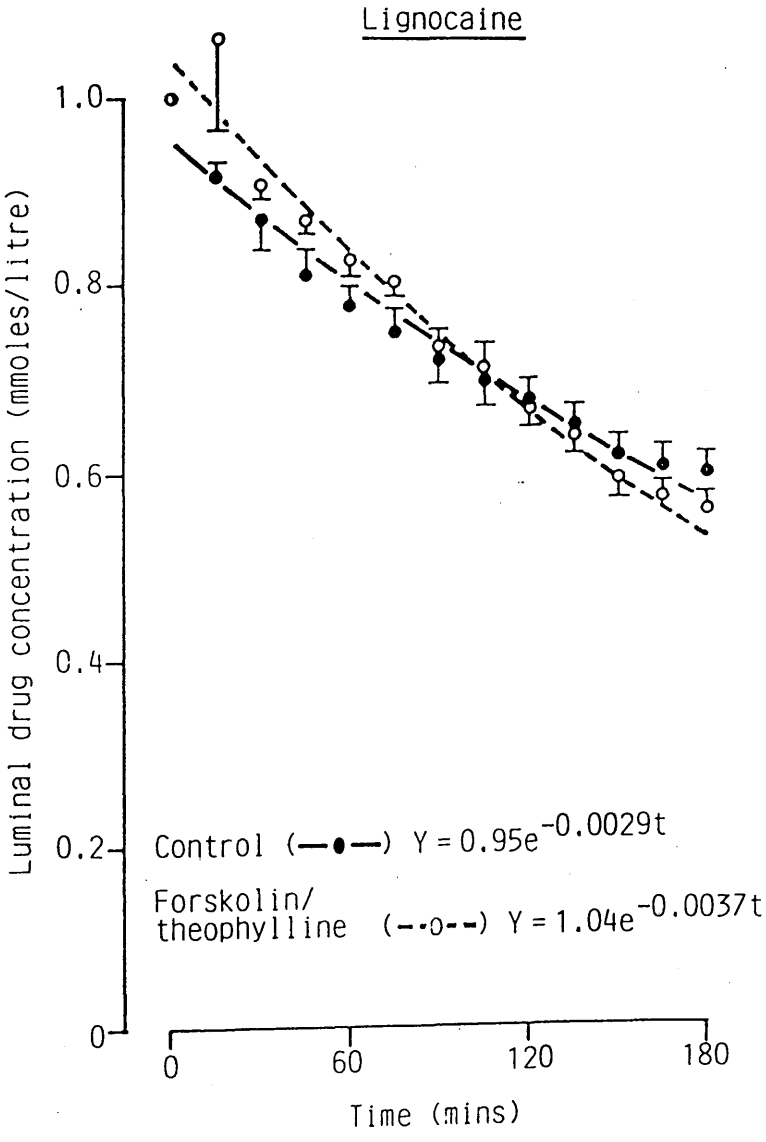


Table 3.6

Model	Parameters	Control	Forskolin	Sig.
$C(t) = A - Bt$	A (mM)	0.934	1.012	
	B (μM/min)	2.08	2.81	*
	r	0.972	0.983	
$C(t) = Ae^{-kt}$	A (mM)	0.954	1.043	
	k ($x10^{-3}$ min^{-1})	2.86	3.71	*
	r	0.985	0.986	

Estimated parameters for zero and first order pharmacokinetic models for lignocaine luminal concentration data (forskolin and theophylline). Correlation coefficients (r) are presented as an index of the degree of fit of each model to the data (* = $P < 0.001$ compared with control).

Figure 3.61 Least squares best fit first order exponential curves for change in luminal lignocaine concentration with time in control (—●—) and forskolin/theophylline (--O--) treated loops. Values are given as mean ± S.E.M. for 9 (control) and 5 (forskolin/theophylline) experiments, one observation per animal at specified times.

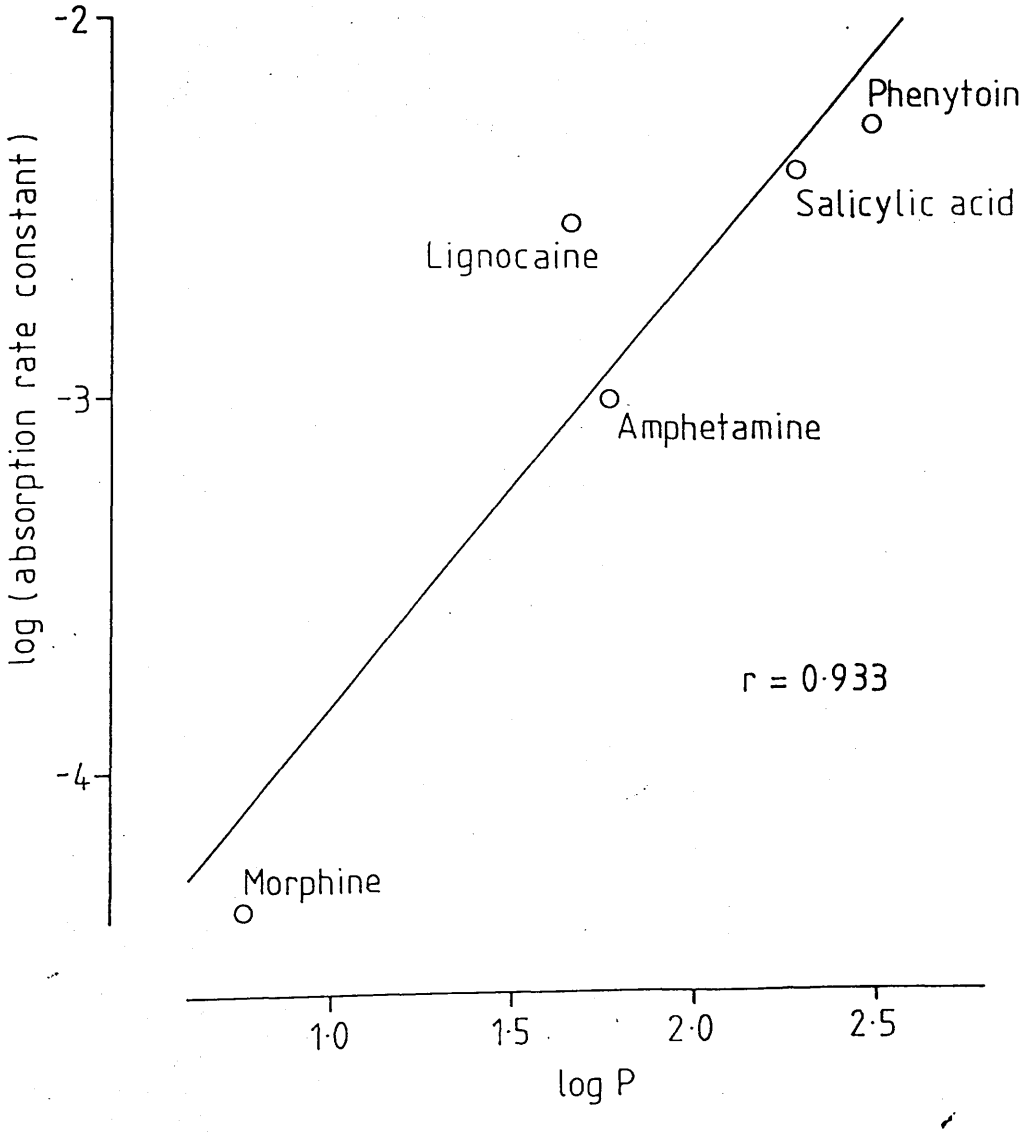


and phenytoin were absorbed at a faster rate than the weak bases, lignocaine, amphetamine and morphine. After exposing the jejunal mucosa to E.coli STa enterotoxin, both weak acids were malabsorbed while all three weak bases had their absorption enhanced. These absorption changes were confirmed by similar changes in the appearance of drug in the peripheral blood, indicating that total drug transfer was affected by STa. Increased weak base uptake occured despite unfavourable enterotoxin-induced fluid secretion. As expected, in all experiments STa abolished luminal acidification.

The combination of forskolin and theophylline resulted in an enhancement of lignocaine absorption very similar to that observed with STa. This was again, despite net fluid secretion. Since both STa and the forskolin/theophylline combination elevate jejunal surface pH, this is further evidence that the reported alterations in weak electrolyte absorption were probably due to changes in the microclimate pH.

More model orientated analysis of the luminal drug data demonstrated that amphetamine, phenytoin, and morphine follow first order absorption kinetics. However, lignocaine and salicylic acid absorption were best described by second order kinetics which are consistent with a compartmental model having an intermediate exchange compartment after the lumen.

Figure 3.62 Correlation of log absorption rate constant with log octanol/water partition coefficient (P). Least squares best fit regression line is shown.



particularly important for these drugs, the preferential route probably being through the cell membrane.

4. DISCUSSION

4.1 Intestinal surface pH measurements

The maintenance of a distinct acid microclimate, as originally proposed by Hogben and co-workers (1959) would firstly require a source of hydrogen ions for secretion into the jejunal lumen. Secondly, diffusion of these hydrogen ions may have to be retarded by intestinal mucus (Blair et al., 1975). It has been known for some time that the rat jejunum acidifies its luminal perfusates and a low surface pH has been demonstrated consistently *in vitro*. However, at the outset of this project it was uncertain whether the acid microclimate could be detected *in vivo*. Previous experiments from this laboratory (Lucas, 1983) demonstrated the existence of a low mucosal surface pH in rat jejunum *in vivo*. The present project undertook to improve on this work by developing a better *in vivo* preparation.

A specially designed *in vivo* perfusion chamber allowed the measurement of rat intestinal mucosal surface pH with ease. Unlike previous techniques (Lucas, 1983; Hogerle & Winne, 1983) the use of this chamber eliminated the problems of blood loss from the tissue under investigation and therefore provided a more stable preparation. When the jejunal mucosa was perfused with Krebs phosphate buffer at pH 7.1 the pH at the mucosal surface was approximately 6.2. This, therefore, confirms the existence of an 'acid microclimate' in the jejunum. The value obtained for this low pH region is consistent with previous measurements of jejunal surface pH both *in vitro* (Lucas et al., 1975; Lucas &

Blair, 1978; Lucas et al., 1980; Daniel et al., 1985; Shiau et al., 1985; Daniel & Rehner, 1986; Shimada, 1987) and *in vivo* (Lucas, 1983; Hogerle & Winne, 1983; Iwatsubo et al., 1986). The present preparation is an improvement on previous attempts at measuring mucosal surface pH *in vivo*. After exposing the mucosa, Lucas (1983) allowed the tissue to continue bleeding in the hope that clotting would be sufficiently rapid and complete to minimise blood loss. The technique adopted by Hogerle and Winne (1983), in which the tissue was compressed beneath a chamber, may have inadvertently reduced the blood flow to the tissue, inducing some anoxia. The chamber utilised in the present experiments is an improvement on both these methods since bleeding is prevented and blood flow is unrestricted. A similar type of chamber preparation has recently been reported (Iwatsubo et al., 1986), however, these workers used antimony pH electrodes to measure surface pH. This introduces additional problems of interpretation as will be discussed below. The glass electrode used in the present study is not subject to the limitations inherent in the use of the antimony electrode. To date, only one group has failed to demonstrate an acid surface pH in the rat jejunum (Rechkemmer et al., 1986). However, it should be noted that only four rats contributed to the latter conclusion and therefore it should not be given too much prominence.

Acidification of jejunal luminal perfusates in vivo, as observed in this study and others (Parsons, 1956; Hubel, 1973; Podesta & Mettrick, 1977) suggests that the low surface pH measured is maintained by a source of hydrogen ions at the mucosal surface. These hydrogen ions would, presumably, diffuse out of the 'microclimate' region to lower the pH of the luminal contents (Blair et al., 1975). The 'microclimate' region probably consists of a surface adhering mucus layer within the unstirred water layer since diffusion of hydrogen ions through mucus would be slower than diffusion through the bulk solution.

The acid microclimate measured in these experiments is not a physiological artefact caused by tissue anoxia. Occlusion of the afferent mesenteric blood vessels supplying the tissue resulted in an elevation of surface pH. This was in agreement with the results of a previous study (Lucas, 1983) and demonstrates that, contrary to a previous suggestion (Flemstrom et al., 1982), anoxia interrupts the processes maintaining the acid surface pH in jejunum. This is presumably due to a cessation of the metabolic processes upon which the the maintenance of an acidic microclimate depends.

Despite taking every possible precaution in the present experiments, some animals inevitably died under the influence of anaesthesia. Rather than let the animals die needlessly it was decided to measure luminal acid-base changes in these preparations. These experiments led to the striking observation

that acidification ceased almost immediately when the heart stopped beating, thereby providing further evidence that acidification is not an anoxic phenomenon. This finding also strongly suggested that luminal acidification and therefore, probably, the maintenance of a low surface pH, requires a metabolically active tissue producing a source of luminally directed protons (or conversely an absorption of bicarbonate anions). This is inconsistent with the hypothesis that mucus alone, acting as an ampholyte, maintains the acid microclimate (Shiau et al., 1985) since, presumably, the mucus would remain after death had ensued. If mucus were responsible for microclimate maintenance acidification should continue, even after death.

The existence of an acid microclimate in the jejunum was first postulated to explain apparent anomalies in intestinal weak electrolyte absorption which did not conform to the pH partition hypothesis (Hogben et al., 1959). Although a low surface pH has been frequently demonstrated, it has been suggested that the acid region occurs at the base of the villi (Jackson, Williamson, Dombrowski & Garner, 1978). Since it is thought that nutrient absorption takes place mainly in the upper third part of the villus (Philips, 1982) it was proposed that the acid microclimate would not affect absorption processes. In the present study, measurements with liquid ion exchange pH microelectrodes have shown that the area of lowest pH can be localised to the region approximately 100-200 um below the villus tips and that even the

tips themselves are bathed in fluid that is substantially more acid than a neutral bulk bathing solution. This finding is in agreement with the findings of a previous study (Daniel et al., 1985) and supports the view that the acid microclimate will affect weak electrolyte absorption. When the pH microelectrode was placed at the villus base the pH measured in this region was considerably higher than that measured in the upper villus confirming the existence of a pH gradient along the jejunal villus-crypt axis as originally measured by Daniel and co-workers (1985) using antimony microelectrodes. The pH values measured in the crypt region in this latter study were much more alkaline than those measured with the liquid ion exchange electrodes. However, antimony electrodes are sensitive to the oxygen tension of the medium in which they are measuring (Tourky & Mousa, 1948; El Wakkad, 1950) and deviations from the pH response have been observed at low PO_2 values (Daniel et al., 1985). Therefore, there is a possibility that there will be a low oxygen tension in the crypt regions which may account for the high pH values measured with the antimony electrodes. Supporting this it was recently shown that when an antimony microelectrode and a liquid ion exchange microelectrode were used to determine the pH profile of the same jejunal villus there was perfect agreement between the values obtained for the upper villus measurements but the values began to deviate towards the crypt region, the antimony electrode giving the higher values (H. Daniel, personal communication). Liquid ion exchange electrodes are not affected

by oxygen tension and therefore the values presented in the present study are probably more representative of the true pH at the villus base. A similar discrepancy between measurements made with liquid ion exchange and antimony electrodes has also been observed in the duodenum (H. Daniel, personal communication). This would suggest that the reported finding that the mucosal surface of rat duodenum is alkaline (Flemstrom & Kivilaakso, 1983) may also be the result of an antimony electrode artefact, since these electrodes were also used in this study.

Mucosal surface pH measurements of the rat ileum indicated that a neutral surface pH exists, which is in agreement with the only other reported measurement of in vivo rat ileal surface pH (Lucas, 1983). This value of surface pH agrees with the insignificant luminal alkalinisation observed in vivo in perfused loops of rat ileum. These findings suggest that in the absence of any stimulus, the rat ileum does not secrete bicarbonate to any significant degree. This is not in agreement with the established view that the rat ileum secretes bicarbonate at all times (Parsons, 1956; Hubel, 1967; Hubel, 1969). However, there is a possibility that the ileum has a capacity for secreting hydogen ions equivalent to its bicarbonate secreting capacity. Indeed this has been suggested on the basis of ion replacement studies (Turnberg et al., 1970). This would produce a net secretion which is neutral and would hence explain both the neutral ileal surface pH measured and the relative failure of the ileum to alter the pH of its luminal perfusates in the present experiments. Where

permanent bicarbonate secretion has been observed, this may have been due to inadvertent stimulation, during the preparative procedures. A reconciling view might be that bicarbonate secretion does not occur all the time but will do when provoked by factors, as yet undetermined. Further evidence for the absence of bicarbonate secretion came from the pH microelectrode studies in vitro. Here, in contrast to the jejunum, when the pH profiles of ileal villi were measured in vitro no striking pH gradient was observed along the villus-crypt axis. This was, again, evidence for the ileum secreting neutral fluid since a pH gradient would not be expected under these conditions. If the tissue were secreting large quantities of bicarbonate alone, higher local pH values than the ones measured might be expected. There are a myriad of possibilities as to what this ileal secretion might be eg. plasma exudate, 'isotonic' bicarbonate secretion etc. However, while these pose interesting questions for further research, the likelihood of bicarbonate secretion in the rat ileum under normal conditions was outside the scope of this thesis.

4.2 Effect of E.coli STa toxin on intestinal mucosal surface pH

Exposing the mucosal surface of rat small intestine to E.coli STa enterotoxin resulted in an elevation of the intestinal microclimate pH to values significantly higher than those measured in unchallenged tissue. In the jejunum, the normally acidic mucosal surface became nearly neutral whereas the neutral

pH measured in the ileum was elevated considerably above the pH of the perfusing buffer. This mucosal surface alkalinisation was both rapid and readily reversible, resembling STa action on intestinal fluid transport (Mullan et al., 1978). These changes are consistent with the changes in luminal acid-base balance observed after STa challenge in in vivo perfused intestinal loops. In these experiments, while inhibiting fluid absorption as expected, STa also abolished luminal acidification in the jejunum and greatly enhanced a low luminal alkalinisation in the ileum. It should be noted, however, that the large and very rapid pH shifts detected at the mucosal surface would not be evident from these perfusate measurements alone.

The cessation of luminal acidification in the STa-challenged jejunum suggests that the enterotoxin is acting by inhibiting luminal hydrogen ion secretion or its converse, bicarbonate anion absorption. Support for this mechanism of toxin action was supplied from the jejunal villus pH profile measurements obtained after STa exposure. These demonstrated that the upper villus region which exhibited the most acid values in untreated tissue was most affected by STa challenge. This can be explained by STa inhibiting the acidification process in the upper villus cells. The relatively low pH values measured at the villus base in the STa-treated jejunum do not favour the concept of STa stimulating bicarbonate secretion from the jejunal crypts as has been demonstrated in the ileum after exposure to cholera toxin (Hallback, Jodal, Sjoquist & Lundgren, 1982).

In the ileum, the mode of action of STa is to cause alkalinisation above bulk phase values but this could occur by several means. Unlike the jejunum, the ileum did not present a striking pH gradient along the villus-crypt axis. Exposure to STa elevated the pH fairly uniformly the villus length. This, coupled with the relatively high pH values attained after STa challenge and the large STa-induced alkalinisation of ileal lumen tends to favour an elevated bicarbonate anion secretion in the ileum, as has been previously demonstrated after exposure to cholera toxin (Leitch & Burrows, 1968; Carpenter et al., 1968; Norris et al., 1969; Moore et al., 1971; Hubel, 1974). However, as mentioned previously, the present results would suggest that the ileum possesses both hydrogen ion and bicarbonate secretory activity and therefore inhibition of hydrogen ion secretion by STa as proposed for the jejunum may have revealed an underlying bicarbonate secretion continuing unaffected by STa.

The mechanism through which STa would affect the acidification process is unknown. However, it has been demonstrated that STa abolishes net sodium absorption in the upper villus cells of the small intestine (Rao & Field, 1984). In the absence of nutrient non-electrolytes which promote sodium ion entry by the well-characterised sodium-cotransport system (Crane, 1962), a substantial component of sodium ion entry could be by exchange with hydrogen ion (Turnberg et al., 1970; Hubel, 1973; Murer et al., 1976; Liedtke & Hopfer, 1977; Podesta & Mettrick, 1977). Inhibition by STa of sodium entry mediated by exchange with

intracellular hydrogen ion should inhibit luminal hydrogen ion secretion resulting in an elevation of the mucosal surface pH. Supporting this view, it has been shown that reducing the sodium concentration in the bulk solution elevates the jejunal mucosal surface pH *in vitro* (Lucas et al., 1980) and *in vivo* (Iwatsubo et al., 1986). It has also been shown that ouabain (Lucas & Blair, 1978; Iwatsubo et al., 1986), chlorpromazine and amiloride (Iwatsubo et al., 1986), all of which impair sodium ion transport, elevate the jejunal surface pH. Therefore, there is evidence to favour STa inhibition of Na^+-H^+ exchange as being the mechanism for STa-induced mucosal surface alkalinisation. However, this evidence is, as yet, circumstantial and other mechanisms could be involved.

STa is believed to act by increasing guanylate cyclase activity in intestinal epithelial cells (Field et al., 1978; Hughes, Murad & Guerrant, 1978a; Guerrant, Chang, Robertson & Murad, 1980). The resulting elevation of intracellular cGMP levels (Hughes et al., 1978; Field et al., 1978) is thought to bring about the changes in fluid and ion transport observed after STa challenge. The evidence for this is as follows: (1) STa effects on both cGMP accumulation and secretory responses in the rabbit (Hughes et al., 1978), mouse (Hughes et al., 1978) and Ussing chamber studies on rabbit ileum (Field et al., 1978) demonstrate a comparable rapid onset, time course and dose response. (2) The 8-bromo analogue of cGMP resembles the STa effect on secretion, including location of intestinal responses (Hughes et al., 1978;

Gianella & Drake, 1979). (3) Agents which prevent STa activation of guanylate cyclase consistently prevent secretory responses, eg. chlorpromazine (Abbey & Knoop, 1979; Greenberg, Chang, Robertson & Guerrant, 1980) and quinacrine (Greenberg, Chang, Robertson & Murad, 1982). STa will only activate the particulate form of guanylate cyclase (Hughes et al., 1978a; Guerrant et al., 1980). In the intestine this form of the enzyme predominates and is localised in the brush border membranes of upper villus cells (Quill & Weiser, 1975; de Jonge, 1975). The proposal that the upper villus cells are the major site of action for STa (de Jonge, 1984) is fully consistent with the present finding that the most prominent change in surface pH in the STa-treated jejunum occured in the upper villous region.

The STa preparation used in this study was produced microbiologically (Mullan et al., 1978). Despite extensive purification there is always a possibility that extraneous products (particularly peptides) may be present. To test for this the response to a highly purified synthetic STa analogue was examined. This analogue, STh(6-19), a fourteen amino acid peptide, has been shown to have the same enterotoxigenic properties as microbiologically produced STa (Aimoto et al., 1983). The observation that the highly purified synthetic peptide induced a virtually identical mucosal surface alkalinisation in the jejunum to that induced by the microbiologically produced STa toxin makes it highly unlikely that the effects observed with the latter were due to anything other than the enterotoxigenic

properties of STa.

Although STa acts through cGMP to alter net fluid transport, almost no biochemical details are known for STa effects on hydrogen ion production. Because of its effects on fluid transport, cGMP seemed the likely mediator of STa-induced mucosal alkalinisation, although the possibility of cAMP involvement also had to be considered. With this in mind an attempt was made to confirm that cGMP is involved in STa action on surface pH. The initial finding that theophylline, a phosphodiesterase inhibitor, prevented the normal reversibility of STa induced mucosal surface alkalinisation provided evidence for cyclic nucleotide involvement in this phenomenon. Theophylline was, presumably, acting by maintaining the elevated intracellular cyclic nucleotide levels after toxin removal. However, since theophylline is a known inhibitor of both cGMP and cAMP phosphodiesterases in intestinal villus cells (Quill & Weiser, 1975) it is not possible, from this result, to differentiate between a cGMP or a cAMP dependent process. To determine which of these cyclic nucleotides was responsible for the observed STa effects the lipid soluble 8-bromo analogues of cGMP and cAMP were applied exogenously to the jejunal mucosa. 8-bromo cGMP induced a mucosal surface alkalinisation very similar to that induced by STa and STh(6-19). In contrast, the same concentration of 8-bromo cAMP caused only a relatively small surface pH shift. From this it must be concluded that cGMP is the likely mediator of STa-induced intestinal mucosal surface alkalinisation.

4.3 Effect of cAMP dependent secretory agents on jejunal mucosal surface pH

The experiments with the cyclic nucleotide analogues suggest that cAMP dependent secretory agents are not so effective at inducing surface pH changes as those dependent on cGMP. To test this hypothesis, the effects of forskolin and cholera toxin, both of which activate intestinal adenylate cyclase (Seamon & Daly, 1981; Schafer, Lust, Sircar & Goldberg, 1970; Guerrant, Chen & Sharp, 1972; Field et al., 1972), on jejunal surface pH were investigated. The mechanisms whereby these compounds activate adenylate cyclase are entirely different. Cholera toxin, an 87 Kdal protein, consists of an A_1 peptide linked by a disulphide bond to an A_2 peptide and five B peptides. The toxin enters the cell at the brush border membrane by interacting with a G_{M1} ganglioside on the cell surface. After gaining entry the A_1 subunit irreversibly modifies the guanine nucleotide binding protein (G protein) leaving adenylate cyclase permanently activated for the lifetime of the cell. These events take a considerable time to occur and account for the normal 30-60 minute delay before any increase in intracellular cAMP levels become evident. In contrast, forskolin does not bind to a membrane receptor, nor does it alter the guanine nucleotide binding protein. The action of forskolin is to stimulate directly the catalytic subunit of the adenylate cyclase complex. The fashion of this action is both rapid and reversible (Seamon &

Daly, 1981). Therefore, the effect of an intracellular elevation of cAMP levels on jejunal surface pH was examined by three different methods: exogenously applied 8-bromo cAMP and mucosal exposure to forskolin or cholera toxin. All three methods resulted in the jejunal surface pH rising to very similar values which were considerably lower than those achieved after exposure to the cGMP elevating agents. The comparatively small surface pH elevation observed with forskolin resembled the reduced, but still significant, luminal acidification observed in perfused jejunal loops after exposure to forskolin. With cholera toxin, there was a three hour delay before any surface pH changes became evident but this was expected given the known delay of cholera toxin induced intesinal fluid secretion (Schaffer et al., 1970; Guerrant et al., 1972). Prolonged exposure to cholera toxin had no effect on the jejunal surface pH. This is more evidence that cAMP dependent secretory agents are far less capable of inducing surface pH changes than those dependent on cGMP.

Why there should be this difference between cGMP and cAMP is puzzling. In mammalian small intestine increased intracellular cGMP levels cause changes in transport similar to the transport changes resulting from increases in cAMP levels (Field et al., 1978; Murad, Arnold, Mittal & Broughler, 1979; Rao & Field, 1984; Rao, 1985). A possible explanation for the apparent difference observed in this study may lie in the respective locations of adenylate and guanylate cyclase in the intestinal epithelial cells. An increasing gradient of guanylate cyclase activity from

the crypt cells to the upper villus cells has been demonstrated in the rat small intestine (de Jonge, 1975; Quill & Weiser, 1975). Conversely, adenylate cyclase activity is greatest in the crypt cells and there is a decreasing gradient of activity from the crypts to the upper villus cells (de Jonge, 1975; Quill & Weiser, 1975). Additionally, guanylate cyclase is located almost exclusively in the brush border membrane whereas adenylate cyclase is located in the basolateral membrane (de Jonge, 1975; Quill & Weiser, 1975). It might be expected from this that an increased guanylate cyclase activity would result in a highly concentrated local elevation of cGMP in the region of the brush border. Therefore it seems reasonable to assume that this would affect brush border transport processes more efficiently than cAMP produced at the basolateral membrane which must diffuse across the cytosol to the luminal membrane before effects on transport could occur. This cAMP would be continuously broken down by cAMP phosphodiesterase, the activity of which is high in the rat jejunum (Loeschke, Farak, Gerzer & Keravis, 1987). There is, therefore, a possibility that the cAMP levels experienced at the brush border membrane are never high enough to elicit a maximal alkalinisation. The finding that forskolin combined with theophylline produced an alkalinisatiion of the mucosal surface pH in the jejunum virtually identical to the response induced by STa toxin is consistent with this hypothesis. Theophylline was, presumably, acting to prevent cAMP breakdown, thereby allowing the cAMP levels at the brush border to rise. The same action

would account for the elevation of surface pH observed when theophylline was applied to the jejunal mucosa after prolonged exposure to cholera toxin. However, theophylline alone had no effect on the jejunal surface pH. This was surprising since an inhibition of cAMP and cGMP phosphodiesterases should lead to an intracellular build up of both these cyclic nucleotides. Therefore, if cGMP and cAMP are responsible for the mucosal surface alkalinisation observed, exposure to theophylline should induce surface pH changes. However, theophylline is a weakly basic compound with a pKa of 8.75 and according to the Henderson-Hasselbach equation, at the pH prevailing at the mucosal surface of the unchallenged jejunum it will exist predominantly in the ionised form. From the pH partition hypothesis (Shore et al., 1957) weak electrolytes only permeate the intestinal epithelium in the unionised form. Therefore, there is a possibility that insufficient theophylline enters the enterocyte to induce any effects. After treatment with forskolin and cholera toxin there was a significant elevation of the jejunal surface pH which would increase the proportion of unionised theophylline presented to the mucosal surface. Theophylline would then enter the cell by non-ionic diffusion helping to increase cAMP levels and causing a further increase in surface pH. This, in turn, would allow more theophylline to enter the cell until a maximal effect was achieved.

The present results do not preclude the possibility that there is a functional difference between cGMP and cAMP. The enhancement of forskolin and cholera toxin-induced mucosal alkalinisation in the presence of theophylline could be equally well explained by an increase in cGMP levels since theophylline inhibits cGMP phosphodiesterase to the same extent as cAMP phosphodiesterase (Quill & Weiser, 1975). The high activity of guanylate cyclase in the intestinal brush border (de Jonge, 1975; Quill & Weiser, 1975) would normally maintain high cGMP levels in this region in the absence of cGMP phosphodiesterase. The finding that high concentrations of mucosally administered 8-bromo cAMP had a relatively minor effect on jejunal surface pH is consistent with cGMP being the more potent effector of surface pH changes. Future experiments measuring tissue cGMP and cAMP levels after jejunal exposure to the various agents tested may provide a more definitive conclusion on the relative effectiveness of cAMP and cGMP. However, the present results raise the possibility that secretory diarrhoea induced by cGMP elevating enterotoxins may be characterised by acid-base changes at the mucosal surface of the upper small intestine which may not be evident with cAMP mediated infection.

4.4 Effect of other secretagogues on jejunal fluid transport and luminal acid-base balance

One of the principle aims of this project was to find methods of efficiently and reliably altering the intestinal microclimate pH so that experiments could be undertaken which tested the microclimate hypothesis. The effects of E.coli STa enterotoxin obviously fulfilled this objective. However, the supplies of STa available were limited since, for much of the period of research, no commercially produced STa existed and the facilities for microbiological production and purification of enterotoxin were not available. Therefore, an attempt was made to find alternative agents which would induce surface pH changes.

In previous in vitro clinical studies of surface pH in the human jejunum (Lucas & Blair, 1978; Lucas et al., 1978), values were obtained from individual cases whose clinical diagnosis was elusive (Lucas, personal communication). This is apparently not unusual as approximately only one half of patients attending a routine gastroenterology clinic have illness which is diagnosed. Frequently, precise diagnosis is not made although treatment alleviates symptoms. One isolated case gave a puzzlingly high value for jejunal mucosal surface pH at the time of investigation and was subsequently found to be suffering from concealed laxative abuse. This is an increasingly recognised syndrome (Moriarty, 1987). The identified laxative was 'Dorbanex', a commercially available anthraquinone. For this reason, the effects of 'Dorbanex' on aspects of intestinal function was

tested.

'Dorbanex' inhibited luminal acidification in the jejunum while inducing mild fluid secretion. However, the surfactant nature of the laxative made its further use impractical. Additional histological evidence suggested that the preparation induced profound structural changes in the intestine and this might complicate the interpretation of any drug absorption study involving 'Dorbanex'. The inhibition of jejunal acidification observed in the 'Dorbanex'-treated jejunum suggests that changes in surface pH may have occurred. The active ingredient of 'Dorbanex' is an anthraquinone derivative, danthron. This class of compounds has been shown to inhibit fluid, sodium and chloride transport in the rat jejunum (Leng-Peschlow, 1980). It is a possibility that the inhibition of luminal acidification observed could be due to an inhibition of the Na^+-H^+ exchange process in the jejunum. Unlike STa, forskolin and cholera toxin, the anthraquinones do not act through cyclic nucleotides, Ca^{2+} being the proposed mediator of laxative action (Donowitz, Wicks & Battisti, 1984). However, the exact nature of Ca^{2+} involvement has yet to be elucidated.

In another series of experiments, Clostridium perfringens enterotoxin failed to alter acidification of the jejunal lumen and only caused a mild inhibition of fluid absorption. This was surprising since a previous study demonstrated that this enterotoxin induced secretion of fluid, sodium and chloride in

the rat ileum (McDonel, 1974). However, when net transport of sodium was resolved into unidirectional fluxes (McDonel & Asano, 1975) enterotoxin was found not to affect sodium absorption but to increase the sodium efflux into the lumen two-fold. This would account for the failure of enterotoxin to alter luminal acidification in the jejunum since presumably the Na^+-H^+ exchange could continue unhindered. The relative failure of enterotoxin to alter fluid transport may also be explained by the finding that enterotoxin is only mildly active in the jejunum and nearly inactive in the duodenum (McDonel & Duncan, 1977). Therefore, in retrospect, the present results obtained from experiments on the effects of C.perfringens enterotoxin are consistent with published observations.

4.5 Effect of E.coli STa toxin on intestinal drug absorption

According to the microclimate hypothesis (Hogben et al., 1959), because weak electrolytes will only partition into the cell membrane by non-ionic diffusion, the pH prevailing at the mucosal surface should determine the rate at which weakly dissociable compounds traverse the intestinal epithelium. An elevation of the microclimate pH, as observed with STa, should promote weak base absorption but hinder weak acid absorption (Figure 3.29). To test this, solutions of five weakly dissociable drugs, two weak acids (salicylic acid and phenytoin) and three weak bases (amphetamine, lignocaine and morphine) were perfused through rat jejunal loops in vivo in the presence and absence of STa. Because the studied

drugs were ^{14}C-labelled compounds it should be noted that monitoring changes in the isotope activity does not necessarilly represent changes in the concentration of the parent molecule. However, the apparatus necessary for a more sophisticated analysis of drug transport was not available and therefore this limitation should be taken into consideration. In the following discussion the five drugs studied will be considered individually.

(i) Salicylic acid

In a previous study from this laboratory (Lynch, 1986) it was demonstrated that salicylic acid absorption is significantly reduced in the presence of STa. However, the method for analysing blood adopted involved burning the samples on filter paper in another laboratory. This technique was unreliable and the subsequent loss of samples was unsatisfactory. Therefore it was decided to repeat the salicylic acid absorption experiments adapting a modified blood processing procedure. By reproducing previous results it was hoped that these experiments would provide a check on the efficacy of the supplied enterotoxin.

Salicylic acid was the most rapidly absorbed drug of the five studied. As reported originally by Schanker and co-workers (1958) this rate of absorption is higher than would be predicted by the pKa of the drug (3.0) at the pH of the luminal perfusate. However, as has been demonstrated in this study, the rat jejunum maintains an 'acid microclimate' at the mucosal surface which

permits weakly acidic compounds to be absorbed at a faster rate than would otherwise be possible. Additionally, salicylic acid is a small molecule (mw 138) and, therefore, there is a possibility that drug permeation through the paracellular pathway might be contributing to the rapid absorption rate observed. Supporting this, it has been demonstrated that salicylic acid absorption can be altered by imposed osmotic loads which may cause solvent movements through the paracellular pathway (Ochsenfahrt & Winne, 1974; Karino, Hayashi, Horie, Awazu, Minawi & Hanano, 1982; Karino, Hayashi, Awazu & Hanano, 1982a). In the presence of STa salicylic acid absorption was reduced by 40%. The similarly lower peripheral blood drug concentrations after STa challenge confirmed that the reduction in drug absorption represented a reduction in total drug transfer rather than just cellular uptake. It is unlikely that these lower blood drug concentrations are due to an STa-increased renal blood clearance since STa has been shown to have no effect on the kidney (Guerrant et al., 1980; Rao, Guandalini, Smith & Field, 1980). This reduced absorption accords with STa elevating the jejunal microclimate pH thereby lowering the proportion of unionised species presented at the mucosal surface. However, the lower rate of uptake could also result from reduced solvent drag, since there was essentially no net fluid absorption in the STa treated jejunum. Both surface pH change and reduced solvent drag probably contributed to the observed reduction to salicylic acid absorption.

(ii) Phenytoin

Salicylic acid is a relatively strong weak acid with a low pKa (3.0), however, no high pKa weak acids had been investigated. The only drug falling into this category which was also available as a ^{14}C-labelled compound was the lipid soluble antiepileptic, phenytoin. Phenytoin is not a carboxylic acid but it readily undergoes keto-enol tautomerism, thereby providing a dissociable group. The pKa of phenytoin is 8.3, and therefore, unlike salicylic acid, at the normal pH prevailing at the jejunal mucosa phenytoin will exist predominantly in the unionised, permeable form. The phenytoin molecule is also almost twice as large as the salicylic acid molecule and therefore solvent drag should not have such a pronounced effect on drug absorption. Phenytoin was absorbed fairly rapidly from the unchallenged jejunal lumen, as would be expected from the pKa of the molecule. After exposure to STa, phenytoin absorption was markedly reduced, although the reduction was not statistically significant. Similarly, peripheral blood drug concentrations were lower in toxin-treated animals, although, again these were not significantly different from controls. However, when the phenytoin data was analysed in terms of a first order pharmacokinetic model, the absorption rate constant obtained for the STa treated intestine was significantly lower than that obtained for the untreated tissue. The high pKa of phenytoin probably accounts for the lack of significance in the STa-induced changes to the drug absorption rate. As has been demonstrated in this study, the mucosal surface pH of rat jejunum

is elevated to near neutral values by STa. However, even with an increase of one pH unit, phenytoin would still exist predominantly in the unionised form. Therefore, it would be expected that a larger elevation of surface pH, as observed in the ileum, might effect more prominent changes in phenytoin absorption rate.

An interesting aspect of phenytoin and some of the other drugs studied is their action on neural function. This is an important consideration since neural control of fluid absorption is well known. There is therefore a possibility that these drugs might affect their own uptake by altering intestinal functions such as fluid transport or surface pH.

Phenytoin caused a modest net fluid secretion into the jejunal lumen in control experiments. Drug-induced inhibition of sympathetic pathways or a stimulation of cholinergic pathways could both result in net fluid secretion. Inhibition of sympathetic nerves would essentially release a 'brake' on fluid secretion which might explain the massive enhancement of STa-induced secretion observed in the presence of phenytoin.

Regardless of mechanism, there is a possibility, as with salicylic acid, that the reduced absorption of phenytoin in the presence of STa might also be due to a reduced fluid movement. However, the magnitude of the STa-induced fluid secretion was very large. If solvent drag made an important contribution to the absorption rate a more pronounced reduction in absorption would

be expected. Therefore, it would appear that solvent drag is not a particularly significant factor in phenytoin absorption. This would suggest that the elevated surface pH after STa challenge resulted in a reduced weak acid uptake due to an increase in the proportion of ionised species in the microclimate region.

(iii) Amphetamine

While the results of the experiments with the weak acids are as predicted by the microclimate hypothesis, they did not provide conclusive evidence in its favour. A test of the hypothesis is to see whether elevation of surface pH is associated with increased weak base absorption. Under these conditions weak base absorption may increase despite unfavourable changes in the direction or magnitude of net fluid transport. This type of experiment to a large extent removes the obscuring effect of change in fluid movement since any effects would tend to work against the hypothesis under test. In attempt to achieve this, jejunal absorption of the weak base, amphetamine, was studied. Amphetamine is a relatively strong weak base (pKa 9.9) and hence at the pH prevailing at the mucosal surface of the unchallenged jejunum it will exist predominantly as the ionised species. Because of this the rate of amphetamine absorption is normally very low compared to salicylic acid and phenytoin. After exposure to STa, amphetamine uptake from the intestinal lumen almost doubled. This increased drug absorption was confirmed by similarly increased amphetamine levels in the peripheral blood.

The enhanced drug uptake was despite a very considerable fluid secretion and therefore, although amphetamine is a relatively small molecule (mw 184), this result cannot be explained in terms of solvent drag. Indeed the possibility exists that this fluid secretion was masking an even greater increase in drug absorption. It therefore, seems most likely that the elevated microclimate pH in the STa-challenged jejunum is responsible for the promotion of amphetamine absorption. This, then, can be considered as stronger evidence for the microclimate hypothesis as it is difficult to explain this result in any other terms.

(iv) Morphine

Morphine was the least well absorbed of the drugs under investigation in this study. In fact, under control conditions morphine absorption from the jejunal lumen was almost negligible. This was expected since morphine was, by far, the most lipophobic molecule of the five drugs studied. As with amphetamine, when the jejunum was exposed to STa toxin morphine absorption was increased almost eight-fold. Despite this apparently massive increase the absorption rate was still relatively low and, as a consequence, the peripheral blood drug levels were also low. There was a significant increase in the blood morphine levels and therefore the total transfer of morphine was increased by STa-challenge. As with amphetamine, elevation of the rate of morphine absorption occured despite unfavourable fluid secretion and therefore, an increase in jejunal mucosal surface pH would

appear to be the most likely explanation of this result providing further evidence for the microclimate hypothesis.

(v) Lignocaine

As it had the lowest pKa (7.9) of the three weak bases tested, lignocaine would be less ionised at the normal jejunal microclimate pH than either amphetamine or morphine. Correspondingly, lignocaine had a higher absorption rate than either amphetamine or morphine under control conditions. After exposure to STa, lignocaine uptake was significantly enhanced, increased blood drug concentrations confirmed that total drug transfer was increased. As with amphetamine and morphine, absorption increased despite ongoing fluid secretion, thereby eliminating solvent drag as a possible cause and providing more evidence for the mucosal surface pH being the primary determinant of drug absorption rate.

However, although E.coli STa toxin seemed to have provided an invaluable tool with which to investigate the microclimate hypothesis there was a possibility that the observed effects on drug absorption were peculiar to the STa-treated jejunum. To ensure that this was not the case, the combined effects of forskolin and theophylline on jejunal lignocaine absorption was investigated. These compounds combined to induce an elevation of the jejunal mucosal surface pH which was virtually identical in both time course and magnitude to the alkalinisation induced by STa. However, since both theophylline and forskolin act on

completely different processes to STa, it would seem unlikely that any effect on drug absorption induced by these compounds would be the result of anything other than surface pH effects. Forskolin and theophylline enhanced lignocaine absorption to levels not significantly different from those observed after STa-challenge. The increased blood drug concentrations were similarly not significantly different from those obtained with STa-treated animals. This, then, was almost incontrovertible evidence that mucosal surface pH changes were the cause of the promoted lignocaine absorption. This conclusion was strengthened by the observation that the forskolin/theophylline combination induced a fluid secretion almost three times greater than that induced by STa, indicating that, for this weak base, surface pH is a much more important determinant of permeation than solvent drag.

Interestingly, in the presence of lignocaine, STa-induced fluid secretion was enhanced when compared to STa-treatment alone. This is contrary to the findings of another study (Eklund, Jodal & Lundgren, 1985) in which jejunal fluid secretion induced by STa was inhibited by serosally administered lignocaine. This was interpreted as evidence that STa-induced secretion may be mediated by local reflexes in the enteric nervous system. Similar evidence for neurally mediated intestinal fluid secretion has also been presented for cholera toxin (Cassuto, Jodal, Tuttle & Lundgren, 1981) and 5-hydroxytryptamine (Cassuto, Jodal, Tuttle & Lundgren, 1982). However, the preparation used in these

experiments was very different from the one adopted in the present study. The intestinal loop under investigation was completely isolated from the remainder of the intestine which was then completely removed. All the extrinsic nerves supplying the loop were removed leaving the tissue devoid of autonomic neural control. In contrast, in the present experiments the gastrointestinal tract was disturbed as little as possible and the intestinal loop preparation had a full nervous supply. There are therefore too many factors which may be involved to make direct comparisons. However, the present results are not consistent with the concept of intrinsic neural involvement in STa-induced intestinal fluid secretion.

Lignocaine, like phenytoin, amphetamine and morphine caused a marked inhibition of fluid absorption from control loops of jejunum. Salicylic acid also reduced fluid uptake but not to such a large extent. There is no obvious reason why these agents should affect intestinal fluid absorption. A possibility is that they all act to some extent on the autonomic nerves affecting the intestine since inhibiting the sympathetic pathways or stimulating the cholinergic pathways would cause a reduction in net fluid uptake as measured by the present method. In the case of lignocaine, it can be envisaged that there might be a local anaesthetic blocking action on all afferent nerves supplying the tissue. It is believed that, under normal conditions, the sympathetic nervous system continuously inhibits an ongoing fluid secretion since small intestinal secretion is enhanced in cats

after sectioning of the splanchnic nerves (Wright, Jennings, Florey & Lium, 1940). Additionally, damage to the sympathetic nerves induced by diabetes mellitus, or chemical ablation with 6-hydroxydopamine, reduces the basal rates of ion and water absorption in the rat ileum and colon (Chang, Bergenstal & Field, 1983). Local anaesthetic action on the sympathetic input to the gut should, therefore result in a marked reduction to fluid absorption as observed with lignocaine. However, phenytoin and amphetamine both induced a significant net fluid secretion. This may have been due to a specific action of these drugs on the sympathetic nerves but it could also be due to drug induced changes to intracellular biochemical processes.

In summary, exposing rat jejunum to E.coli STa enterotoxin resulted in an enhanced absorption of three weakly basic drugs, amphetamine, morphine and lignocaine, while reducing the absorption of two weakly acidic drugs, salicylic acid and phenytoin. In all cases the increased weak base absorption occured despite considerable unfavourable fluid secretion thereby excluding solvent drag from accounting for the observed changes. These results are in accordance with prediction using the microclimate hypothesis in that the alterations to weak electrolyte absorption are consistent with an STa induced elevation of the jejunal microclimate pH. The observation that the forskolin/theophylline combination induced a very similar enhancement of lignocaine absorption to that observed with STa confirmed this conclusion. The additional finding that STa also

alkalinises the ileal mucosal surface suggests that the changes observed in the jejunum would also occur in the ileum.

According to the Henderson-Hasselbach equation, an elevation of mucosal surface pH of the magnitude measured in this study after STa-challenge would cause a five-fold change in the ratio of ionised:unionised species presented at the mucosal surface. However, with the exception of morphine, the changes in drug absorption observed were considerably less than this. This was not unexpected since the unstirred layer and other second order interactions will prevent the microclimate from exerting its full effect. There was a tendency for the less lipid soluble molecules to have their absorption rates affected more by STa-challenge, although their absorption rates from the untreated tissue were lower than those of the more lipid soluble molecules. It was demonstrated in the present study that, under control conditions, there is good correlation between lipid solubility and the rate at which the individual molecules were absorbed. Therefore, it would appear that the mucosal surface pH and lipid solubility are the important factors governing weak electrolyte absorption. Solvent drag seems to be only important for small molcules, such as salicylic acid, suggesting that the majority of transport occurs across the cell membrane.

It has been proposed (Dietschy et al., 1971; Winne, 1977) that the unstirred water layer can account for all deviations in weak electrolyte absorption from the pH partition hypothesis. In the

present experiments reduced weak acid uptake could be explained by a thickened unstirred layer in the presence of enterotoxin. However, the same enterotoxin would have to thin the unstirred layer to cause enhanced weak base absorption. It seems unlikely that STa would have specific effects on unstirred layer thickness depending on whether the absorbed molecule is a weak acid or weak base. Therefore, although the possibility exists, the practicalities of the situation would tend to dismiss the unstirred layer as being the effector of the changes to weak electrolyte absorption observed in this study.

Another possibility is that STa in some way changes the permeability of the brush border membrane to the ionised species permitting ionised permeation. However, while increased cation permeability might explain the observed effects of STa on weak base absorption, there would also need to be a reduced anion permeability to explain the reduced weak acid uptake. This mechanism seems unnecessarily complex when mucosal surface pH changes, which have been measured, can adequately account for both situations.

4.6 Pharmacokinetic analysis of drug absorption

In an attempt to derive some further information about the mechanism of drug absorption the luminal drug concentration data were subjected to pharmacokinetic analysis. Two pharmacokinetic models were applied in an attempt to describe the drug absorption

process. The first of these, in which drug absorption occurs by diffusion across a single barrier (the intestinal epithelium) provided a better model for drug absorption for most of the drugs investigated, in both the control and toxin-treated intestine, than the simple zero order model. The latter would apply only if absorption were occurring at a constant rate and the luminal concentration declined linearly with time. However, in the present experiments the decline over time of luminal drug concentration resembled an exponential process and this provided a better fit for the data. Using a first order exponential model, first order absorption rate constants were obtained for both control and toxin treatments. In the case of the two weak acids, there was a significant reduction in the absorption rate constants after STa-challenge whereas significant increases were obtained with the three weak bases. This confirmed the results obtained using the more empirical zero order analysis and provided a significant difference for phenytoin absorption which was not observed with the zero order model.

However, if the microclimate played an important role in the absorption process it might be expected that absorption kinetics could be modelled incorporating the microclimate as an exchange compartment. A mathematical consideration of such a system provides a solution which incorporates two exponential terms. An attempt was made to fit this model to luminal drug concentration data. With amphetamine, morphine and phenytoin, the curve fitting defaulted to a first order, single exponential model. This is not

unusual and means that no additional variance in the data could be accounted for by proposing a separate 'reservoir' compartment at the surface. This does not contradict the idea of a microclimate but simply indicates that apparent change is represented adequately by a change in one permeability term. However, with salicylic acid and lignocaine two exponential processes described both the control and toxin-treated data and provided a better fit to the data than the simpler single exponential expression. From the estimated parameters, it was possible to calculate the ratio of the rate constants k_{23}/k_{21} which compares the rate of absorption into the cell (k_{23}) with back diffusion out of the surface compartment (k_{21}). In the case of salicylic acid, STa reduced this ratio whereas the ratio for lignocaine was increased. This was further evidence for the microclimate pH controlling absorption since this is what would be expected with an elevated surface pH.

4.7 Implications for human intestinal disease

The present study has demonstrated that exposure to the heat-stable enterotoxin of E.coli (STa) results in an elevation of the small intestinal microclimate pH and that this surface pH shift alters the rate at which weakly dissociable drugs permeate the intestinal epithelium. Similar findings to these have been observed with some types of human intestinal disease. Like the rat, the human jejunum maintains an acidic microclimate at the mucosal surface. This has been measured directly with

miniaturised pH electrodes on jejunal biopsy samples taken from healthy human subjects (Lucas & Blair, 1978; Lucas et al., 1978; Kitis et al., 1982). Recently these measurements were confirmed by direct *in situ* measurement of human jejunal surface pH (Rawlings et al., 1987). However, in patients with coeliac or Crohn's disease the jejunal surface pH is significantly higher than in normal subjects (Lucas et al., 1978; Rawlings et al., 1987). In accordance with the microclimate hypothesis, increased uptake of the weak bases, propranolol (Schneider et al., 1976; Parsons, 1978), quinine and trimethoprim (Matilla et al., 1973) have been measured in patients suffering from these disease states. Therefore, from the present results, there is a possibility that in certain types of diarrhoea mediated by enterotoxigenic bacteria, especially *E.coli*, the mucosal surface pH of human small intestine may be alkalinised. This could have important implications for the administration of any weakly dissociable drugs under such conditions. An elevation of surface pH may reduce the therapeutic efficacy of weakly acidic drugs but also may allow more absorption of weak bases, which may be undesirable if these are harmful. Although changes in weak electrolyte absorption rates may be the most obvious consequence of a surface pH shift, many non-dissociable compounds have pH dependent uptake mechanisms while several membrane-associated digestive processes could also be affected.

The observed pH changes may also be relevant for the formulation of oral rehydration solutions. These glucose-electrolyte solutions take advantage of the carrier mediated transport in the intestine (Crane, 1962) to improve fluid balance in diarrhoeal disease states (Philips, 1964; Nalin, Cash, Islam, Molla & Philips, 1968). This simple therapy removes the need for intravenous fluid administration for the rehydration of diarrhoea sufferers and allows the possibility of treatment in the field. The therapy is inexpensive and has saved countless lives since its introduction. A recent development in oral rehydration therapy is the supplementation of oral rehydration solutions with weak acids such as acetate. These are assumed to enter the epithelial cells as neutral forms which dissociate to provide a proton for luminal $Na^+:H^+$ exchange (Forsyth, Kapitany & Hamilton, 1981). The increased net sodium ion transport energises fluid transport and presumably attenuates the net fluid secretion caused by enterotoxins. However, citrate and bicarbonate oral rehydration solutions seem only to enhance fluid absorption in choleraic but not non-choleraic secretion (WHO, 1985; Hoffman, Moechtar, Simanjuntak, Punjabi, Kumala, Sutoto, Silalahi, Sutopo, Kuncoro, Soriano, Plowe, Paleologo, Edman & Laughlin, 1985), although even the efficacy of weak acids in cholera toxin-promoted secretion is disputed (Newsome, Burgess & Holman, 1983; Rolston, Kelly, Borodo, Bown, Farthing, Clark & Dawson, 1984). Whether a given weak acid is useful could depend on whether the mucosal surface pH is elevated in a given diarrhoeal

disease. To be effective, weak acids must permeate the enterocytes as neutral forms. This process will not function efficiently if the mucosal surface pH is elevated since more weak acid will exist as the non-absorbable anion. Individual differences between enterotoxins, as has been demonstrated in this study, might explain why weak acid supplemented oral rehydration solutions are less effective in some diarrhoeal disease (WHO, 1985).

4.8 Conclusions

In conclusion, the results of the present study have demonstrated that the pH prevailing at the mucosal surface of rat small intestine is a major, if not the primary, determinant of weak electrolyte absorption rate, providing substantial evidence in favour of the microclimate hypothesis. There is evidence to suggest that the human and rat intestine behave similarly and therefore the present results may have important implications for drug absorption in human intestinal disease. The current findings additionally suggest that diarrhoeal disease mediated by cGMP and cAMP dependent bacterial enterotoxins may affect the intestinal surface pH differently. This may prove an important factor requiring consideration in the formulation of oral rehydration solution supplements.

4.9 Future Work

Due to limitations of time and finance, certain experiments which

should have been undertaken as part of this project had to be ignored. This was unavoidable, however any future investigation into this area should probably take account of the following proposals. With any hypothesis, the more evidence presented in its favour, the more credence the hypothesis will receive. This is also true for the microclimate hypothesis. Although the list of drugs which conform to the hypothesis is growing there may still be many drugs which do not conform. Therefore, more drugs with as wide a variety of structures, lipid solubilities and pKa values as possible should be tested. It would also be useful to measure the villus-crypt axis pH in vivo rather than in vitro as with this study. This would allow a more direct comparison with the surface pH measurements obtained and would eliminate any uncertainties about the viability of isolated tissue. The problem of knowing which cyclic nucleotide is responsible for the surface pH shift when theophylline is combined with forskolin or cholera toxin could be solved by measuring tissue cGMP and cAMP levels under these conditions. Additionally other bacterial enterotoxins should be used to try and change the mucosal surface pH in the hope that a more general statement about drug absorption in diarrhoeal disease could be made. Finally, studies should be undertaken to measure human intestinal surface pH in situ under diarrhoeal disease conditions and, if possible, this should be compared with the absorption of weakly basic drugs to determine whether the present results and conclusions are directly applicable to the healthy and disturbed human small intestine.

5 REFERENCES

ABBEY, D.M. & KNOOP, F.C. (1979). Effect of chlorpromazine on the secretory activity of Escherichia coli heat stable enterotoxin. Infect. Immun. 26, 1000-1003.

AIMOTO, S., TAKEDA, T., TAKEDA, Y. & MIWATANI, T. (1983). Chemical synthesis of a highly potent and heat-stable analog of an enterotoxin produced by a human strain of enterotoxigenic Escherichia coli. Biochem. Biophys. Res. Com. 112, 320-326.

AMMAN, D., SCHULTHESS, P., SHIJO, Y. & SIMON, W. (1981). Neutral carrier based hydrogen ion selective microelectrode for extra- and intracellular studies. Anal. Chem. 53, 2267-2269.

BLAIR, J.A., HILBURN, M.E., LUCAS, M.L. & SAID, H.M. (1982). The effects of various metabolic inhibitors and substrates on the maintenance of surface pH in vitro in rat proximal jejunum. Biochem. Soc. Trans. 10, 470-471.

BLAIR, J.A., LUCAS, M.L. & MATTY, A.J. (1975). Acidification in the rat proximal jejunum. J. Physiol. 245, 333-350.

BOIGE, N., AMIRANOFF, B., MUNCK, A. & LABURTHE, M. (1984). Forskolin stimulates adenylate cyclase in human colonic crypts: interaction with VIP. Eur. J. Pharm. 101, 111-117.

BRIDGES, J.W., PARKE, D.V., SHILLINGFORD, J.S. & UPSHILL, D.G. (1976). Gastrointestinal absorption of carbenoxolone in the rat determined in situ: deviations from the pH partition hypothesis. J. Pharm. Pharmacol. 28, 117-126.

CARPENTER, C.C.J., SACK, R.B., FEELEY, J.C. & STEENBERG, R.W. (1968). Site and characteristics of electrolyte loss and effect of intraluminal glucose in experimental canine cholera. J. Clin. Invest. 47, 1210-1220.

CASSANO, G., STEIGER, B. & MURER, H. (1984). Na/H- and Cl/OH- exchange in rat jejunal and rat proximal tubular brush-border membrane vesicles. Studies with acridine orange. Pflug. Arch. 400, 309-317.

CASSUTO, J., JODAL, M., TUTTLE, R. & LUNDGREN, O. (1981). On the role of intramural nerves in the pathogenesis of cholera toxin-induced intestinal secretion. Scand. J. Gastroenterol. 16, 377-384.

CASSUTO, J., JODAL, M., TUTTLE, R. & LUNDGREN, O. (1982). 5-Hydroxytryptamine and cholera secretion. Scand. J. Gastroenterol. 17, 695-703.

CHANG, E.B., BERGENSTAL, R.M. & FIELD, M. (1983). Diabetic diarrhea: Loss of adrenergic regulation of intestinal fluid and electrolyte transport. Gastroenterol. 84, 1121.

CRANE, R.K. (1962). Hypothesis for mechanisms of intestinal active transport of sugars. Fed. Proc. 21, 891-895.

CROUTHAMEL, W.G., TAN, G.H., DITTERT, L.W. & DOLUISIO, J.T. (1971). Drug absorption IV: Influence of pH on absorption kinetics of weakly acidic drugs. J. Pharm. Sci. 60, 1160-1163.

DANIEL, H., NEUGEBAUER, B., KRATZ, A. & REHNER, G. (1985). Localization of acid microclimate along intestinal villi of rat jejunum. Am. J. Physiol. 248, G293-298.

DANIEL, H. & REHNER, G. (1986). Effect of metabolizable sugars on the mucosal surface pH of rat intestine. J. Nutr. 116, 768-777.

DE JONGE, H.R. (1975). Properties of guanylate cyclase and levels of cyclic GMP in rat small intestinal villous and crypt cells. FEBS Letters 55, 143-152.

DE JONGE, H.R. (1984). The mechanism of action of Escherichia coli heat-stable toxin. Biochem. Soc. Trans. 12, 180-184.

DIETSCHY, J.M., SALLEE, V.L. & WILSON, F.A. (1971). Unstirred water layers and absorption across the intestinal mucosa. Gastroenterol. 61, 932-934.

DONOWITZ, M. & WELSH, M.J. (1987). Regulation of mammalian small intestinal electrolyte secretion. In Physiology of the Gastrointestinal Tract (Vol 2), Ed. L.R. Johnson. Raven Press, New York, pp1351-1388.

DONOWITZ, M., WICKS, J. & BATTISTI, L. (1984). Senokot inhibits active Na and Cl transport in rat descending colon by a Ca^{2+} dependent mechanism. Gastroenterol. 87, 503-512.

DUNCAN, C.L. & STRONG, D.H. (1969). Ileal loop fluid accumulation and production of diarrhea in rabbits by cell-free products of Clostridium perfringens. J. Bacteriol. 100, 86-94.

EKLUND, S., JODAL, M. & LUNDGREN, O. (1985). The enteric nervous system participates in the secretory response to the heat stable enterotoxins of Escherichia coli in rats and cats. Neuroscience 14, 673-681.

EKLUND, S., JODAL, M. & LUNDGREN, O. (1986). The net fluid secretion caused by cyclic 3'5'-guanosine monophosphate in the rat jejunum in vivo is mediated by a local nervous reflex. Acta Physiol. Scand. 128, 57-63.

EL WAKKAD, S.E.S. (1950). The electrochemical behaviour of the antimony electrode. J. Chem. Soc., 2894-2896.

FIELD, M., FROMM, D., AL-AWQATI, Q. & GREENOUGH, W.B. (1972). Effect of cholera enterotoxin on ion transport across isolated ileal mucosa. J. Clin. Invest. 51, 796-804.

FIELD, M., GRAF, L.H., LAIRD, W.J. & SMITH, P.L. (1978). Heat-stable enterotoxin of Escherichia coli: In vitro effects on guanylate cyclase activity, cyclic GMP concentration, and ion transport in small intestine. Proc. Natl. Acad. Sci. USA 75, 2800-2804.

FLEMSTROM, G., NYLANDER, O., HURST, B.C. & HEYLINGS, J.R. (1982). Surface epithelial HCO_3 transport by mammalian duodenum in vivo. Am. J. Physiol. 243, G348-358.

FLEMSTROM, G. & KIVILAAKSO, E. (1983). Demonstration of a pH gradient at the luminal surface of rat duodenum in vivo and its dependence on mucosal alkaline secretion. Gastroenterol. 84, 787-794.

FORSYTH, G.W., KAPITANY, R.A. & HAMILTON, D.L. (1981). Organic acid proton donors decrease intestinal secretion caused by enterotoxins. Am. J. Physiol. 241, G227-234.

GIANELLA, R.A. & DRAKE, K.W. (1979). Effect of purified Escherichia coli heat-stable enterotoxin on intestinal cyclic nucleotide metabolism and fluid secretion. Infect. Immun. 24, 19-23.

GRAHAM, W.R. & EMERY, E.S. (1928). The reaction of the intestinal contents of dogs fed on different diets. J. Lab. Clin. Med. 13, 1097-1108.

GRAYZEL, D.M. & MILLER, E.G. (1927). pH concentration of intestinal contents of dog, with special reference to inorganic metabolism. Proc. Soc. Exp. Biol. Med. 24, 668-672.

GREENBERG, R.N., CHANG, B., ROBERTSON, D.C. & MURAD, F. (1982). Inhibition of Escherichia coli heat-stable enterotoxin effects on intestinal guanylate cyclase and fluid secretion by quinacrine. Biochem. Pharm. 31, 2005-2009.

GREENBERG, R.N., CHANG, B., ROBERTSON, D.C. & GUERRANT, R.L. (1980). Inhibition of Escherichia coli heat-stable enterotoxin by indomethacin and chlorpromazine. Infect. Immun. 29, 908-913.

GUERRANT, R.L., CHEN, L.C. & SHARP, G.W.G. (1972). Intestinal adenylate cyclase activity in canine cholera: correlation with fluid accumulation. J. Infec. Dis. 125, 377-381.

GUERRANT, R.L., CHANG, B., ROBERTSON, D.C. & MURAD, F. (1980). Activation of rat and rabbit intestinal guanylate cyclase by the heat-stable enterotoxin of Escherichia coli: Studies on tissue specificity, potential receptors and intermediates. J. Infec. Dis. 142, 220-228.

HALLBACK, D.A., JODAL, M., SJOQVIST, A. & LUNDGREN, O. (1982). Evidence for cholera secretion emanating from the crypts. A study of villus tissue osmolality and fluid and electrolyte transport in the small intestine of the cat. Gastroenterol. 83, 1051-1056.

HAMILTON, D.L., FORSYTH, G.W., ROE, W.E. & NIELSEN, N.O. (1978). Effect of heat stable and heat labile Escherichia coli enterotoxins and cholera toxin in combination with theophylline on unidirectional sodium and chloride flux in the small intestine of weanling swine. Can. J. Comp. Med. 42, 316-321.

HILDEMAN, B., HAASE, W., BARAC-NIETO, M. & MURER, H. (1980). Sodium ion/L-lactate co-transport in rabbit small intestinal brush-border membrane vesicles. Biochem. J. 186, 169-176.

HOBER, R. & HOBER, J. (1937). Experiments on the absorption of organic solutes in the small intestine of rats. J. Cell. Comp. Physiol. 10, 401-422.

HOFFMAN, S.L., MOECHTAR, M.A., SIMANJUNTAK, C.H., PUNJABI, N.H., KUMALA, S., SUTOTO, SILILALAKI, P., SUTOPO, B., KUNCORO, Y.S., SORIANO, M., PLOWE, C., PALEOLOGO, F.P., EDMAN, D.C. & LAUGHLIN, L.W. (1985). Rehydration and maintenance therapy of cholera patients in Jakarta: Citrate-based versus bicarbonate-based oral rehydration salt solutions. J. Infec. Dis. 152, 1159-1165.

HOGBEN, C.A.M., SCHANKER, L.S., TOCCO, D.J. & BRODIE, B.B. (1957). Absorption of drugs from the stomach II. The human. J. Pharm. Exp. Ther. 120, 540-545.

HOGBEN, C.A.M., TOCCO, D.J., BRODIE, B.B. & SCHANKER, L.S. (1959). On the mechanism of intestinal absorption of drugs. J. Pharm. Exp. Ther. 125, 275-282.

HOGERLE, M.L. & WINNE, D. (1983). Drug absorption by the rat jejunum perfused in situ. Dissociation from the pH partition theory and role of microclimate-pH and unstirred layer. Naun. Schmied. Arch. Pharm. 322, 249-255.

HUBEL, K.A. (1967). Bicarbonate secretion in rat ileum and its dependence on intraluminal chloride. Am. J. Physiol. 213, 1409-1413.

HUBEL, K.A. (1969). Effect of luminal chloride on bicarbonate secretion in rat ileum. Am. J. Physiol. 217, 40-45.

HUBEL, K.A. (1973). Effect of luminal sodium concentration on bicarbonate absorption in the rat jejunum. J. Clin. Invest. 52, 3172-3179.

HUBEL, K.A. (1974). The mechanism of bicarbonate secretion in rabbit ileum exposed to choleragen. J. Clin. Invest. 53, 964-970.

HUGHES, J.M., MURAD, F., CHANG, B. & GUERRANT, R.L. (1978). Role of cyclic GMP in the action of heat-stable enterotoxin of Escherichia coli. Nature 271, 755-756.

HUGHES, J.M., MURAD, F. & GUERRANT, R.L. (1978a). Studies to elucidate the mechanism of action of heat-stable enterotoxin of Escherichia coli. Clin. Res. 26, 524A.

IWATSUBO, T., YUASA, H., IGA, T. & HANANO, M. (1986). Effects of potential damaging agents on the microclimate-pH in the rat jejunum. J. Pharm. Sci. 75, 1162-1165.

JACKSON, M.J. & AIRALL, A.A. (1978). Transport of heterocyclic acids across rat small intestine in vitro. J. Mem. Biol. 38, 255-269.

JACKSON, M.J., SHIAU, Y.F., BANE, S. & FOX, M. (1974). Intestinal transport of weak electrolytes. Evidence in favor of a three compartment system. J. Gen. Physiol. 63, 187-213.

JACKSON, M.J., WILLIAMSON, A.M., DOMBROWSKI, W.A., GARNER, D.E. (1978). Intestinal transport of weak electrolytes. Determinants of influx at the luminal surface. J. Gen. Physiol. 71, 301-327.

KAPRAL, F.A., O'BRIEN, A.D., RUFF, P.D. & DRUGEN, W.J. (1976). Inhibition of water absorption in the intestine by S.aureus d toxin. Infect. Immun. 13, 140-145.

KARINO, O., HAYASHI, M., AWAZU, S. & HANANO, M. (1982). Solvent drag effect on drug intestinal absorption. II studies on drug absorption clearance and water influx. J. Pharm. Dyn. 5, 670-677.

KARINO, O., AWAZU, S., MINAWA, H. & HANANO, M. (1982a). Solvent drag effect in drug intestinal absorption. I studies on drug and D_2O absorption clearances. J. Pharm. Dyn. 5, 410-417.

KINNE-SAFFRAN, E. (1985). Transport function of renal cell membrane: ATP driven transport system. In "Renal Biochemistry; Cells, Membranes, Molecules"., Ed. R. Kinne, Elsevier, Amsterdam, pp143-174.

KITIS, G., LUCAS, M.L. BISHOP, H., SARGENT, A., SCHNEIDER, R.E., BLAIR, J.A. & ALLAN, R.N. (1982). Surface pH and drug absorption in coeliac disease. Clin. Sci. Mol. Med. 63, 373-380.

KREBS, H.A. & HENSELEIT, K. (1932). Untersuchungen ueber die Harnstoffbildung im Tierkoerper. Z. Physiol. Chem. 210, 33-66.

LEITCH, G.J. & BURROWS, W. (1968). Experimental cholera in the rabbit ligated intestine: ion and water accumulation in the duodenum, ileum and colon. J. Infec. Dis. 118, 349-359.

LENG-PESCHLOW, E. (1980). Inhibition of intestinal water and electrolyte absorption by senna derivatives in rats. J. Pharm. Pharmacol. 32, 330-335.

LIEDTKE, C.M. & HOPFER, U. (1977). Anion transport in brush border membranes isolated from rat small intestine. Biochem. Biophys. Res. Com. 76, 579-585.

LOESCHKE, K., FARACK, U.M., GERZER, R. & KARAVIS, T. (1987). Evidence that the turnover rather than the concentration of cAMP determines cholera toxin induced fuid secretion in rat intestine. Z. Gastroenterol. 25, 388.

LONNROTH, I. (1983). Cholera toxin. In "Intestinal Secretion", Ed. L.A. Turnberg, SK&F Publications, pp58-62.

LUCAS, M. (1983). Determination of acid surface pH in vivo in rat proximal jejunum. Gut 24, 734-739.

LUCAS, M.L. (1976). The association between acidification and electrogenic events in the rat proximal jejunum. J. Physiol. 257, 645-662.

LUCAS, M.L. (1984). A contribution to analysis of three compartment models for intestinal weak electrolyte absorption. Am. J. Physiol. 247, G463-467.

LUCAS, M.L. & BLAIR, J.A. (1978). The magnitude and distribution of the acid microclimate in proximal jejunum and its relation to luminal acidification. Proc. R. Soc. Lond. A 200, 27-41.

LUCAS, M.L. & CANNON, M.J. (1983). Measurement of sodium ion concentration in the unstirred layer of rat small intestine by polymer Na^+-sensitive electrodes. Biochem. Biophys. Acta 730, 41-48.

LUCAS, M.L., COOPER, B.T., LEI, F.H., HOLMES, G.K.T., JOHNSON, I.T., BLAIR, J.A. & COOKE, W.T. (1978). Surface pH in Crohn's and coeliac disease: a model for folic acid absorption. Gut 19, 735-742.

LUCAS, M.L., LEI, F.H. & BLAIR, J.A. (1980). The influence of buffer pH, glucose and sodium ion concentration on the acid microclimate in rat proximal jejunum in vitro. Pflug. Arch. 385, 137-142.

LUCAS, M.L., SCHNEIDER, W., HABERICH, F.J. & BLAIR, J.A. (1975). Direct measurement by pH-microelectrode of the pH-microclimate in rat proximal jejunum. Proc. R. Soc. Lond. B 192, 39-48.

LYNCH, J. (1986). The effect of experimental malabsorption on the absorption of weak electrolytes. PhD thesis, University of Glasgow.

LYNCH, J. & LUCAS, M.L. (1984). The effect of E.coli toxin on intestinal drug absorption. Biochem. Soc. Trans. 12, 263.

MATILLA, M.J., JUSSILA, J. & TAKKI, S. (1973). Drug absorption in patients with intestinal villous atrophy. Arz. Forsch. 23, 583-585.

McDONEL, J.L. (1974). In vivo effect of Clostridium perfringens enteropathogenic factors on the rat ileum. Infect. Immun. 10, 1156-1162.

McDONEL, J.L. (1986). Toxins of Clostridium perfringens types A, B, C, D and E. In "Pharmacology of Bacterial Toxins", Eds. F.Dorner & J. Drews, Pergammon Press, pp477-517.

McDONEL, J.L. & ASANO, T. (1975). Analysis of unidirectional sodium fluxes induced by Clostridium perfringens enterotoxin in the rat terminal ileum. Infect. Immun. 11, 526-529.

McDONEL, J.L. & DUNCAN, C.L. (1977). Regional localization of activity of Clostridium perfringens type A enterotoxin in the rabbit ileum. J. Infec. Dis. 136, 661-666.

McNEIL, N.I., LING, K.L.E. & WAGER, J.W. (1987). Mucosal surface pH of the large intestine of the rat and of normal and inflamed large intestine in man. Gut 28, 707-713.

McROBERT, G.R. (1928). Observations on the hydrogen ion concentration of the alimentary canal of the albino rat. Ind. J. Med. Res. 16, 545-552.

MELTZER, S.J. (1896). On the absorption of strychnine and hydrocyanic acid from the mucous membrane of the stomach. - An experimental study in rabbits. J. Exp. Med. 1, 529-536.

MILNE, M.D., SCRIBNER, B.H. & CRAWFORD, M.A. (1958). Non-ionic diffusion and the excretion of weak acids and bases. Am. J. Med. 24, 709-729.

MOORE, P.A. (1981). Preparation of whole blood for liquid scintillation counting. Clin. Chem. 27, 609-611.

MOORE, W.D., MOROWASKI, S.F., FINKELSTEIN, R.A. & FORDTRAN, J.S. (1971). Ion transport during cholera induced ileal secretion in the dog. J. Clin. Invest. 50, 312-318.

MORIARTY, K.J. (1987). Laxatives - use and abuse. Gastroenterol. Pract. 3, 15-24.

MULLAN, N.A., BURGESS, M.N. & NEWSOME, P.M. (1978). Characterization of a partially purified, methanol soluble heat-stable Escherichia coli enterotoxin in infant mice. Infect. Immun. 19, 779-784.

MURAD, F., ARNOLD, W.P., MITTAL, C.K. & BROUGHLER, J.M. (1979). Properties and regulation of guanylate cyclase and some proposed actions for cGMP. Adv. Cyc. Nuc. Res. 11, 175-204.

MURER, H., HOPFER, U. & KINNE, R. (1976). Sodium/Proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. Biochem. J. 154, 597-604.

NALIN, D.R., ISLAM, R., MOLLA, M. & PHILIPS, R.A. (1968). Oral maintenance therapy for cholera in adults. Lancet (ii), 370-372.

NEWSOME, P.M., BURGESS, M.N. & HOLMAN, G.D. (1983). Stimulation of ileal absorption by sodium citrate. Scand. J. Gastroenterol. 18, Suppl. 87, 119-121.

NEWSOME, P.M., BURGESS, M.N. & MULLAN, N.A. (1978). Effect of Escherichia coli heat-stable enterotoxin on cyclic GMP levels in mouse intestine. Infect. Immun. 22, 290-291.

NOGAMI, H. & MATSUZAWA, T. (1961). Studies on absorption and excretion of drugs: I kinetics and penetration of acidic drug, salicylic acid, through intestinal barrier in vitro. Chem. Pharm. Bull. 9, 532-540.

NOGAMI, H. & MATSUZAWA, T. (1962). Studies on absorption and excretion of drugs: II kinetics and penetration of basic drug, aminopyrine, through intestinal barrier in vitro. Chem. Pharm. Bull. 10, 1055-1060.

NORRIS, H.T., CURRAN, P.F. & SCHULTZ, S.G. (1969). Modification of intestinal secretion in experimental cholera. J. Infec. Dis. 119, 117-125.

OCHSENFAHRT, H. & WINNE, D. (1974). The contribution of solvent drag to the intestinal absorption of the basic drugs amidopyrine and antipyrine from the jejunum of the rat. Naun. Schmied. Arch. Pharm. 281, 175-196.

OCHSENFAHRT, H. & WINNE, D. (1974). The contribution of solvent drag to the intestinal absorption of the acidic drugs benzoic acid and salicylic acid from the jejunum of the rat. Naun. Schmied. Arch. Pharm. 281, 197-217.

OSER, B.L. (1928). The intestinal pH in experimental rickets. J. Biol. Chem. 80, 487-497.

OVERTON, E. (1902). Beitrage zur allgemeinen Muskel-und Nervenphysiologie. Pflug. Arch. Ges. Physiol 92, 115-280.

PARSONS, D.S. (1956). The absorption of bicarbonate-saline solutions by the small intestine and colon of the white rat. Q. J. Exp. Physiol. 41, 410-420.

PARSONS, R.L., RAYMOND, K., TROUNCE, J.R. & TURNER, P. (1976). Absorption of propranolol and practolol in coeliac disease. Gut 17, 139-143.

PHILIPS, A.D. (1982). Small intestinal mucosa in childhood in health and disease. In Basic Science in Gastroenterology; Structure of the Gut., Eds. J.M. Polak, S.R. Bloom, N.A. Wright & M.J. Daly, Glaxo Group Research Ltd., pp65-85.

PHILIPS, R.A. (1964). Water and electrolyte losses in cholera. Fed. Proc. 23, 705-712.

PODESTA, R.B. & METTRICK, D.F. (1977). HCO_3^- transport in rat jejunum: relationship to NaCl and H_2O transport in vivo. Am. J. Physiol. 232, E62-68.

POWELL, D.W., SOLBERG, L.I., PLOTKIN, G.R., CATLIN, D.H., MAENZA, R.M. & FORMAL, S.B. (1971). Experimental diarrhea III. Bicarbonate transport in rat salmonella enterocolitis. Gastroenterol. 60, 1076-1087.

QUILL, H. & WEISER, M.M. (1975). Adenylate and guanylate cyclase activities and cellular differentiation in rat small intestine. Gastroenterol. 69, 470-478.

RAO, M.C. (1985). Toxins which activate guanylate cyclase: Heat stable enterotoxins. Cib. Found. Symp. 112, 74-87.

RAO, M.C. & FIELD, M. (1984). Enterotoxins and ion transport. Biochem. Soc. Trans. 12, 177-180.

RAO, M.C., GUANDALINI, S., SMITH, W.J. & FIELD, M. (1980). Mode of action of heat-stable E.coli enterotoxin: tissue and subcellular specificities and role of cGMP. Biochem. Biophys. Acta 632, 35-46.

RAWLINGS, J.M. & LUCAS, M.L. (1985). Plastic electrodes for the measurement of gastrointestinal pH. Gut 26, 203-207.

RAWLINGS, J.M., LUCAS, M.L. & RUSSEL, R.I. (1987). Measurement of jejunal surface pH in situ by plastic pH electrode in patients with coeliac disease. Scand. J. Gastroenterol. 22, 377-384.

RECHKEMMER, G., WAHL, M., KUSCHINSKY, W. & VON ENGELHARDT, W. (1979). pH-microclimate at the surface of the intestine in guinea pig and rat. Pflug. Arch. 382, 31R.

RECHKEMMER, G., WAHL, M., KUSCHINSKY, W. & VON ENGELHARDT, W. (1986). pH-microclimate at the luminal surface of the intestinal mucosa of guinea pig and rat. Pflug. Arch. 407, 33-40.

REDMAN, T., WILLIMOT, S.G. & WOKES, F. (1927). LXXXIII. The pH of the gastrointestinal tract of certain rodents used in feeding experiments and its possible significance in rickets. Biochem. J. 21, 589-605.

ROBINSON, C.S., LUCKEY, H. & MILLS, H. (1943). Factors affecting the hydrogen ion concentration of the contents of the small intestine. J. Biol. Chem. 147, 175-181.

ROLSTON, D.D.R., KELLY, M.J., BORODO, M.M., BOWN, R., FARTHING, M.J.G., CLARK, M.L. & DAWSON, A.M. (1984). Should acetate and bicarbonate be included in oral rehydration solutions? Gastroenterol. Clin. Biol. 8, 871.

SCHAFER, D.E., LUST, W.D., SIRCAR, R. & GOLDBERG, N.D. (1970). Elevated concentration of adenosine 3',5'-cyclic monophosphate in intestinal mucosa after treatment with cholera toxin. Proc. Natl. Acad. Sci. USA 67, 851-856.

SCHANKER, L.S., SHORE, P.A., BRODIE, B.B. & HOGBEN, C.A.M. (1957). Absorption of drugs from the stomach I. The rat. J. Pharm. Exp. Ther. 120, 528-539.

SCHANKER, L.S., TOCCO, D.J., BRODIE, B.B. & HOGBEN, C.A.M. (1958). Absorption of drugs from the rat small intestine. J. Pharm. Exp. Ther. 123, 81-88.

SCHNEIDER, R.E., BABB, J., BISHOP, H., MITCHARD, M., HOARE, A.M. & HAWKINS, C.F. (1976). Plasma levels of propranolol in treated patients with coeliac disease and patients with Crohn's disease. Br. Med. J. 2, 794-795.

SEAMON, K.B. & DALY, J.W. (1981). Forskolin: A unique diterpene activator of cyclic AMP-generating systems. J. Cyc. Nuc. Res. 7, 201-224.

SHIAU, Y.F., FERNANDEZ, P., JACKSON, M.J. & McMONAGLE, S. (1985). Mechanisms maintaining a low-pH microclimate in the intestine. Am. J. Physiol. 248, G608-617.

SHIMADA, T. (1987). Factors affecting the microclimate pH in rat jejunum. J. Physiol. 392, 113-127.

SHORE, P.A., BRODIE, B.B. & HOGBEN, C.A.M. (1957). The gastric secretion of drugs. A pH partition hypothesis. J. Pharm. Exp. Ther. 119, 361-369.

SIMON, W. (1977). Mathematical techniques for biology and medicine. MIT Press, Cambridge, Mass. USA.

THOMSON, A.B.R. & DIETSCHY, J.M. (1984). The role of the unstirred layer in intestinal permeation. In "Pharmacology of Intestinal Permeation II", Ed. T.Z. Csaky Springer Verlag, Berlin, pp165-269.

TOURKY, R. & MOUSA, A.A. (1948). Studies on some metal electrodes. III Does the antimony electrode behave simply as a metal-metal oxide electrode in air? J. Chem. Soc., 752-755.

TRAVELL, J. (1940). The influence of the hydrogen ion concentration on the absorption of alkaloids from the stomach. J. Pharm. Exp. Ther. 69, 21-33.

TURNBERG, L.A., FORDTRAN, J.S., CARTER, N.W. & RECTOR, F.C. (1970). Mechanism of bicarbonate absorption and its relationship to sodium transport in the human jejunum. J. Clin. Invest. 49, 548-556.

WALKER, J.L. (1971). Ion specific liquid ion exchanger microelectrodes. Anal. Chem. 43, 89-93A.

WHO.DDC document /SER/84.7 Rev. 1 (1985).

WILSON, T.H. (1953). Lactate and hydrogen ion gradients developed across rat intestine in vitro. Biochem. Biophys. Acta 11, 448-449.

WILSON, T.H. (1956). Concentration gradients of lactate, hydrogen and some other ions across the intestine in vitro. Biochem. J. 56, 521-527.

WILSON, T.H. & KAZYAK, L. (1957). Acid base changes across the wall of hamster and rat intestine. Biochem. Biophys. Acta 24, 124-132.

WILSON, T.H. & WISEMAN, G. (1954). The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol. 123, 116-125.

WINNE, D. (1977). Shift of pH absorption curves. J. Pharm. Biopharm. 5, 53-94.

WRIGHT, R.D., JENNINGS, M.A., FLOREY, H.W. & LIUM, R. (1940). The influence of nerves and drugs on secretion by the small intestine and an investigation of the enzymes in intestinal juices. Q. J. Exp. Physiol. 30, 73-120.