Characterisation of the Mechanisms Involved in the Transcellular Transport of Monocarboxylates across the Equine Large Intestine

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Synopsis

Short chain fatty acids (SCFA) are produced by anaerobic fermentation of dietary fibre in the equine large intestine. These SCFA contribute enormously to the horse's energy requirements. They also have profound biological effects especially on growth, differentiation and apoptosis of colonic epithelial cells.

The current study was undertaken to characterise the mechanisms for transcellular transport of SFCA across equine colonic luminal and basolateral membrane vesicles (LMV and BLMV).

Two methods were developed for the preparation of LMV and BLMV from equine colon. The membrane vesicles were then characterised in term of purity and origin using enzyme assays and immuno-detection of marker proteins. Transport of butyrate, as a SFCA model, into LMV and BLMV was characterised. The results demonstrated that:

❖ In the LMV: **1)** Butyrate was transported in the presence of an outward directed bicarbonate gradient, **2)** The rate of butyrate uptake was enhanced in the presence of pH gradient (pH_{in} 7.5, pH_{out} 5.5), **3)** Butyrate uptake was markedly inhibited in the presence of butyrate analogues, acetate, propionate and lactate, and by specific inhibitors of MCT1, 4CHC and phloretin, **4)** The uptake was saturable with an apparent Michaelis constant of 5.6 mM, **5)** Experimental evidence suggested MCT1 may be the transporter of butyrate in these membranes, **6)** oligonucleotide primers to MCT1 were designed and the full equine MCT1 was cloned and sequenced. The MCT1 cDNA encodes 500 amino acid protein having 87 % identity with human MCT1.

❖ In the BLMV: The results are consistent with the existence of a butyrate/HCO_3^- exchange process. **1)** The transport of butyrate reached an optimum rate at extravesicular medium pH of 5.5, **2)** The transport of butyrate was inhibited by the structural analogues of butyrate as well as formate and oxalate, **3)** Transport of butyrate was insensitive to inhibitors of MCT1, 4CHC and phloretin, but was markedly decreased in the presence of high concentration SITS and DIDS indicating the existence of a transport protein distinct from MCT1, **4)** Butyrate transport was a saturable process with an apparent K_m of 12.2 mM, **5)** Extravesicular chloride inhibited butyrate uptake suggesting the potential involvement of an anion exchanger (AE) family in the transport, **6)** It was shown that AE2 is the only family member expressed in the equine colon. Equine AE2 cDNA was cloned and sequenced, it encodes for 1237 amino acid protein, **7)** It was shown, using transport studies and inhibitory properties, that AE2 (Cl^-/HCO_3^- exchanger) does not mediate the transport of butyrate in the equine basolateral membranes. The results rather indicate the co-localisation of both butyrate/HCO_3^- and Cl^-/HCO_3^- on the basolateral plasma membrane of equine colon.

This study is the first to show the existence of two carriers distinct transporters mediating SCFA transport in the equine colon and providing an opportunity to develop an overall model for SCFA absorption in the equine large intestine.

List of Abbreviations

Alk.P.	Alkaline phosphatase
APS	Ammonium persulfate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BBMV	Brush-border membrane vesicles
BLMV	Basolateral membrane vesicles
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDS	Coding sequence
cpm	Counts per minute
DIDS	4,4`-di-isothiocyanato-2,2`-stilbenedisulphonic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
E_m	Enzyme activity coefficient
ER	Endoplasmic reticulum
GLUT	Facilitative glucose transporter
Hepes	(N-[2-Hydroxyethyl]piperazine-N`-[2-ethanesulphonic acid])
IPTG	Isopropyl 1-thio-β-D-galactopyranoside
kb	Kilobase
kDa	Kilodalton
k_m	Michaelis constant
LB	Liquid broth
LMV	Luminal membrane vesicles
MCT	Monocarboxylate transporter
Mes	(2-[N-Morpholino] ethanesulfinic acid)
Mops	(3-[N-Morpholono]propanesulfonic acid)
mRNA	Messenger RNA
MW	Molecular weight
NaBt	Sodium butyrate
NHE	Na^+/H^+ exchanger
O.D.	Optical density
PAGE	Polyacrylamide-gel-electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P_i	Inorganic phosphate
PMSF	Phenylmethylsulfonamide
pNPP	Para-nitrophenyl phosphate
RNase	Ribonulcease
RT	Reverse transcriptase

RT-PCR	Reverse transcription polymerase chain reaction
S.E.M	Standard error of the mean
SCFA	Short chain fatty acid
SDS	Sodium dodecyl sulphate
SGLT1	Sodium-dependent glucose co-transpoter1
SITS	4-acetamido-4`-isothiocyanostilbene-2,2`-disulphonic acid
SSC	Standard saline citrate
TAPS	N-[Tris(hydroxymethyl)methyl]-3-aminopropane-sulfonic acid
TEMED	N,N,N`,N`-tetramethylethylenediamine
TEN	Tris/ EDTA/ NaCl
Tris	(tris[hydroxymethyl]aminoethane)
TTE	Tris-TAPS-EDTA buffer
U	units
UTR	Untranslated region
Vmax	Maximum velocity
UV	Ultra violet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER I

Introduction

I.1 Structure and function of the equine gastro-intestinal tract

I.1.1 Anatomy of the digestive tract

Horses are herbivores with a digestive system designed for constant consumption of plant food. Unlike most herbivores, the horse is considered to be monogastric rather than a ruminant (Evans *et al.* 1990). The digestive system includes the stomach, small intestine and large intestine. The stomach and the small intestine are commonly referred to as the upper gut whereas the large intestine is known as the hindgut (Frape, 1998., Hintz, 1994).

The picture bellow is a schematic representation for the digestive tract of the horse.
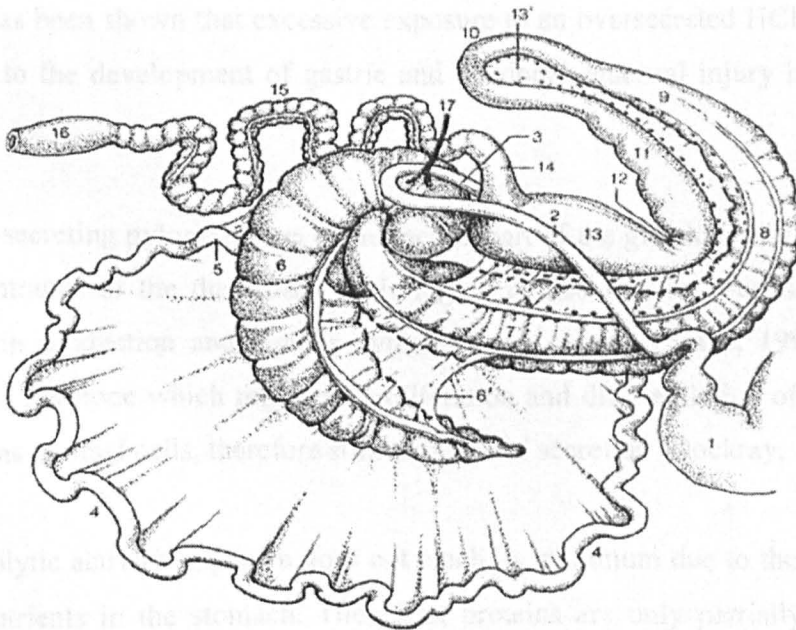
Figure I.1: The equine digestive tract (seen from the right). 1; Stomach, 2 & 3; Duodenum, 4; Jejunum, 5; Ileum, 6; Caecum, 6'; Cecocolic fold, 7; Right ventral colon, 8; Ventral diaphragmatic flexure, 9; Left ventral colon, 10; Pelvic flexure, 11; Left dorsal colon, 12; Dorsal diaphragmatic flexure, 13; Right dorsal colon, 14; Transverse colon, 15; Small colon, 16; Rectum, 17; Cranial mesenteric artery. (Adopted from Dyce, Sack and Wensing, "Textbook of veterinary anatomy". Second edition (1990), by W.B. Saunders Company.

a) Stomach

The stomach of the horse is a small organ and represents about 8 – 10 % of the capacity of the total digestive tract. The stomach stores, only for short period, mixes, digests and propels nutrients into the small intestine. The enterance of the stomach is monitored by a powerful muscle called "the cardiac sphincter" which prevents vomiting to occur.

Histologicaly, the stomach is divided into: 1) squamous mucosa which is relatively a continuation of the oesophagus since it is functionally inert. This part is termed *the saccus cecus* 2) glanduluar mucosa, which in turn consists of parietal, zymogen and enterochromaffin-like (ECL) cells-rich fundic region maintaining the production and secretion of hydrocloric acid, pepsin and histamine respectively (Merrit, 1999., Frape, 1998). It has been shown that excessive exposure to an oversecreted HCl and pepsin may contribute to the development of gastric and duedonal mucosal injury in foals (Murray, 1999).

3) Gastrin-secreting pyloric region is the second part of the glandular mucosa. It extend to join the entrance of the duedenum. This region is also rich in D-cells, responsible for somatostatin production and G-cells, which secrete gastrin (Frape, 1998). Gastrin is a polypeptide hormone which regulates proliferation and differentiation of gastic epithelial cells such as parietal cells, therefore stimulating acid secretion (Dockray, 1999).

The proteolytic activity of pepsin does not reach its maximum due to the short residential time of nutrients in the stomach. Therefore, proteins are only partially digested in the stomach and yields a small quantity of lactic acid (Frape, 1975., Evans *et al.* 1990). Carbohydrates and fats are only slightly hydrolysed before food reaches the upper segment of the small intestine (Frape, 1998).

b) Small intestine

The small intestine of the horse is about 20 – 25 metre in length and accounts for about 75 % of the total length of the gut (Hintz, 1994). It provides approximately 30 % of the capacity of the gastro-intestinal tract (Evans *et al.* 1990).

The small intestine extends from the pyloric sphincter and comprises; duodenum, jejunum and ileum. The duodenum is approximately 2 metre in length. It represents a pool where large quantities of bile and pancreatic juice are excreted. The jejunum is the longest segment of the small intestine, measuring about 17 – 22 metre. It is distinguished by its high mobility. The ileum represents the far end of the small intestine. It is a continuation of the jejunum and ends at the ileo-caecal orifice. The ileum is a tiny portion and has much thicker wall and firmer consistency compared to the jejunum (Dyce *et al.* 1987., Frape, 1998).

Histologically the small intestine is made up of: 1) the mucosa which in turn consists of epithelium and lamina propria, 2) submucosa which is blood vessels-rich connective tissues, 3) the muscularis consisting of two layers of smooth muscles and 4) the serosa.

The epithelium of the small intestine is folded into finger-like projections packed together into which line the predominant cell types. These cells include absorptive cells (enterocytes), mucus-secreting cells (goblet cells) and hormone-secreting cells (entero-endocrine cells). They are responsible for the general functions of the small intestine.

The small intestine plays a vital role in food digestion and absorption. Although hydrolysis of dietary proteins is initiated in the stomach, the major activity (60 – 70 %) takes place in the small intestine (Frape, 1975., Hintz, 1975). High proportions of fats and carbohydrates are also hydrolysed and absorbed in the small intestine, mainly in the proximal segment (Dyer *et al.* 2002), before they reach the large intestine (Roberts, 1975).

Only materials escaping enzymatic digestion in the small intestine attain the hindgut. This consists of fibrous residues, undigested starch as well as protein, micro-organisms, intestinal secretions and cell debris (Frape, 1998).

c) Digestion and absorption in the small intestine

The small intestine is the primary site of nutrient digestion and absorption. In non-ruminant species complex soluble carbohydrates are digested in the small intestine through the action of pancreatic enzymes and intestinal brush border hydrolases (Shirazi-Beechey, 1995). The capacity of the equine small intestine to digest and absorb these carbohydrates was determined by assessing the expression of sucrase, maltase and lactase in equine small intestine (Dyer *et al.* 2002). It has been shown that these enzymes hydrolyse disaccharides to monosaccharides (D-glucose, D-galactose and D-fructose) which are then absorbed across the small intestinal enterocytes by specific transporters (Shirazi-Beechey, 1995).

It is well established that, in most species including horse, D-glucose and D-galactose are transported by the sodium-dependent glucose co-transporter (SGLT1) (Shirazi-Beechey, 1995). However, absorption of D-fructose across the brush border membranes occurs via the sodium-independent glucose transporter (GLUT5) (Shirazi-Beechey, 1996; Levin, 1994).

SGLT1 transports glucose by coupling glucose uptake with that of Na^+. This protein works in synergy with basolateral sodium pumps which maintain the trans-membrane Na^+ gradient ((Shirazi-Beechey, 1995).

Western blotting analysis and immunohistochemical studies have shown that SGLT1 is located on the luminal membranes of the small intestine (Thompson & Wild, 1997II), whereas in the parotid secretory acinar cells the Na^+-glucose co-transporter has been localised to the basolateral membranes (Vayro *et al.* 1991; Tarpey *et al.* 1995).

Rabbit SGLT1 was cloned by Hediger *et al.* (1987; 1989). Subsequently, ovine SGLT1 was cloned and characterised by Wood *et al.* (1994; 1999).

So far, several isoforms of SGLT family have been identified (Wright, 2001; Wood & Trayhurn, 2003; Wood *et al.* 2000). They have different functional properties and tissue distribution. The activity and abundance of intestinal SGLT1 was found to be modulated in response to luminal sugars (Dyer *et al.* 1997).

The equine intestinal SGLT1 was cloned and sequenced by Dyer *et al.* (2002), it is a high affinity, low capacity transporter (Dyer *et al.* 2002).

Monosaccharides such as D-glucose, D-fructose and D-galactose exit the enterocyte to the bloodstream down their concentration gradient by a facilitative Na^+-independent mechanism through GLUT2 isoform (Thompson & Wild, 1997I).

In the diarrhoeal state SGLT1 plays a vital role for oral re-hydration therapy. The principle lies in the fact that sodium and glucose can enhance the osmotic re-absorption of water across the enterocytes. This therapy requires operational SGLT1 in the apical membrane and Na^+-K^+-ATPase in the basolateral membrane (Field, 2003; Zeuthen *et al.* 2001).

d) Large intestine

The large intestine of the horse represents about 65% of the overall volume of the gastro-intestinal tract. It is approximately 25 feet long and includes all segments distal to the ileo-caecal sphincter. The large intestine is made up of the caecum, the large colon and the small colon.

The caecum is a blind sac of about 1 metre in length with an average capacity of 33 litres; this represents 25 – 30 % of the large intestine capacity (Evans *et al.* 1990., Jones *et al.* 1998). The caecum is a massive organ consisting of base, body and apex and has extensive contacts with other abdominal organs (Dyce *et al.* 1987).

The large colon begins from the caecocolic orifice and extends distally beyond this point. It is further sub-divided into right ventral colon, left ventral colon, left dorsal colon and right dorsal colon, all of which are arranged in four parallel limbs separated by three bends called "flexures" (ventral diaphragmatic flexure, pelvic flexure and dorsal diaphragmatic flexure), transverse colon, small colon which ends with the rectum (Dyce *et al.* 1987).

The caecum and the colon are jointed together to the surrounding intestinal wall with a wide distinct bands or taeniae. The large intestine has a distinct pattern of motility that pushes the ingesta along the tract (Sellers and Lowe, 1986). At the end of the right dorsal colon, the calibre decreases sharply and continues to become the transverse colon (Dyce *et al.* 1987).

The small colon is few metres long and represents the last segment of the large intestine. It continues to the rectum, which is distinguished by its pelvic location (Dyce *et al.* 1987., Jones *et al.* 1998).

I.1.2. Histology of the large intestine

The histological features of the equine large intestine relates to the general functions attributed to it. The large intestine has similar structural characteristics to the small intestine with the presence of few distinctive features:

- Absence of villi.
- The intestinal glands are long and straight.
- The mucosa is relatively thicker than the small intestine due to increased length of intestinal glands.
- Substantial increase of the number of goblet cells.
- Absence of Paneth cells.

Similar to the rest of the alimentary tract, the large intestine is arranged in 4 layers; however it is often difficult to distinguish between them.

a) **Mucosa**: it contains numerous straight tubular intestinal glands called "crypt of Liberkuhn" and is divided into epithelium and lamina propria.

Each crypt is made of three main cell lineages: colonocytes (absorptive cells), which function to re-absorb water and electrolytes. They are scattered on the crypt surface and interspersed with numerous goblet cells at irregular intervals. These latter cells produce mucus that lubricates the bowel, facilitating the passage of solid contents. The third cell types found in the colonic crypt are enteroendocrine cells. These cells are present throughout the gut and form about 0.4 % of cells in the colon. Enteroendocrine cells are distinguished by the accumulation of granules in the infranuclear cytoplasm. Moreover they synthesise and store peptide hormones.

The production of the aforementioned cell types is achieved by stem cells located at the crypt base.

The space between the crypts is filled by lamina propria which is a loose connective tissue containing some elastic fibers and lymphatic nodules.

b) **Submucosa**: consists of bundles of collagenic fibers linked to the mucosa from one side and to the muscularis from the other side. It contains blood vessels, lymphatic vessels as well as submucosal glands and nerve fibers.

c) **Muscularis**: consists of two layers of smooth muscles; the inner circular and the outer longitudinal separated by a thin connective tissues. One of the characteristic features of the horse colon is that the outer longitudinal layer forms a large, flat muscle bands containing copious elastic fibers (*taenia coli*). The muscularis produces peristaltic contractions which result in a distal movement of the colonic contents.

d) **Serosa**: a layer of connective tissue containing collagenic and elastic fibers, enclosing and preventing over-extension of the colon. (Dellmann and Eurell, 1998., Dellmann, 1971).

I.1.3 Structure of the colonic epithelium

The colonic crypts are lined by numerous polarised epithelial cells. These cells exhibit a regular model of spatial organisation from the base of the crypt to the free surface. Along the length of the crypt several stages of differentiating cell types were found (Kaye *et al*. 1973). One of them is columnar absorptive cells.

a) Absorptive cells

The large intestinal absorptive cells or colonocytes are located predominantly on top of the crypt and are associated with the transepithelial absorption of water and substrates. They are polarised epithelial cells consisting of two asymmetrical membrane domains; apical or luminal domain facing the lumen content and a basolateral domain facing the bloodstream separated by tight junctions (Zonula occludens), which allows selective passage of substrates between cells and mediates inter-cellular communication (Gerike *et al.* 1998).

The absorptive cells are characterised by the presence of microvilli which are less prominent than those of the small intestinal absorptive cells, the tight junctions are well developed, apical mitochondrial localisation and presence of multivesicular bodies such as lipid droplets and lysosomes.

b) Cell turnover

Understanding the renewal of the colonic epithelial cells is fundamental and recent studies have shed light upon this since it is tightly correlated with the functional properties of the organ such as absorption, digestion and secretion (Karam, 1999).

It has been reported that the epithelium of the large intestine undergoes constant renewal. The epithelial cell types of the colon originate from stem cells which are located at the crypt base (Grant & Specian, 2001). Stem cells are characterised as being undifferentiated and exhibit embryogenic cell-like features; high nucleus to cytoplasm ratio, much diffuse chromatin. They have an amazing potential to proliferate whilst maintaining constant numbers (self maintenance) (Marshaman *et al.* 2002).

The site and the rate of cell proliferation have been demonstrated by the ability of the cells to incorporate [^{3}H]-thymidine and or its analogue BrdU during DNA synthesis (Grant & Specian, 2001).

Epithelial cells produced by stem cells migrate towards the surface of the colonic crypt. This migration is characterised by acquisition of structural and functional features (differentiation) and develops the ability to digest and absorb nutrients before being shed (Kim & Shibata, 2002). Differentiation of stem cells to several cell lineages is controlled by systemic factors (hormones and growth factors) and maintained by the presence of food in the bowel (Wong & Wright, 1999; Thompson *et al.* 2000; Gericke *et al.* 1998).

It has been agreed that the dynamics of colonic stem cells vary, however epithelial cell turnover is rapid and essentially all cells except stem cells are renewed within a week (Kim & Shibata, 2002), with an average turnover time of 4 – 6 days (Karam, 1999; Marshaman *et al.* 2002).

I.1.4 Luminal and basolateral intestinal membrane markers

Epithelial cells of the small and large intestine are polarised cells characterised by the presence of two distinct functional anatomic membrane domains separated by tight junctions located in the lateral plasma membrane. What makes these two domains different is the presence of a highly distinct protein composition on the two membranes. These proteins are responsible for the vectorial transport of different molecules, such as a variety of organic and inorganic nutrients, across the epithelium.

During sub-cellular fractionation the population of integral and peripheral proteins can be used as markers to confirm the origin of isolated luminal (LMV) or basolateral (BLMV) membrane vesicles and to assess the purity of the fraction of interest from any potential contamination by other organelle membranes.

Table I.1: Proteins of the luminal and basolateral membranes of the intestinal epithelial cells

Protein	Luminal	Basolateral
Villin	X	
GLUT2		X
Na-K-2Cl		X
Na^+-K^+-ATPase		X
MCT1	X	
Alkaline Phosphatase	X	
B-actin	X	
RLA class I (Histocompatibility antigen)		X

So far, many attempts to isolate LMV or BLMV have been carried out. Varied approaches have been used to obtain pure membrane vesicles, because the cross-contamination certainly affects the experimental results. None of the investigators managed to isolate 100% pure LMV or BLMV, the presence of negligible contamination is always certain.

In order to determine the purity and origin of luminal and basolateral membrane vesicles isolated from equine colonocytes, the activity of marker enzymes and the expression of marker proteins in these membranes have been assessed, since the validity and significance of the data depend upon the cellular origin of the membrane vesicles.

a) Villin

Villin is a calcium-regulated actin-binding protein (Craig & Powell, 1980). It is the major structural component of the microvilli cytoskeleton (Bretscher & Weber, 1980). Each microvillus is made of a bundle of about 20 longitudinally oriented actin filaments. Villin as well as fimbrin bind the F-actin filaments along its length (Ho, 1992).

The 95 KDa molecular weight villin was shown to be abundant in the epithelium of the small and large intestine (West et al. 1988; Robine et al. 1985). Studies on the human tissue have confirmed that villin is expressed on the luminal membranes of differentiated and undifferentiated intestinal cells (Pringault et al. 1986), thus it is considered as a reliable marker.

Immunohistochemical studies have localised villin exclusively to the apical membrane of healthy colonic epithelial cells (Ho, 1992). Further studies have revealed that villin is also expressed in carcinoma colonic tissue (Grone et al. 1986) and HT-29 cell line (Pringault et al. 1986). These studies confirm that villin can be used as a marker of colonic apical membranes.

b) Na^+-K^+-ATPase

The Na^+-K^+-ATPase also called sodium the pump is ubiquitously expressed in the basolateral membrane of epithelial cells including small and large intestinal epithelial cells where it serves as the major regulator of intra-cellular ion homeostasis (Mobasheri et al. 2000; Vagnerova et al. 1997; Zemelman et al. 1992). It has also been identified on the luminal membrane of the choroid plexus (Quinton, 1973) and the retinal pigmented epithelium (Ghosh et al. 1990).

Na^+-K^+-ATPase exchanges 3 Na^+ molecules outside the cell for 2 K^+ molecules inside the cell with hydrolysis of one ATP molecule (Scheiner-Bobis, 2002). Therefore, it is responsible for maintaining Na^+ and K^+ gradients across the plasma membrane (Pacha, 2000) and for maintaining the resting membrane potential of -35 mV inside the cell (Spiller, 1994).

The sodium pump is a heterodimer protein consisting of three subunits (α, β and γ). The α subunit (100 – 112 KDa), referred to as the catalytic, is responsible for ion transport, whereas the β subunit (45 – 55 KDa), referred to as the regulatory unit, is a glycoprotein involved in the assembly of the sodium pump (Geering, 1990; Mobasheri et al. 2000). The γ subunit is located extracellularly with unclear function. Na^+-K^+-ATPase possesses a catalytic site for ATP at the cytoplasmic face of the membrane and is inhibited by ouabain at the extracellular face (Forbush III, 1983).

In addition to Na^+-K^+-ATPase, other ATPase has been identified in mammalian colon. These include Ca^{+2}-ATPase, involved in Ca^{+2} extrusion across the basolateral membrane (Bronner, 2003), and K^+-H^+-ATPase, involved in the absorption and excretion of K^+. K^+-H^+-ATPase is the closest relative of the sodium pump (Jaisser and Beggah, 1999). Two different types of K^+-H^+-ATPase have been detected on the luminal membrane of the colon depending on their sensitivity towards ouabain (ouabain-sensitive K^+-H^+-ATPase and ouabain-insensitive K^+-H^+-ATPase) (Scheiner-Bobis et al. 2002, Kunzelmann and Mall, 2002).

c) Facilitative glucose transporters

The facilitative glucose transporters (GLUT family) are energy-independent carriers (passive carriers) that transport glucose down its concentration gradient (Brown, 2000). The ubiquitous use of glucose for the intracellular metabolism is linked to the widespread distribution of glucose transporters in most tissues including

liver, kidney, brain, erythrocytes, small and large intestine, skeletal muscle and adipose tissue (Mueckler, 1994; Thorens *et al.* 1990; Bird *et al.* 1996).

GLUTs belong to a super family of genes whose protein products span the plasma membrane 12 times and have cytoplasmic disposition of both COOH and NH_2 termini (Mueckler, 1994).

13 mammalian glucose transporter isoforms have been identified thus far, four of which are well characterised designated GLUT1 – 4 (Brown, 2000) displaying distinct functional and kinetic properties and different tissue distribution.

a. GLUT1 is very abundant in erythrocytes (5 % of the total membrane) and nervous tissue (Brown, 2000; Mueckler, 1994). Recently it has been shown to be expressed at the mRNA and protein levels in colon carcinoma (Noguchi et al. 2000).

b. GLUT2 is a low affinity transporter (K_m 30 mM), found primarily in β-cells of the pancreas, hepatocytes, enterocytes, colonocytes and kidney cells (Thorens, 1996; Wu *et al.* 1998; Thorens *et al.* 1990).

In the intestine, GLUT2 is restricted to the basolateral membrane of enterocytes (Thomson & Wild, 1997 I) and colonocyte (Pinches *et al.* 1993). It has been cloned from many species including horse small intestine. GLUT2 is responsible for the exit of monosaccharides (glucose, galactose and fructose) into the bloodstream but can work in the opposite direction (Olson and Pessin, 1996). It is specifically inhibited by cytochalasin B (Axelrod & Pilch, 1983).

c. GLUT3 is a prominent glucose transporter in the brain (Gould et al. 1992), it is also expressed in the placenta of rat (Zhou & Bondy, 1993). It has a high affinity for glucose (Brown, 2000).

d. GLUT4 is the insulin-sensitive glucose transporter found predominantly in cardiac and skeletal muscles as well as adipose tissue (James *et al.* 1989; Fukumoto *et al.* 1989).

e. GLUT5 is a fructose transporter on the brush border membrane of the small intestinal epithelial cells. It is only 39 – 40 % identical to the other glucose transporter isoforms (Thorens, 1996).

d) Monocarboxylate transporter 1 (MCT1)

MCT1 is a member of the monocarboxylate transporter (MCT) family, which mediates the transport of acetate, propionate and butyrate as well as pyruvate and lactate across the plasma membrane in a variety of mammalian cell types (Halestrap and Price, 1999). It has been shown that MCT1 plays a pivotal role in the transport of monocarboxylates in the colon (Ritzhaupt *et al.* 1998b).

It has also been shown that MCT1 is expressed at both mRNA and protein levels in human and pig colonic tissue and the protein is located on the apical membrane of the colonocytes (Ritzhaupt *et al.* 1998b; Lambert *et al.* 2002).

A number of recent studies have indicated that MCT1 is the transporter of monocarboxylates in a colon-derived cell line (Caco-2) and sheep ruminal epithelium (Hadjiagapiou *et al.* 2000; Muller *et al.* 2002).

Apical localisation of MCT1 on the colonic absorptive cells can be used as a reference to determine the luminal origin of the colonic membrane vesicles.

I.1.5. Microbiology of the colon

The large intestine of mammals is warm, moist, anaerobic and filled with nutrients. This characteristic makes the large intestine the most favourable place for the growth of microorganisms. The microbial ecosystem of the equine hindgut contributes enormously in the host's health and performance, including energy requirement since up to 75 % of the horse's body energy is provided by the end products of microbial fermentation (Argenzio, 1975; Jones *et al.* 1998), nitrogen provision (Frape, 1975; Mackie & Wilkins, 1988) in addition to the stimulation of both immune response and gene expression in the host epithelial cells (Tran *et al.* 1998).

However disruption of this ecosystem makes the horse exposed to disorders (colic, laminitis) which might put at risk the health of the horse (Medina *et al.* 2002).

It has been found that the principal inhabitants of the equine large intestine are bacteria, fungi (flora) and protozoa (fauna) (Hintz, 1994). Several inter-relationships occur among members of the ecosystem; commensalism, mutualism, competition, and predation (Bergman, 1990).

It was suggested that the flora of the horse's large intestine is qualitatively comparable to that of the rumen of sheep and cow (Hintz, 1975). Mackie & Wilkins (1988), and Bergman (1990) have reported that the major genera of bacteria dwelling in the colon includes *Bacteroides, Eubacterium, Bifidobacterium, Streptococci, Lactobacilli, Enterobacteria, Methonobacterium, Fusobacterium.* These bacterial populations distribute differently along the large intestine. High numbers were found in the caecum and ventral colon, most of them cellulolytic bacteria and about 20 % proteolytic, compared to other parts of the large intestine (Frape, 1998).

Varying bacterial counts in the hindgut were revealed deponding on the segmental differences and the nature of food. A high bacterial count was obtained from the caecum and the colon with $0.5 - 5 \times 10^9$ per gram of content (Mackie and Wilkins, 1988; Frape,

1998). In addition, fungi represented 2×10^2 to 25×10^2 units per gram of content most of which are cellulolytic.

Work carried out in our laboratory by Daly *et al.* (2001) used analysis of PCR-amplified 16S ribosomal RNA gene sequence to describe the microbial population in the equine large intestine. This method is considered to be a powerful tool to quantitatively characterise the predominant microbial populations inhabiting the equine large intestine (Daly and Shirazi-Beechey, 2003). The authors found that 72 % of all sequences were members of low % G + C gram positive (LGCGP), 20 % *Cytophaga-Flexibacter-Bacteroides* (CFB) and the remaining 8 % was associated with others such as *Spirochaetaceae*. 37 % of all sequences were affiliated with one Clostridial group, cluster XIVa. Only 5 % of all bacterial population in the equine hindgut corresponded to the sequence of known organism in the public database. A further 6 % corresponded to the unidentified sequences and the majority of sequences (89 %) with no correspondence, which clearly demonstrated that the equine colon contained a novel non-recognisable microflora (Daly *et al.* 2001).

Protozoa are also members of the intestinal ecosystem with an amount of $0.5 – 1.5 \times 10^5$ per ml of content (Frape, 1998). Despite protozoa being much larger than bacteria, their removal from the hindgut did not disrupt the metabolism, leading to the suggestion that protozoa do not play a major task in the fermentation in the equine large intestine (Moore & Dehority, 1993).

I.1.6. Functions of the colon

The equine colon plays an important role in the body homeostasis. It serves to maintain fluid balance since tremendous amounts of water, electrolytes and other nutrients are absorbed. The colon is the site of fermentation and bacterial fiber digestion hence the site of organic acids (SCFA) production and absorption. Furthermore, the colon secretes potassium and bicarbonate ions and stores faeces for excretion (Argenzio *et al.* 1974; Hintz, 1975).

The significance of the colon was realised following surgical studies. It was found that removal of the caecum had no nutritional consequences, however removal of 95 % of the equine colon resulted in decreased fiber digestibility and body performance (Hintz, 1994).

a) Microbial digestion (fermentation)

It is well documented that the small intestine is the primary site of nutrients (proteins, amino acids, carbohydrates, fats, vitamins...etc) digestion (Hintz, 1975). However nutrients escaping small intestinal enzymatic digestion will be fermented by the resident microflora of the large intestine. Similar to cattle, horses receive a fibrous diet, but differently to cattle, is predominantly digested in the hindgut whereas in cattle fibre is predominantly digested in the rumen (Bayley, 1978). The undigested nutrients entering the colon consist of polysaccharides of which resistant starch, non-starch polysaccharides NSP (cellulose, hemicellulose, pectin). In addition, substrates of endogenous origin such as exfoliated epithelial cells, mucin, dead bacteria are found in the colon (McFarlane & Cummings, 1991).

The principal structural polysaccharides of the cell wall of the plants are cellulose, hemicellulose and pectin. The intestinal microflora can hydrolyse the β-1,4-linkes polymers of cellulose which the horse's digestive enzymes cannot do (Frape, 1998).

The microbial fermentation of dietary fiber yields substantial amounts of short chain fatty acids (SCFA) commonly referred to as monocarboxylates (acetate, propionate and butyrate) previously known as volatile fatty acids (VFA). Moreover, acetate, propionate and butyrate are not the only products, minor side products are produced by microbial fermentation as well e.g. lactate, and gases (H_2, CO_2 and CH_4) (Bugaut, 1987). The gas is normally evacuated as its retention will cause distention of the gut accompanied with colic.

The ratio of SCFA generally ranges from 70:20:10 to 75:15:10 for acetate, propionate and butyrate respectively (Hintz and Cymbaluk, 1994). Similar end products of microbial fermentation were found in the rumen with relatively varying ratio (Bergman, 1990). The amount of butyrate has been reported to be greater than propionate in rabbit (Hintz *et al.* 1978). Hintz *et al.* (1978) reported that microbial digestion in the hindgut of equine is less efficient (2/3) compared to that in the rumen of the cow. This is due to the fact that starch can be digested and absorbed in the equine small intestine prior to ingesta reaching the large intestine. Another explanation was referred to the high rate of digesta passage in the horse colon with regard to the rate of passage in the rumen. This is consistent with what has been previously reported by Bergman (1990).

A formula equation was adapted by Bergman (1990) to estimate the fermentation in the rumen based on the molar ratio of acetate, propionate and butyrate (65:20:15).

$$58\ C_6H_{12}O_6 \longrightarrow 65\ \text{acetate} + 20\ \text{propionate} + 15\ \text{butyrate} + 60\ CO_2 + 35\ CH_4 + 25\ H_2O$$
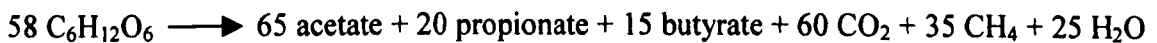
Taking into consideration the molar ratio of SCFA in the hindgut of the horse (70:20:10), the same equation might be applied with slight adjustment to acetate and butyrate concentrations.

Argenzio *et al.* (1974) reported that the highest concentrations of SCFA are produced in the caecum and ventral colon and tends to decrease to lower values in the other segments. Concentration of 100 mmol.l^{-1} was found in the caecal content of most mammals with existence of certain variations (Engelhardt *et al.* 1998). The table below summarises the proportion of SCFA in digesta to bodyweight (BW) in four herbivores.

Table I.2 The proportion of SCFA in digesta to bodyweight (BW) in four herbivores.

	g SCFA / Kg BW
Horse	1.0
Sheep	1.5
Ox	1.5
Rabbit	0.5

Reproduced from Frape, D "Equine Nutrition and Feeding" 2nd edition (1998) Blackwell Science.

Microbial fermentation in the colon produced not only acetate, propionate and butyrate but also branched-chain fatty acids for instance isobutyrate, isovalerate and 2-methylbutyric acid (Bergman, 1990).

After their production, SCFA are readily absorbed from the colonic lumen into the bloodstream through the colonocytes. Approximately 95 % of the butyrate produced by colonic bacteria is transported across the epithelium (Pryde *et al.* 2002).

b) Absorption

♦ Water

Water is considered as the most critical element for the function of the large intestine. It is important for microbial fermentation and absorption. It was demonstrated that tremendous amounts of water pass the ileo-caecal junction (Frape, 1998). Using polyethelene glycol (PEG-4000) as a marker to study digesta and water passage in the equine large intestine, Argenzio *et al.* (1974) found that the liquid marker reached the caecum 2 hours after its administration. The volume of water entering the large intestine is almost equivalent to the animal's total extracellular fluid volume (Hintz *et al.* 1978).

Although a substantial amount of water is absorbed from the colon, the caecum is considered as the primary site of water recovery (Frape, 1998; Evans *et al.* 1998). In a pony weighing 160 kg, 19.4 liters of water are received by the large intestine 95 % of which is reabsorbed and only 1.5 liters are excreted in the faeces (Argenzio *et al.* 1974). Interestingly the type of diet does not influence the amount of water recovered (Frape, 1998). On the other hand, little difference in the faecal water content of horses that were fed grain or grass was noticed (Evans *et al.* 1998).

Absorption of water by the colonic epithelial cells is due to the osmotic pressure gradients caused by solute uptake. The amount of water taken up is directly proportional to the rate of Na^+ absorption and indirectly to SCFA (Engelhardt *et al.* 1998).

Is has been postulated that water can be driven via a paracellular route through tight junctions (Masyuk *et al.* 2002). Using in situ hybridisation (ISH) and immunohistochemistry (IHC) techniques, Koyama *et al.* (1999) have shown that the rat colon expresses water channels AQP1, AQP3 and AQP8 with basolalateral location of AQP3 and AQP4 (Kunzelmann & Mall, 2002).

♦ **Electrolytes**

The equine large intestine plays a vital role in electrolytes balance. It has been revealed that 96 % of the sodium (Na^+) and chloride (Cl^-) and 75 % of the soluble potassium (K^+) and phosphate (PO_4^{-2}) passing the ileo-caecal junction were absorbed (Frape, 1998). This is approximately 115 meq of Na^+ and 30 meq of K^+ per liter of water were absorbed (Hintz et al. 1987). Previous studies undertaken in human and animals indicate an inter-relationship between SCFA and colonic Na^+, Ca^{2+}, Mg^{2+} absorption and K^+ secretion (Clarke et al. 1992).

Sodium can enter the cell via an electroneutral Na^+/H^+ exchanger (NHE). This protein secretes protons and removes Na^+ from the luminal fluid (Jones *et al.* 1998). Perfusion studies in a number of species demonstrated that Na^+ absorption was inter-dependent from SCFA absorption. In addition, segmental disparity in Na^+ absorptive mechanism in the equine large intestine was noted (Clarke et al. 1992). This might correlate with variation in fermentation potential in different compartments of the hindgut (Argenzio and Stevens, 1975). Na^+ absorption is mediated by three types of Na^+/H^+ exchanger since expression of NHE1, NHE2 and NHE3 have been detected in the colonic epithelium (Kunzelmann & Mall, 2002).

Sodium is then removed from the cell by the basolateral sodium pump (Na^+-K^+-ATPase). Evidence for the absorption of Na^+ via the bumetanide-sensitive Na:K:2Cl cotransporter (NKCC) on the basolateral side of the colonocytes was shown to exist (D'Andrea *et al.* 1996).

It has been shown that Cl^- absorption is concomitant with secretion of K^+ and HCO_3^- in the colon. Chloride can enter the colonocyte via Cl^-/OH^- exchanger. It was also shown that the epithelial cells in the colonic crypt possess Cl^- channels that are regulated by intra-cellular hormones and neuro-transmitters (Liedtke, 1989). Cl^- can also be transported by the NKCC. On the other hand, Rajendran and Binder (2000) have

proposed the involvement Cl^-/HCO_3^- exchanger in chloride absorption. Cl^- secretion in rat and human colon was shown to be inhibited by SCFA. Reduced secretion was associated with decreased synthesis of cAMP (Engelhardt *et al.* 1998; Resta-Lenert *et al.*2001) and decreased expression of basolateral NKCC cotransporter (Resta-Lenert *et al.* 2001).

The equine colon contributes to maintain K^+ homeostasis. K^+ is vitally important for many cellular functions: intra-cellular chemical reactions, muscular contraction and conductance of nervous impulses (Philip & McCabe, 1984). It was proposed that potassium enters the colonocytes actively, coupled to H^+ on the luminal membranes (K^+-H^+-ATPase) (Foster *et al.* 1984; Philip & McCabe, 1984), and coupled to Na^+ on the basolateral membranes (Na^+-K^+-ATPase) (Philip & McCabe, 1984). Other studies have described the presence of luminal and basolateral K^+ channels for K^+ recycling. These channels are also important to maintain the hyperpolarised membrane voltage and the electrical driving force that is required for the activation of Na^+-K^+-ATPase (Kunzelmann & Mall, 2002).

Another function of the equine large intestine is the absorption of the phosphate entering from the ileum (Argenzio and Stevens, 1975).

Martin *et al.* (1996) have reported that 26 % of the urea reaching the hindgut was not accounted in either faecal or urinary nitrogen. This would amount up to 88 g of urea per day in a 500 Kg horse (Frape, 1975). These investigators proposed that high proportion of urea was converted into ammonia, a proportion of which was used by the host tissue (Hintz *et al.* 1978). The other fraction was utilised by bacteria, thereby maintaining microbial fermentation in the hindgut as a result of providing a supply of amino acids for bacteria and SCFA for the host (Martin et al. 1996; Frape, 1975/ 1998).

The efficiency of the equine large intestine to utilise vitamins remains controversial. While most authors stress the idea that vitamins are recovered by the small intestine (Evans *et al.* 1998), others demonstrated that significant amounts of group B vitamins are synthesised in the large intestine especially vitamin B_{12} which is taken up by the horse's large intestine (Hintz *et al.* 1978).
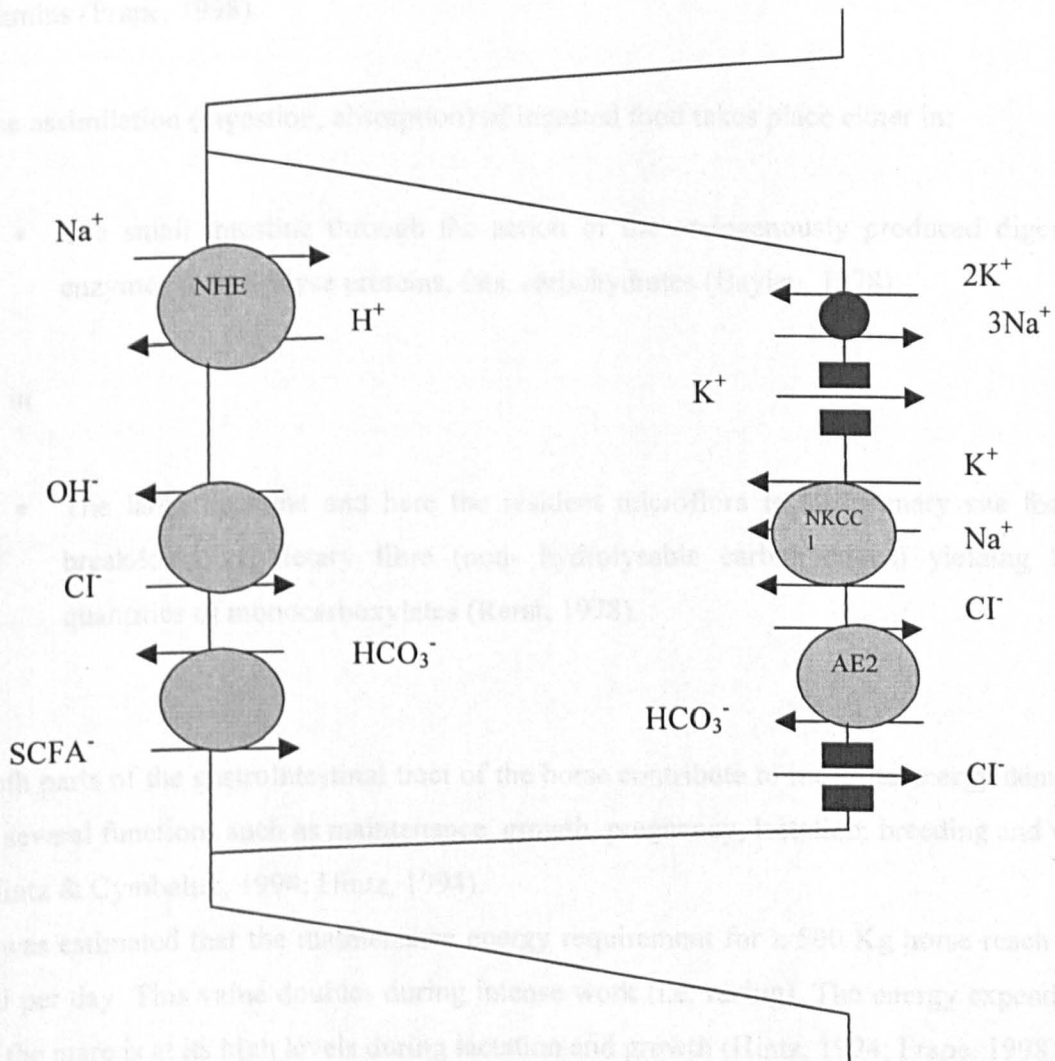
Figure I.2 Cellular model of electrolytes absorption in the colon

I.2. Nutrition of the horse

The horse is a non-ruminant herbivore consuming a variety of feeds (plant materials) with different physical forms ranging from forage to grain to hay. The chemical composition of horse's diet consists of water, soluble proteins, hydrolysable carbohydrates (i.e. starch), non- hydrolysable carbohydrates (i.e. cellulose) in addition to minerals and vitamins (Frape, 1998).

The assimilation (digestion, absorption) of ingested food takes place either in:

- The small intestine through the action of the endogenously produced digestive enzymes to hydrolyse proteins, fats, carbohydrates (Bayley, 1978).

Or in

- The large intestine and here the resident microflora is the primary site for the breakdown of dietary fibre (non- hydrolysable carbohydrates) yielding large quantities of monocarboxylates (Rerat, 1978).

Both parts of the gastrointestinal tract of the horse contribute to meet the energy demands of several functions such as maintenance, growth, pregnancy, lactation, breeding and work (Hintz & Cymbaluk, 1994; Hintz, 1994).
It was estimated that the maintenance energy requirement for a 500 Kg horse reach 68.6 MJ per day. This value doubles during intense work (i.e. racing). The energy expenditure of the mare is at its high levels during lactation and growth (Hintz, 1994; Frape, 1998).

In order to fulfil the increasing energy demands, concentrate-based diet has been introduced. This will help to maintain the hindgut microbial population and to render the digestion more efficient. Supplementation of compounded nuts or coarse mixes ensures a

complete diet since they are made of several ingredients including minerals and vitamins. These products are commercially manufactured and encompass many forms suitable for different states for instance gestation, lactation.

From what has been said earlier, it is clear that the nutrition of the horse is essential and digestion of nutrients is a determining issue. However many different factors may influence the digestion for instance processing of feeds (pelleting and rolling of roughage), work, maturity of the plant, time of watering, individuality (Hintz, 1975; Evans *et al.* 1998). Digestive disorders such as colic, acidosis, gastric ulcer, strongyles…etc are likely to affect the digestion (Nadeau *et al.* 2000; Love, 1992).

In summary, there is much evidence for the role of nutrition to enhance the well being and the longitivity of the equine and to decrease the risk of injuries. However it is recommended that a proper balance of dietary fibre, soluble carbohydrates, fat, protein is required (Hintz, 1994).

I.3 Short chain fatty acids

I.3.1 Definition and properties

Short chain fatty acids (SCFA), previously known as volatile fatty acids (VFA) because they are volatile in aqueous solutions at acid pH, are now referred to as monocarboxylates. They have a chain length ranging from one to six carbon atoms (C1 – C6) (McFarlane and Cummings, 1991).

Name	Chemical formula	Number of carbon atom
Formate	H-COOH	C1
Acetate	CH3-COOH	C2
Propionate	CH3-CH2-COOH	C3
Butyrate	CH3-$(CH2)_2$-COOH	C4
Valerate	CH3-$(CH2)_3$-COOH	C5
Caproate	CH3-$(CH2)_4$-COOH	C6

Table I.3. Names and chemical formula for SCFA.

The production process of SCFA is accomplished by different species of microflora (see section I.1.5) residing in the large intestine (non-ruminants) or the rumen (ruminants). The process involves a number of discrete steps starting with hydrolysis of polysaccharides and ending by fermentation (Hill, 1995).

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SCFA are produced in the equine colon by anaerobic fermentation of substrates from the diet (dietary fibre) and substrates of endogenous origin (i.e. exfoliated cells) (Blaut, 2002). The amount of SCFA produced is highly variable along the digestive tube and between species (30 – 240 mM) (Bergman, 1990) with an average rate of production of 100 mM (Engelhardt *et al.* 1998). In man, the daily concentration of SCFA produced has been measured in the range of 300 mmol – 600 mmol (Hoverstad, 1986).

It has been shown that acetate, propionate and butyrate are the major end products of the microbial fermentation. The molar proportions of acetate to propionate to butyrate have been found to be approximately 70:20:10 (Bergman, 1990). The molar ratio of the three main SCFA in different organs of the human is summarised in table 1.4.

	Acetate	Propionate	Butyrate
Right colon	57	22	21
Left colon	57	21	22
Portal vein	71	21	8
Hepatic vein	81	12	7
Peripheral blood	91	5	4

Table I.4. Molar ratio of SCFA (%). (Adapted from Bugaut, (1987)).

SCFA have the following properties:

- Can be found in acid or anion form
- SCFA are weak acids with pKa of 4.79, 4.87 and 4.81 for acetate, propionate and butyrate respectively.
- At normal colonic pH (6 – 7), 90 – 99 % of SCFA are present as anions.
- SCFA are stable at low pH and remain active after incubation at pH 1.0.
- SCFA are rapidly absorbed throughout the length of the large intestine and extensively metabolised either locally by the colonic epithelium or systemically after entering the bloodstream.
- SCFA execute many biological effects during normal and disease states (Bugaut and Bentejac, 1993; Wachtershauser and Stein, 2000; Ahmed *et al.* 2000; Perrin *et al.* 2001).

I.3.2 Absorption of SCFA

It has been reported that SCFA are rapidly absorbed from all segments of the lower digestive tract. (Bergman, 1990; Dijkstra *et al.* 1993) with an average of about 95 % of the total SCFA produced (Titus and Ahearn, 1992). It was revealed that the rate of SCFA absorption in the colon exceeds that of sodium (Cummings, 1984). The rate of total SCFA absorption in horse was 8 $\mu mol/cm^2/h$ (Argenzio *et al.* 1974; Cummings, 1984). In ruminants and non-ruminants SCFA absorption is determined by pH and SCFA concentration (Bergman, 1990; Dijkstra *et al.* 1993).

Two different mechanisms for the absorption of SCFA have been proposed. The first involved the transport of the protonated form and the second was related to the ionic form.

I.3.2.1 Passive diffusion

In this system SCFA acrosses the colonic luminal membranes in a protonated form. Two mechanisms have been postulated to explain the source of the proton:

- In the first, protons originate from the colonic lumen after formation of H^+ and HCO_3^- from CO_2 by microbial fermentation.

- In the second, H^+ originate intra-cellularly and come from the apical carrier mediated process which exchange Na^+ for H^+ (NHE) and or potassium – hydrogen exchanger (K^+-H^+-ATPase) (Macfarlane and Cummings, 1991; Ruppin *et al*. 1980; Argenzio *et al*. 1977; Titus and Ahearn, 1992). At the same time, SCFA entry decreases intra-cellular pH and activates the Na^+-H^+ exchanger (Sellin & DeSoignie, 1998).

I.3.2.2 Carrier mediated mechanism

The second mechanism was proposed to explain the transport of anionic form of SCFA. There is increasing evidence that the transport of SCFA occurs predominantly via this mechanism, since at colonic pH, 95 % of SCFA are in anionic from and can not diffuse through the plasma membrane. This model was also proposed based on observation of increasing concentration of bicarbonate in the colonic lumen.

Carrier mediated transport is characterised by saturation kinetics, competitive substrate inhibition and cotransport with associated anion, (Titus and Ahearn, 1992). All these characteristics have been uncovered while studying the mechanism of SCFA transport across the colonic luminal membranes (Mascolo *et al*. 1991; Harig *et al*. 1996; Ritzhaupt

et al. 1998a). These authors concluded that a SCFA/HCO$_3^-$ exchange process exists and is the main mechanism for SCFA to cross the apical membranes of the colon (Ramaswamy *et al.* 1994).

Work from our laboratory has shown that a monocarboxylate transporter (MCT1) is involved in butyrate transport across the luminal side of the colonocytes (Ritzhaupt *et al.* 1998b). A number of recent studies have confirmed the finding (Stein et al. 2000; Hadjiagapiou *et al.* 2000).

Recent studies have established the existence of a functional Na-monocarboxylates cotransporter on the colonic luminal membranes of the human and mouse colon. This transporter is SLC5A8 of the SCL5 gene family (Miyauchi *et al.* 2004., Coady *et al.* 2004., Gopal *et al.* 2004)..

Once inside the cell, some of SCFA is metabolised by the colonocytes and the remainder passes into the bloodstream. It has been suggested that the intra-cellular concentration of SCFA is high and may reach 50 mM (Sellin, 1999).

In the basolateral membranes, SCFA transport has been shown not to be associated with nonionic diffusion but via a carrier mediated process in exchange with bicarbonate (Reynolds *et al.* 1993; Tyagi *et al.* 2002) however the SCFA/HCO$_3^-$ exchangers on both apical and basolateral are different.

I.3.2.3 SCFA transport *in vivo*

Many experimental approaches have been used to investigate the mechanism of SCFA transport, one of which is *in vivo* perfusion. The colon from different species, human, guinea pig and rat (Ruppin *et al.* 1980; Schmitt *et al.* 1976; Fleming *et al.* 1991) was perfused with solutions containing acetic, propionic and butyric acids with different

ranges of concentration. The absorption of SCFA was also measured *in vivo* using the dialysis bag technique (Hoverstrad, 1986).

These techniques have shown that SCFA absorption is rapid, concentration dependent, not easily saturable and accompanied with stimulation of Na^+ absorption and bicarbonate secretion (Ruppin *et al.* 1980). Based on these findings, two explanations have been considered: 1) the presence of a carrier mediated $SCFA/HCO_3^-$ or 2) non-ionic diffusion of protonated SCFA (Sellin, 1994).

On the other hand, Rechkemmer *et al.* (1995) observed saturation of SCFA in the large intestine of guinea pig, indicating the presence of a carrier mediated mechanism.

The use of the aforementioned techniques is limited because only changes in the luminal contents are studied (Hoverstrad, 1986).

I.3.2.4 SCFA transport using the Ussing chamber

Transepithelial flux measurement under short circuit condition was employed to determine SCFA absorption. Pieces of stripped intestinal mucosa were mounted in Ussing chamber and the isotopic flux was measured over successive periods of time. Then the unidirectional mucosal to serosal and serosal to mucosal fluxes were calculated.
Compared with ion transport studies of Na^+, K^+, Cl^-, where this technique provided useful data, less information has been produced while investigating SCFA transport.

The data gained from Ussing chamber studies showed that SCFA were transported via passive diffusion (Sellin, 1994; Sellin and DeSoignie, 1990; Rechkemmer *et al.* 1995; Charney *et al.* 1998). It has been reported that SCFA were transported in all segments of guinea pig large intestine in protonated form and were related to the H^+ released from the Na^+/H^+ exchanger (Musch *et al.* 2001). The authors found that amiloride inhibited SCFA

absorption in the caecum and proximal colon of guinea pig, whereas in the distal colon SCFA absorption was more ouabain sensitive.

I.3.2.5 SCFA transport using isolated membrane vesicles

The technique using isolated membrane vesicles has been developed to investigate the mechanism of transport of a number of electrolytes (i.e. Na^+, K^+, Cl^-, HCO_3^-) in a number of tissues (i.e. kidney, small and large intestine, parotid gland) from a number of species (i.e. human, rabbit, rat, pig, guinea pig).

The mechanism of SCFA transport was analysed using purified colonic apical or basolateral membrane vesicles. A number of studies have consistently proved that SCFA transport in LMV occurred via a carrier mediated process and provided strong evidence for $SCFA/HCO_3^-$ anion exchange. It has also been shown that non-ionic diffusion represent a negligible fraction of SCFA absorption (Harig *et al.* 1990; Mascolo *et al.* 1991; Harig *et al.* 1996; Ritzhaupt *et al.* 1998a).

These authors have shown that butyrate transport into colonic LMV was insensitive to the anion exchange inhibitor 4,4`-di-isothiocyanato-2,2`-stilbenedisulphonic acid (DIDS) and displayed several characteristics for the $SCFA/HCO_3^-$ exchanger. The transport process was stimulated by low extra-vesicular medium pH, inhibited by other butyrate analogues and display saturation kinetics but with distinct Km values.

Isolated membrane vesicles methodology represented an ideal model to study the mechanism of SCFA transport across the colonic basolateral membranes as well.

SCFA transport across the basolateral membrane vesicles (BLMV) was first studied by Reynolds *et al.* (1993) using rat colon, then by Tyagi *et al.* (2002) using human colon. It has been demonstrated that the uptake of butyrate is stimulated in the presence of bicarbonate gradient, supporting the evidence of $SCFA/HCO_3^-$ anion exchange. The

transporter presented characteristic features of a carrier mediated process which is different from those of $SCFA/HCO_3^-$ anion exchange in the LMV.
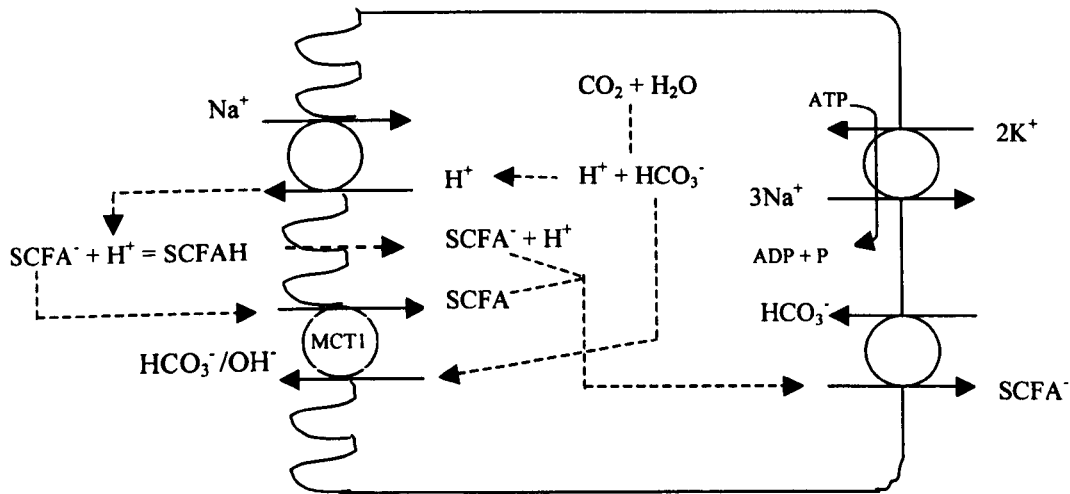
Figure I.3 Cellular model of SCFA absorption in the colon.

I.4. Effects of Short chain fatty acids

Data from early research studies showed that dietary fibre played an important role in maintaining health of colonic mucosa and in preventing colonic diseases (Topping & Clifton, 2001). More recent studies have attributed these effects to the products of dietary fibre fermentation, short chain fatty acids (SCFA) (Rodriguez-Cabezas *et al*. 2002; Key *et al*. 1996). SCFA, mainly acetate, propionate and butyrate are the predominant organic anions in the colonic content produced by microbial fermentation of dietary fibre (Bugaut & Bentejac, 1993).

Beside its importance as a source of energy for colonic epithelial cells (Roediger, 1994), SCFA, especially butyrate, exerts a number of biological effects on cell proliferation,

differentiation and apoptosis (Cuff *et al.* 2002). These effects have been shown by *in vivo* and *in vitro* studies.

It is interesting to understand that cell proliferation is initiated at the bottom of the colonic crypt by division of stem cells. The mitotic pressure forces the newly formed cells to move upward to the surface. During this movement, the cells undergo differentiation and ultimately apoptosis (programmed cell death) on the surface (Marshman *et al.* 2002). It is imperative that the balance between cell proliferation, differentiation and apoptosis has to be maintained since deregulation of this balance leads to the development of cell cancer (Lupton, 1995).

I.4.1. SCFA and cell proliferation

A paradoxal effect of SCFA on normal colonic epithelial cells and on cancer cell lines was observed (Engelhardt *et al.* 1998). While stimulating the proliferation of normal rat colonocytes (Sakata & Engelhardt, 1983), butyrate was reported to be anti-proliferative agent for cancer cell line (Clausen, 1995). An increase in normal colonic mucosal mass villous height, surface area and crypt depth was seen after infusion of SCFA. This effect might at first be linked to the oxidation of SCFA for the energy, however recent studies indicate that SCFA stimulate the enteric nervous system and induce growth factors release which in turn modulate mucosal blood flow (Cook and Sellin, 1998; Frankel *et al.* 1994; Scheppach *et al.* 1997; Singh *et al.* 1997).

Galfi & Neogrady (2001) have reported that butyrate inhibits cell division *in vitro*. This effect has been ascribed to reduce pH value after incubation with butyrate. The mechanisms underlying the paradoxal effects of butyrate between normal mucosa and cancer cell lines are unclear.

In vitro studies have shown that butyrate inhibits proliferation of a number of colorectal cancer cell lines by inducing cell cycle arrest in the G0/G1 phase (Luciano *et al.* 1996) and by modulating the expression of a number of genes involved in the regulation of cell cycle

such as $p21^{WAF1/CIP1}$, which encodes a cyclin-dependent kinase inhibitor protein known to inhibit cell cycle progression (Archer *et al.* 1998; Siavoshian *et al.* 2000).

I.4.2. SCFA and cell differentiation

If has been shown that treatment of colon carcinoma cell line, HT29, with butyrate stimulated differentiation of the cells (Augeron *et al.* 1984) to a phenotype more consistent with normal cells (Schappach, 1994). Cell differentiation is judged by the increase in expression of marker of cell differentiation, alkaline phosphatase, (Rickard *et al.* 1999; Mariadason *et al.* 2001) and decrease of growth rate (Lupton, 1995). Cell differentiation is also associated with reduce colonic paracellular permeability (Kinoshita *et al.* 2002).

I.4.3. SCFA and cell apoptosis

Butyrate is also known to induce apoptosis in a number of transformed colorectal cell lines. Evidence revealed that butyrate-induced cell apoptosis may be mediated by changes in gene expression. Butyrate inhibited the expression of Bcl-2 (an integral protein which blocks apoptotic cell death) and stimulated the expression of pro-apoptotic genes, Bak and Bax (Hague *et al.* 1997; Emenaker *et al.* 2001) and Cdx2 (Domon-Dell *et al.* 2002).

Other studies have shown that butyrate induces apoptosis through the activation of intra-cellular cascades. Different pathways have been proposed:

- Activation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) – caspace route (Hernandez *et al.* 2001).
- Activation of β-catenin-Tcf pathway (Bordonaro *et al.* 1999; Augenhicht *et al.* 2002).

- Inactivation of transcription factor NF-κB which is a critical regulator of apoptosis (Feinman *et al*. 2001).
- Down regulation of $\alpha_2\beta_1$ integrin thus perturbing cell adhesion (Buda *et al*. 2003).

At the molecular level butyrate supplementation was shown to modulate expression of *c-myc*, *c-jun* and *c-fos* genes associated with cell cycle and regulate the normal growth of gastrointestinal mucosa (Tappenden and McBurney, 1998). Whereas in colon carcinoma cells, butyrate inhibits the activity of histone deacetylase, therefore causing histone hyperacetylation and activation of transcription factors such as p21 (Hinnebusch *et al*. 2002).

I.4.4. Other effects of SCFA

Besides the contribution to enhance electrolytes absorption (Choshniak and Mualem, 1997; Vidyasagar and Ramakrishna, 2002), SCFA have further effects on the colonic mucosa.

- Locally SCFA stimulates mucus release from the colon (Willemsen *et al*. 2003) that is believed to protect the epithelium against mechanical and chemical damage (Shimotoyodome *et al*. 2000).
- It has also been shown that SCFA stimulates colonic smooth muscles contraction therefore enhancing intestinal motility. The mechanism underlying muscle motility is unclear but may be mediated through neuronal or hormonal pathways ((Rondeau *et al*. 2003).
- It was observed that acetic acid, as well as propionic and butyric acids, causes vasodilatation in many vascular beds (Aalkjær, 2002). Study from Knock *et al*. (2002) has shed the light on the vasodilatator effect of SCFA and have shown that endothelium-derived hyperpolarizing factor (EDHF) is behind it.
- It has been shown that propionate is an important substrate for gluconeogenesis in horse (Ford and Simons, 1985).

- Ketone bodies production from butyrate in the large intestine of mammals has been revealed (Bugaut, 1987; Miehinen and Huhtanen, 1996).

- Glucagons and insulin releasing effect of SCFA on the pancreas of sheep was also reported (Mineo *et al.* 1994).

I.4.5. Butyrate and colon cancer

Despite the large number of epidemiological studies showing the existence of negative correlation between dietary fibre and colon cancer, the mechanism of action is still unclear.

Colon cancer is one of the most frequent cancers in the world (Faivre *et al.* 1995; Levi *et al.* 1995). Cancer cells are not under normal growth regulation (Jass *et al.* 2002) and have the capacity to invade surrounding tissues (Kim *et al.* 1977).

It was found that butyrate slows proliferation and promotes differentiation of cancer cell population (Clausen, 1995). This is supported by the successive preventive action of butyrate in ulcerative colitis (Scheppach *et al.* 1995).

A conflicting opinion on the chemopreventive effect of butyrate exists. Accordingly, it has been proposed that cellular sensitivity towards butyrate is different between *in vivo* and *in vitro* environment (Lupton, 2004).

I.5 Monocarboxylate transporter (MCT) family

MCTs are membrane carriers mediating the transport of important monocarboxylates such as lactate, pyruvate, acetate, propionate and butyrate as well as the branched-chain amino acids derived from leucine, valine and isoleucine (Halestrap and Price, 1999).

MCTs are characterised by being: 1) either H^+-monocarboxylates cotransporter or OH^-/ monocarboxylates exchanger. 2) The carriers are sensitive to specific anion exchange inhibitors such as phloretin, 4-hydroxycinnamate (4-CHC) and *p*-chloromercuribenzoate (pCMB).

So far, several members (14 isoform) of MCT family have been identified and cloned, each of them having slightly different properties and tissue distribution (Halestrap and Meredith, 2004). It has been reported that monocarboxylate transporters play a promising role to improve epithelial drug absorption in the intestine and other organs (Lee, 2000).

The proposed topology for MCTs indicates that the protein have 12 transmembrane domains (TMD) with large intra-cellulare loop between TMD6 and 7. Both amino and carboxy termini are in the cytoplasmic side of the membrane (Halestrap and Meridith, 2004).

I.5.1 Isoforms and expression

a) MCT1

MCT1 is the most studied isoform, because it has a very broad tissue distribution. Kim *et al.* (1992), was the first to clone and sequence MCT1 from Chinese hamster ovary cells. MCT1 has been functionally expressed in the breast tumour cells and demonstrated that it catalysed inhibitor-sensitive monocarboxylate transport, leading to its nomination as MCT1 (Garcia *et al.* 1994).

MCT1 is found in many tissues including colon, ovary, rumen, muscle, placenta, brain, heart, erythrocytes, retina, and in many species such as human, rat, mouse and horse (Price *et al.* 1998; Muller *et al.* 2002; Gerhart *et al.* 1999; Koho *et al.* 2002). It

was also found that in colon carcinoma its expression was significantly reduced (Lambert *et al.* 2002).

MCT1 transports a wide range of monocarboxylates either linked to proton (Hadjiagapiou *et al.* 2000), linked to Na^+ (Poole and Halestrap, 1993) or in exchange with anion (Ritzhaupt *et al.* 1998b).

Recent studies have shown that MCT1 as well as MCT4 require a membrane glycoprotein, CD147, for their correct plasma membrane expression and function, since co-localisation of MCT1, MCT4 and CD147 was revealed (Kirk *et al.* 2000; Wilson *et al.* 2002).

In the colon, MCT1 transports SCFA via an anion exchange mechanism. The transport process is inhibited by known anion inhibitors (4CHC, pCMB). Western blot analysis demonstrated the presence of MCT1 protein in the luminal membranes of the colon from human and pig (Ritzhaupt *et al.* 1998b). Basolateral location of MCT1 in rat jejunum has also been demonstrated (Orsenigo *et al.* 1999).

b) MCT2

Later, the second isoform, named MCT2, was cloned and sequenced (Garcia *et al.* 1995). MCT1 and MCT2 share 65 % amino acid sequence identity (Price *et al.* 1998). MCT2 is a high affinity pyruvate transporter (25 µM), several time lower than for MCT1, suggesting that MCT2 may participate in conditions of low substrate concentrations where rapid transport is indispensable (Halestrap and Price, 1999)

MCT2 expression has been found in fewer tissues compared to MCT1, liver, kidney, brain and testis (Juel and Halestrap, 1999). It has been demonstrated that MCT2 protein catalysed the basolateral H^+-lactate transport (Eladari *et al.* 1999). MCT2 gene has been cloned from many species, with human MCT2 being mapped to chromosome band 12q13 (Lin *et al.* 1998).

c) MCT3

In human, MCT3 is expressed at the mRNA level in the muscle, placenta and testis with an expected size of 2.0 Kb (Price *et al.* 1998). The protein location is to the basolateral membranes of the retinal pigment epithelium (Philip *et al.* 1998). MCT3 shares 43 % and 45 % amino acid sequence identity with MCT1 and MCT2 respectively (Halestrap and Price, 1999).

d) MCT4

MCT4 is characterised as low affinity L-lactate transporter (K_m 20 mM). MCT4 sequence is highly related to that of MCT3. The protein has a limited tissue distribution, heart, brain, skeletal muscle and while blood cells (Enoki *et al.* 2003; Rafiki *et al.* 2003; Juel and Halestrap, 1999). Indeed MCT4 has been shown to co-localise with MCT1 in the plasma membrane of rat cardiac myocytes, in association with a chaperon protein (CD147) facilitating their cell surface expression (Kirk *et al.* 2000).

e) MCT5, MCT6 and MCT7

Northern blot analysis showed that mRNAs of these isoforms are differently expressed in human tissues. MCT5 is mainly located in the prostate, placenta and muscle, MCT6 in the kidney and the brain whereas MCT7 in the colon, liver, heart and other tissues (Price *et al.* 1998).

They share 23 – 30 % amino acid sequence identity with MCT1. Their role has never been elucidated (Halestrap and Price, 1999).

f) MCT8

Originally named XPCT and has low degree of conservation with other MCT isoforms. Yet, MCT8 has not been functionally characterised as proton-monocarboxylate co-transporter (Halestrap and Price, 1999). The expression of MCT8 in xenopus oocytes, however, revealed that this isoform is an active transporter for thyroid hormone (Friesema *et al.*2003).

g) Other MCTs

New isoforms have been identified as a members of MCT family, this includes; MCT9, TAT1, MCT11, MCT12, MCT13 and MCT14. All these members are functionally uncharacterised. TAT1 was shown to be involved in the transport of aromatic amino acids (Halestrap and Meredith, 2004).

I.5.2 MCT1 and SCFA

SCFA are the end products of bacterial fermentation of undigested carbohydrates. They are the predominant anions in the colonic lumen of many species including horse (Bugaut, 1987). They cannot cross the luminal membranes by passive diffusion. Accordingly, a number of studies indicated that SCFA are transported via an anion exchange mechanism with enhanced activity at low extravesicular medium pH (Mascolo *et al.* 1991; Harig *et al.* 1996; Ritzhaupt *et al.* 1998a).

Functional and kinetics studies revealed that SCFA transport exhibits specific features which are in accordance with the characteristics of MCT1. This suggestion was confirmed using xenopus laevis oocytes as a functional expression system (Ritzhaupt *et al.* 1998b).

The involvement of MCT1 as a potential transporter for monocarboxylates was further emphasised by Stein *et al.* (2000) and Hadjiagapiou *et al.* (2000).

I.5.3 Regulation of MCT1

The level of nutrient absorption is directly regulated by the abundance of dietary substrates in the intestinal lumen (Karasov and Diamond, 1987). As major anions in the colonic contents, SCFA rate of absorption is controlled by the amount of production. It has been reported that MCT1 has the ability to transport SCFA including butyrate (Ritzhaupt *et al.* 1998b).

It has been shown that treatment of cultured colonic epithelial cells (AA/C1) with butyrate upregulated the expression of the carrier mediated butyrate transport, MCT1, in a concentration and time dependent manner (Cuff *et al.* 2002). The authors have concluded that this regulation resulted from control of MCT1 expression at the level of gene transcription and mRNA stability.

In human, significant reduction of MCT1 expression was found to occur in the colon during transition from normality to malignancy. This was interpreted as a result of reduce availability of butyrate (Lambert *et al.* 2002) and/or deregulation of cellular events controlling MCT1 expression.

It has been reported that human and rat skeleton muscle MCT1 is up-regulated during exercise followed by an increase in the rate of lactate transport (Juel and Halestrap, 1999).

Human gene	Protein name	Predominant substrates	Transport type	Tissue distribution	Human gene locus
SLC16A1	MCT1	Monocarboxylates	Cotransporter/ Exchanger	Ubiquitous	1q13.2
SLC16A2	MCT8	T3, T4	Facilitated transporter	Liver, heart, brain, thymus, intestine, ovary, prostate, pancreas, placenta	Xq13.2
SLC16A3	MCT4	Monocarboxylates	Cotransporter/ Exchanger	Skeletal muscle, chondrocytes, leukocytes, testis, lungs, placenta, heart	17q25.3
SLC16A4	MCT5	Uncharacterised	–	Brain, muscle, liver, kidney, ovary, placenta, heart	1p13.3
SLC16A5	MCT6	Uncharacterised	–	Kidney, muscle, brain, heart, pancreas, prostate, lung, placenta	17q25.1
SLC16A6	MCT7	Uncharacterised	–	Brain, pancreas, muscle	17q24.2
SLC16A7	MCT2	Monocarboxylates	Cotransporter/ Exchanger	Kidney, brain	12q14.1
SLC16A8	MCT3	Lactate	Cotransporter/ Exchanger	Retinal pigment epithelium, choroids plexus	22q13.1
SLC16A9	MCT9	Uncharacterised	–	Endometrium, testis, ovary, breast, brain, kidney, adrenal, retina	10q21.2
SLC16A10	TAT1	Aromatic amino acids	Facilitated transporter	Kidney, intestine, muscle, placenta, heart	6q21-q22
SLC16A11	MCT11	Uncharacterised	–	Skin, lung, ovary, breast, pancreas, retinal pigment epithelium, choroids plexus	17p13.2
SLC16A12	MCT12	Uncharacterised	–	Kidney	10q23.3
SLC16A13	MCT13	Uncharacterised	–	Breast, bone marrow stem cells	17p13.1
SLC16A14	MCT14	Uncharacterised	–	Brain, heart, ovary, breast, lung, pancreas, retinal pigment epithelium, choroids plexus	2q36.6

Table I.5. The characteristics of monocarboxylates transporter (MCT) family. (Adapted from Halestrap & Meredith (2004)).

I.6. Anion exchanger family

I.6.1 Properties

Anion exchanger family of proteins consist of Cl^-/HCO_3^- exchangers mediating the transport of one Chloride molecule for one bicarbonate molecule cross the plasma membrane (Sterling and Casey, 2002; Romeo *et al.* 2004). These proteins are encoded by the SLC4 gene family which contains a least three Na^+-independent anion exchanger genes, SLC4A1, SLC4A2 and SLC4A3 giving product for AE1, AE2 and AE3 proteins respectively (Alper *et al.* 2002).

They are broadly distributed, tissue specific, integral plasma membrane glycoproteins. They play an important role to maintain electrolytes balance and regulate cell volume and intra-cellular pH (Kopito, 1990; Puceat, 1999; Merot *et al.* 1997; Guizouarn *et al.* 2001). The most stricking feature of all members of this family is their high affinity for the stilbene derivatives, SITS and DIDS, (Cabantchik and Rothstein, 1972/1974). They first bind reversibly to the inhibitors through weak ionic interactions, which become irreversible when the covalent reaction takes place (Janas *et al.* 1989).

I.6.2 Isoforms

a) Anion exchanger 1 (AE1)

AE1 (band 3) is the most studied isoform of the AE family, being the most abundant protein in the erythrocyte (eAE1) plasma membrane (25 % of the total membrane protein) (Casey and Reithmeier, 1998). However, recent work from Guizouarn et al. (2002) has proposed the presence of all isoforms (AE1, 2 and 3) at the RNA level in the skate erythrocytes. AE1 protein is also highly expressed in the basoateral membranes of intercalated cells of the kidney (kAE1) (Merot *et al.* 1997). Kopito and Lodish (1985) were the first to clone and sequence AE1 gene which is located on the chromosome 17 encoding 100 KDa protein. Anion exchangers have the proposed topology of 12 – 14

transmembrane spanning domains with both amino and carboxy termini facing the cytosol (Casey and Reithmeier, 1998; Alper *et al.* 2002).

In addition to its role as Cl^-/HCO_3^- exchanger, the AE1 protein catalyses the exchange of divalent anion sulfate but at slower rate (Sekler *et al.* 1995). It is strongly inhibited at low concentration of DIDS (Salhany, 1998). Furthermore it has been revealed that band 3 functions as an anchor for the cytoskeleton, haemoglobin and glycolytic enzymes, and as senescence antigen (Wang, 1994).

b) Anion exchanger 2 (AE2)

AE2 is the most widely expressed form of AEs and in almost all epithelial cells has basolateral membrane location (Alper *et al.* 2002). It was found in the kidney (Eladari *et al.* 1998; ; Castillo *et al.* 2000), liver (Garcia *et al.* 1998), gastric mucosa (Stuart-Tilley *et al.* 1994; Rossmann *et al.* 2001), epididymis (Jensen *et al.* 1999), Colon (Ikuma *et al.* 2003; Rajendran *et al.* 2000), coroid plexus epithelium (Lindsey *et al.* 1990), and pancreas (Hyde *et al.* 2000). In the small intestine, AE2 localisation remained controversial between brush border membranes (Chow *et al.* 1992) and basolateral membranes (Alper *et al.* 1999). A Golgi form of AE2 has recently been detected in fibroblasts (Holappa *et al.* 2001).

AE2 gene, which contains 22 introns and 23 exons (Medina *et al.* 1997), has been found to encode 5 N-terminal variant AE2 subtypes (AE2a, AE2b1, AE2b2, AE2c1 and AE2c2) (Alper et al. 2002) transcribed from at least 3 promoters (Rossmann *et al.* 2000).

AE2 exhibits 80 % homology to the AE1 whereas the three AE isoforms share 65 % amino acid sequence identity in the membrane-associated transport domains (Puceat, 1999).

AE2 protein (130 – 180 KDa) appears to be composed of a 700aa N-terminal cytoplasmic domain (it is approximately 320 – 400aa in AE1 and 700aa in AE3) followed by 500aa transmembrane domain and a short C-terminal cytoplasmic domain (Apler *et al.* 2002).

AE2 protein is well characterised anion exchanger mediating the electroneutral Cl⁻ for HCO₃⁻ across the plasma membrane. In the parietal cell of the human gastric mucosa, AE2 is linked to basolateral cytoskeleton through protein called ankyrin. This close association might stabilise AE2 to this domain and prevent it from unspecific endocytosis (Jons and Drenckhahn, 1998).

c) Anion exchanger 3 (AE3)

AE3 is highly expressed in excitable tissues including brain, heart and retina. It is also found elsewhere in the gut, muscle and kidney (Alper *et al.* 2002).

The AE3 gene uses two promoters to generate two C-terminal variants (bAE3 and cAE3). The bAE3 protein is 1232aa long but the cAE3 is about 200aa shorter.

AE3 proteins exchange Cl⁻ for HCO₃⁻ in a 1:1 electroneutral anion exchange process and are inhibited reversibly and irreversibly by DIDS (Puceat *et al.* 1995). AE3 and pH sensitivity of transport have been demonstrated in a study carried out by Sterling and Casey, (1999). The authors have shown that AE3 isoform remained insensitive to change in pH over a range of pH 6.0 – 9.0 but contributed to pH recovery after cellular-acid loading.

I.6.3 AE2 and SCFA

The functional properties of monocarboxytes transport mediated by AE2 were assessed using transient AE2 expression in HEK 293 cell and uptake studies (Yabuuchi *et al.* 1998). The result demonstrated that AE2 transfected cells exhibited a significant rate of uptake of monocarboxylates with regard to the control cells. This data suggested that AE2 is functionally involved in the transport mechanism of monocarboxylates.

I.6.4 Regulation of AE2

It was demonstrated that AE2 is highly sensitive to changes to the medium pH. Data from Zhang and colleagues suggested that transmembrane domain serves as a pH sensor to regulate the protein (Zhang *et al.* 1996). However, recent studies reported that AE2 was inhibited by intracellular acidification and activated by intracellular alkalinisation, and the NH_2-terminal was most critical for AE2 regulation (Stewart *et al.* 2001)

Human gene symbol	Protein name	Predominant substrates	Transport type	Tissue distribution	Human gene locus
SLC4A1	AE1 (band3)	Cl^-/HCO_3^-	Exchanger	Erythrocyte, intercalated cell, heart, colon	17q21-q22
SLC4A2	AE2	Cl^-/HCO_3^-	Exchanger	Basolateral membranes in most epithelial cells	7q35-q36
SLC4A3	AE3	Cl^-/HCO_3^-	Exchanger	Brain, retina, heart, kidney muscle and GI tract	2q36

Table I.6 Characteristics of anion exchanger (AE) family. (Adapted from Romeo *et al.* (2004)).

I.7 Aims

A large proportion (60 – 70 %) of the horse's energy requirement is provided by short chain fatty acids, SCFA, absorbed from the large intestine. Despite such an important role, very little is known about the mechanisms of transcellular transport of SCFA across the colonic epithelial cells; neither there is any information on the nature of the membrane transport proteins involved.

The aim of this study was: (i) to investigate the mechanisms of butyrate transport across the luminal and basolateral membranes of equine colonocytes and (ii) to characterise the membrane proteins involved.

CHAPTER II

Materials & Methods

II.1 Materials

II.1.1 chemicals, reagents and radioisotopes

All chemicals and solvents were of highest analytical grade and obtained from Sigma-Aldrich (UK), Fisher Scientific (Leicester, UK) and BioRad laboratories (Herts, UK) unless otherwise stated. DNA enzymes were purchased from Promega, Strategene (STRATEGENE, UK).

$[\alpha\text{-}^{32}P]$-dCTP (specific activity 3000 $Ci.mmol^{-1}$) was obtained from the radiochemical centre (Amersham, Bucks, UK) $[U\text{-}^{14}C]$-α-D-glucose with specific activity of 297 $mCi.mmol^{-1}$ was purchased from (Amersham Life Science, UK) and sodium $[U\text{-}^{14}C]$-butyrate (specific activity of 16.0 $mCi.mmol^{-1}$) was obtained from (Sigma, UK).

II.1.2 Antibodies

Polyclonal antibody to MCT1 was raised in rabbit against the C-terminus region of human MCT1 (CQKDTEGGPKEEESPV). The GLUT2 antibody was raised in rabbit against a peptide corresponding to the C-terminus region of the horse intestinal GLUT2. The monoclonal antibody against the chicken villin was obtained from The Binding Site (UK). The anion exchanger 2 (AE2) antibody was raised in rat and was purchased from Alpha Diagnostic International, UK. Antibody to human anion exchanger 1 (band 3), raised in rabbit, was supplied by Dr. R. Kopito (Stanford University, CA, USA). Secondary antibodies (swine anti-rabbit and rabbit anti-mouse) conjugated to horseradish peroxidase were purchased from Dako (DAKO Ltd, Bucks, UK).

II.1.4 Oligonucleotides

All oligonucleotide primers were designed using the Vector NTi software package (INFORMAX, USA) and synthesised commercially by Eurogentec (UK)

The sequences of the antisense and sense primers against horse AE2 were:
Sense: 5' RACE UAP; Antisense: 5'-TGTAGGGGGAGATGGGCTGGT-3'

Sense: 5'-AGAGCTAGCGGGTTATGCCTCC-3'; Antisense: 5'-CGTCCTCAAACCGGTGACT-3'

Sense: 5'-TACCACCGCCAGTCCTCC-3'; Antisense: 5'-ATCTCGTGGTCGTCCACGTTGG-3'

Sense: 5'-CCAAGTCCAAGCACGAGCT-3'; Antisense: 5'-GGCCAAAGAGGGCACAGATGC-3'

Sense: 5'-ATCACCACGCTGATCATCTCC-3'; Antisense: 5'-AGGATGAGGATGAAGGGGAAG-3'

Sense: 5'-ATCACCACGCTGATCATCTCC-3'; Antisense: 3' RACE UAP

The sequence of the antisense primer against equine AE1 (band 3) cDNA was 5'-TCACCTTCGGCGGCCTCCTGG-3', and the sense primer had the sequence 5'-AGAGGAGGGCTGTGTTGGGCA-3'.

The antisense primer complementary to equine bAE3 cDNA had the sequence 5'-TCAGGAACAGCCGCTTCCTG-3', and the sequence of the sense primer was 5'-CTGGATCTGGGGCTTGTCAC-3'.

II.1.3 Bacterial strains

The E. coli JM109 (recA1, endA1, gyrA96, thi, hsdR17, relA1, Δ(lac-proAB). [F` traD36, proAB⁺, lacIᵖ, lacZΔM15]) was used as a bacterial strain for transformation with recombinant plasmid.

II.1.5 Plasmid and cDNA

pGEM-T cloning vector was purchased from Promega (UK) and used for direct cloning for polymerase chain reaction (PCR) products.

II.2 Equine tissue

II.2.1 Removal and storage of the equine tissue

Horse colonic tissue was obtained from the abattoir (Nantwick, Cheshire) within 10 min of slaughter of the animals. The colon was rapidly removed and flushed with ice cold 0.9% (w/v) NaCl (pH 7.0) to remove faecal content. The tissue was then everted, further washed in saline then gently blotted with a paper towel to remove adherent materials (mucus). The colon mucosa was harvested by placing the everted tissue upon a glass plate placed on ice, and gently scraping using two glass microscope slides. The scrapings were wrapped in aluminium foil and immediately immersed in liquid nitrogen. Afterwards, frozen intestinal scrapings were stored at -80°C until use.

II.3 Preparation of plasma membrane vesicles

II.3.1 Isolation of luminal membrane vesicles (LMV)

The isolation of the horse colonic LMV was done as described by Ritzhaupt *et al.* (1998a) using a cation precipitation and differential centrifugation technique. All steps were carried out at 4^0C. Mucosal scrapings were weighed and thawed in a buffer containing 100 mM mannitol, 2 mM Hepes/ Tris (pH 7.1), 0.2 mM benzamidine and 0.2 mM PMSF at 1 g scraping per 10 ml buffer. A polytron (Ystral Polytron, Scientific Instruments Cambridge, UK) was used to homogenise the scraping at setting 4 for 2 min, then the homogenate was filtered through nylon gauze to remove excess mucus. $MgCl_2$ was added to a final concentration of 10 mM and stirred on ice for 20 min. A centrifugation step was performed at 1000g for 15 min (Sorvall RC5 SS-34 rotor) to sediment of the nuclei and large cellular fragments. The resulting pellet was discarded and the supernatant was filtered again through nylon gauze to remove any remaining fat. In the following centrifugation (30,000g for 25 min) the supernatant was discarded. The pellet was collected and re-suspended in a buffer containing 100 mM mannitol, 20 mM Hepes/ Tris (pH 7.4) and 0.1 mM $MgSO_4$, and homogenised using 40 strokes, in a Dounce homogeniser (Jencons Ltd, UK) with tight fitting

Teflon pestle. The homogenate was centrifuged at 30,000g for 35 min and the resulting pellet was re-suspended in 500 µl of the final buffer (300 mM mannitol, 20 mM Hepes/ Tris pH 7.4 and 0.1 mM MgSO₄) and homogenised by passing several times through a 27G needle. The membrane vesicles were aliquoted and stored in liquid nitrogen until use.

II.3.2 Isolation of basolateral membrane vesicles (BLMV)

BLM vesicles were isolated from horse colon by using sucrose density gradient and differential centrifugation techniques based on that described by Pinches *et al.* (1993) and Wiener *et al.* (1989) with some modifications. All steps were carried out at 4°C. The mucosal scraping was weighed before being thawed in a solution which contained 20 mM Hepes/ Tris pH 8.3, 250 mM sucrose, 25 mM choline chloride, 1 mM dithiotheil and 0.2 mM phenylmethylsulphonylfluoride (1g scraping per 10 ml buffer). The scrapings were homogenised using Ystral polytron homogeniser (Ystral Scientific Instruments, UK) at setting 5 for 3 min. The homogenate was filtered through nylon gauze to remove fat and mucus, then centrifuged (Sorvall RC5, SS-34 rotor) rapidly at 500 x g for 15 min to obtain an initial pellet (P₁) and supernatant (S₁) (see figure II.1). S₁ was collected and saved on ice. P₁ was brought up to 30 ml using the same buffer and dounced 10 strokes using a hand held dounce homogeniser (Jencons Ltd, UK) and spun at 500 x g for 10 min (P₂, S₂) (see figure II.1). P₂ was discarded while S₁ and S₂ were combined and further centrifuged at 100 000g for 35 min (Beckman SW-36 rotor using a Beckman Ultracentrifuge) to produce the pellet (P₃). This fraction was suspended in buffer containing 250 mM sucrose and 20 mM Hepes/ Tris, pH 7.4 by 100 strokes using dounce tissue disperser with a tight fitting pestle (pestle A). Approximately 800 µl of the membrane suspension was applied directly on a continuous sucrose gradient [25-55% (w/v) sucrose in 10 mM Hepes/ Tris, pH 7.4]. The gradient was spun until the average g-force reached 100,000 x g for 1 hour (Beckman SW-36 rotor using a Beckman Ultracentrifuge). A discrete band near the top of the gradient was collected by Pasteur pipette and re-suspended in a buffer containing 300 mM mannitol, 0.1 mM MgSO₄ and 20 mM Hepes/ Tris, pH 7.4 followed by centrifugation at 100,000 x g for 45 min. The pellet was again carefully removed free of sucrose and re-suspended in 300 to 400 µl of suspension buffer by passing through a 27-gauge needle. Finally, the vesicles were aliquoted (50 µl) and stored in liquid nitrogen for later studies.

Colonic mucosal scrapings

Homogenate Suspend in buffer, homogenise using polytron homogenizer and filtere through nylon gauze.

Centrifuge 500xg for 15 min (SS-34, Sorvall).

Pellet (P_1)   Supernatant (S_1)

Re-suspend in 30 ml, homogenise 10 strokes and centrifuge 500xg for 10 min.

Supernatant (S_2)

Combine and centrifuge 100,000xg for 35 min.

Pellet (P_2)

Pellet (P_3)   Supernatant (S_3)

Re-suspend 250 mM sucrose and 20 mM Hepes/ Tris, pH 7.4

100 Strokes Dounce (pestle A)

Load onto 25-55% (w/v) sucrose gradient

Centrifuge 100,000xg for 60 min

Re-suspend in 300 mM Mannitol, 0.1 mM MgSO$_4$, 20 mM Hepes/ Tris, pH 7.4 and centrifuge 100,000xg for 45 min.

Pellet   Supernatant

Resuspend in suspension buffer

Figure.II.1 Flow diagram showing isolation of basolateral membrane vesicles (BLMV) from horse colon

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II.4 Protein estimation

The method described by Bradford, 1976 (ability of the protein to bind Coomassie Blue) was used to assess the membrane protein concentration. A commercially available kit (Bio-Rad Laboratories Ltd) was used. A standard calibration curve (0 - 150 µg) was prepared by diluting pig gamma globulin (1.5 mg.ml^{-1}) in a suspension buffer to a final volume of 100 µl. Samples (5 µl) were also diluted in suspension buffer (95 µl). The dye reagent was diluted 5-folds in dd-H$_2$O and then filtered through Whatman filter paper. In test tubes, 5 ml of the reagent was added to each sample. The tubes were vortexted and after 10 min the absorbance was read at A$_{595}$nm for both standards and membrane samples against the blank.

II.5 Enzyme assays

II.5.1 Cysteine-sensitive alkaline phosphatase

Reaction buffer:

- 34 mM glycine, pH 9.3

- 3.4 mM MgCl$_2$

- 0.34 mM ZnSO$_4$

- 5 mM *p*-nitrophenyl phosphate

Cysteine-sensitive alkaline phosphatase activity, a marker for the luminal plasma membrane from large intestine (Gustin and Goodman, 1981), was determined by the method of Brasitus and Keresztes (1984) using *p*-nitrophenyl phosphate (Sigma) as a substrate. In a test tube, 100 µg of protein from either homogenate or BLM fraction was incubated in 900 µl of reaction buffer with or without the addition of 10 mM L-cysteine. The tubes were incubated at 38°C for 30 min before the addition of 2 ml of 1 M NaOH to stop the reaction. The absorbance was read at A$_{410}$nm (E$_{mM}$ = 17.0). The activity was calculated as the difference in substrate hydrolysed in the presence and absence of L-cysteine and was

expressed as nmol product. (mg protein)$^{-1}$. min^{-1}. The appearance of p-nitrophenol was shown to be linear with time and protein concentration under the parameters of the assay.

II.5.2 α-Mannosidase

Reaction buffers:

- ❖ Citrate buffer:

    - 50 mM citric acid

    - 50 mM sodium citrate, pH 4.5

- ❖ Borate buffer:

    - 0.2 M boric acid

    - pH 9.8 with NaOH

The method of Tulsiani *et al.* (1976) was used to determine the activity of α-mannosidase activity, a marker of the Golgi apparatus. The activity was assayed by the measuring of the hydrolysis of p-nitrophenyl α-D-mannopyranoside (substrate). 100 μg of protein from either homogenate or BLM fraction was incubated in a 500 μl citrate buffer. The reaction was initiated by the addition of 500 μl of 10 mM substrate. After incubating for 30 min at 38°C, the reaction was stopped by the addition of 2 ml 0.2 M borate buffer. The release of p-nitrophenol was measured photometrically at A_{410nm} ($E_{mM} = 18.5$) against no protein blank buffer. Enzyme activity was expressed as nmol product. (mg protein)$^{-1}$. min^{-1}. The appearance of p-nitrophenol was shown to be linear with time and protein concentration under the parameters of the assay.

II.5.3 Succinate dehydrogenase

Reaction buffer (2X):

    - 100 mM Sodium succinate

    - 50 mM Sucrose

    - Potassium phosphate buffer (50 mM KH_2PO_4 and K_2HPO_4, pH 7.4)

Succinate dehydrogenase, a marker of the mitochondria, was measured according to the method of Pennington (1961). 100 µg of protein from either homogenate or BLM fraction was temperature equilibrated at 38°C in 750 µl reaction buffer diluted 1:1 in ddH$_2$O and containing p-iodotetrazolium violet at a concentration of 1 mg.ml^{-1}. The reaction was carried out in glass tubes for 30 min and then was terminated by the addition of 750 µl of 10% (w/v) trichloroacetic acid. 3 ml ethyl acetate was added to the reaction mix to extract the formazan dye-product. The tube content was mixed and allowed to separate. The upper layer was measured photometrically at A$_{490nm}$ (E$_{mM}$ = 20.1) against no protein blank.

II.5.4 Tris-resistant α-glucosidase

Reaction buffers

- ❖ Tris buffer:
    - 35 mM Tris/ HCl, pH 8.0
    - 55 mM Glutathione

- ❖ Substrate buffer:
    - 0.21 mM p-nitrophenyl α-D-glucoside
    - 0.1 mM Na$_2$HPO$_4$, pH 8.0
    - 0.1% (w/v) Triton X-100

Tris-resistant α-glucosidase activity, a marker of the endoplasmic reticulum, was determined as described earlier by Peters (1976). 100 µl of protein from either homogenate or BLM fraction was incubated in 50 µl of Tris buffer for 15 min on ice. After addition of 250 µl of substrate buffer, the reaction was conducted at 38°C for 20 min. The absorbance was read at A$_{410nm}$ (E$_{mM}$ = 18.5) against a buffer blank. Specific activity was expressed as nmol. (mg protein)$^{-1}$. min^{-1}. The appearance of p-nitrophenol was shown to be linear with time and protein concentration under the parameters of the assay.

II.5.5 Na$^+$/K$^+$/ATPase assay

❖ Assay mix:

- 120 mM NaCl

- 4 mM Na$_2$ATP

- 4 mM MgCl$_2$

- 60 mM Tris/ HCl, pH 7.5

- 1 mM EDTA

❖ Developing solution (to be prepared fresh)

- 1.15 M H$_2$SO$_4$

- 1 % (w/v) Ammonium molybdate

- 4 % (w/v) Ferric sulphate

The activity of the Na$^+$-K$^+$-ATPase, a marker for the basolateral plasma membranes, was determined as described by Forbush (1983) and Rajendran *et al.* (2000). Prior to the assay basolateral membrane vesicles were permeabilised using saponin at a concentration of 0.010 (w/v). A range from 0-720 µM of standard calibration curve for K$_2$HPO$_4$ was made in a final volume of 0.5 ml ddH$_2$O.

A sample of 100 µg of protein from either homogenate or BLM fraction was set to a final volume of 100 µl and temperature equilibrated at 38°C. The reaction was started by the addition of 400 µl of the assay mix in the presence or absence (control) of ouabain (5 mg. ml^{-1}). After 10 min, 1 ml of developing solution was added to terminate the reaction, which was left up to 1 hour for full colour to develop. The absorbance was measured at A$_{690nm}$ using a spectrophotometer. Na$^+$-K$^+$-ATPase activity was determined from the difference between the P_i released in control tubes and that released in tubes containing ouabain. Specific activity was expressed as nmol. (mg protein)$^{-1}$. min^{-1}.

II.6 SDS-PAGE and western blotting

II.6.1 SDS-PAGE

a) Preparation of the sample

Denaturing sample buffer:

- 62.5 mM Tris/ HCl, pH 6.8

- 0.1 % (w/v) glycerol

- 2 % (w/v) SDS

- 0.05 % (w/v) β-mercaptoethanol

- 0.00125 % (w/v) Bromophenol bleue

In a 0.5 ml Eppendorf, 20-25 μg protein (100 μg protein in AE2 case) was diluted 1:4 in denaturing sample buffer and heated for 3 min at 65°C. The sample/ sample buffer were centrifuged. High molecular weight markers (Sigma) were treated as aforementioned and loaded onto gel alongside the samples.

b) Preparation of the gel

Running gel 8%:

- 2.5 ml of running gel buffer (1.5 M Tris/ HCl, pH 8.8)

- 2.65 ml of 30% (w/v) Acrylamide

- 50 μl of 20% (w/v) SDS

- 50 μl of 20% (w/v) APS

- 5 μl TEMED

- 5.4 ml ddH$_2$O

Stacking gel 4%:

- 2.5 ml of stacking gel buffer (0.5 M Tris/ HCl, pH 6.8)

- 1.3 ml of 30% (w/v) Acrylamide

- 50 µl of 20% (w/v) SDS

- 50 µl of 20% (w/v) APS

- 10 µl TEMED

- 6.4 ml ddH$_2$O

c) Setting the gel

Prior to the experiment, the glass plates were cleaned using propanol and separated with 1mm spacers. Preparation of the SDS-PAGE was performed as previously described by Laemmli (1970) using a vertical mini protean II gel apparatus (Bio-Rad laboratories Ltd, UK) according to the manufacturer's recommendations.

After assembly of the apparatus, the 8% gel was poured between the glass plates using Pasteur pipette and leaving enough space for the comb. The gel was covered with a thin layer of butanol-saturated ddH$_2$O, to produce a level surface of the gel, and allowed to polymerise. Afterwards, the butanol-saturated ddH$_2$O was tipped off and the gel was dried using Whatman paper. The comb was inserted into the top of the stacking gel, and was allowed to polymerise.

d) Loading and running the gel

TANK Buffer:

- 0.3% (w/v) Trizma-base (Sigma)

- 1.44% (w/v) glycine

- 0.1% (w/v) SDS

After polymerisation, the gel was placed in a buffer chamber and left to equilibrate in Tank buffer for 10 min. The samples were loaded in wells and the gel electrophoresed at 22mA (11 mA for one gel) using Bio-Rad power pack (model 1000/ 500). The gel was run until the bromophenol blue dye reached the bottom of the gel.

II.6.2 Electrotransfer

Transfer buffer:

- 20% (v/v) Methanol

- 20 mM Trizma-base

- 150 mM Glycine

1) A PVDF membrane was cut to same size as the gel and soaked in methanol for 30 seconds and then equilibrated in transfer buffer together with two pieces of 3mm filter paper (size of the gel) (Whatman, UK).

2) The polyacrylamide gel was carefully removed from the glass plates. The stacking gel was discarded and the running gel was soaked in transfer buffer.

3) The gel and the membranes were assembled in air bubble-free transfer cassette as follow: fibre pad, 3mm filter paper, gel, PVDF membrane, 3mm filter paper, fibre pad.

4) The cassette alongside with a block of ice were placed in the buffer chamber and submerged with transfer buffer.

5) A stirring rod was put in the buffer chamber, which was placed on a magnetic stirrer (both the ice and the stirring cool down the heat generated during the transfer process).

6) The transfer was performed at 100 volts for 1 hour.

II.6.3 Staining with Ponceau red

The PVDF membranes were removed from the cassette and stained using Ponceau red S (1% (w/v) in 3% (w/v) trichloroacetic acid) to visualise protein bands. The membranes were immerged in the stain for 2 min then rinsed with ddH$_2$O. Usually, molecular weight marker was cut off and kept for later reference. The membrane was further rinsed with ddH$_2$O to remove the stain, wrapped in cling film and stored at -20°C until use.

II.6.4 Immuno-detection

a) Villin

Prior to any immuno-blotting PVDF membranes were removed from the freezer and emerged in ethanol for few seconds, and then they were washed thoroughly with distilled water. All incubations and washes were performed on a rocking platform.

The PVDF membranes were incubated in PBS solution containing 5% (w/v) non-fat dried milk (Oxoid, UK), 0.1 mM EDTA and 0.5% (v/v) Triton X-100. Incubation was carried out for 1 hour at room temperature. Membranes were then incubated with the primary antibody diluted 1:1000 in a milk-free blocking solution at room temperature for 1 hour. After being washed 3 x 10 min in washing solution consisting of PBS, 1% (w/v) non-fat dried milk, 0.1 mM EDTA and 0.5% (v/v) Triton X-100, the membrane were incubated with the goat anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO, UK) diluted 1:2000 in milk-free blocking solution for 1 hour. The membranes were washed 3 times for 10 minutes each with 10 ml washing solution. Immunoreactive bands were visualised as described in section II.6.5.

b) Na$^+$/K$^+$/ATPase

The membranes were blocked with 5% (w/v) non-fat dried milk in PBS containing 0.1 mM EDTA and 0.5% (v/v) Triton X-100 and then incubated for 2 hours with the Na$^+$/K$^+$/ATPase polyclonal antibody diluted 1:1000 in PBS-ET. Sheets were washed as

described before. The secondary antibody was a swine anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:2000 in PBS-ET. The site of antigen-antibody cross-reaction was visualised as described in section II.6.5.

c) Monocarboxylate transporter isoform 1 MCT1

Expression of MCT1 in equine colon was accomplished by determining the purity of the basolateral membrane vesicles (BLMV). Non-specific protein binding sites on the PVDF membranes were blocked by incubation in PBS-TM (5% (w/v) non-fat dried milk, 0.05% (v/v) Tween 20 in PBS) for 1 hour at room temperature with the appropriate primary antibody. The membranes were washed 3 x 10 min in washing solution (PBS, 0.5% (w/v) non-fat dried milk and 0.05% (v/v) Tween 20). Swine anti-rabbit peroxidase-linked secondary antibody (DAKO, UK) was diluted 1:2000 in washing solution and incubated for 1 hour. Sheets were rinsed 3 x 10 min in 10 ml washing solution. Cross-reactions were visualised as described in section II.6.5.

d) Glucose transporter isoform 2 (GLUT2)

To determine the abundance of GLUT2, a marker of the colonic basolateral membranes (Thorens, 1996; Brown, 2000) in the equine BLM fraction, non-specific protein binding sites on the PVDF membranes were blocked by incubation in TTBS containing 5% (w/v) non-fat dried milk. Primary antibody was used at the dilution 1:500 in TTBS. Following 3 x 10 min washes in TTBS, the membranes were incubated in secondary antibody coupled to horseradish peroxidase at a concentration of 1:2000 for 1 hour at room temperature. Subsequently, 3 x 10 min washes were completed in 10 ml TTBS and the immunoreactive bands were visualised as described in section II.6.5.

e) Anion exchanger isoform 2 (AE2)

For the immunodetection of AE2 protein, samples were loaded at 100 µg protein per lane onto SDS-polyacrylamide 4-8% gels and electroblotted into PVDF membrane. The membrane was blocked for 1 hour at room temperature with TTBS (0.05% Tween 20, tris-buffered saline pH 7.4) containing 5% (w/v) non fat dried milk. Immunoblot was incubated

with a 1:1000 dilution of AE2 antibody for 2 hours followed by incubation in a 1:2000 dilution peroxidase conjugated swine anti-rabbit secondary antibody for 1 hour. Washes between and after incubations were done in a TTBS solution containing 1% non fat dried milk. Detection of antibody binding was visualised as described in section II.6.5.

II.6.5 Development of the blots

An enhanced chemiluminescence kit (ECL; Amersham, UK) was used to visualise immunoreactive bands. The PVDF membranes were placed in a mixture made of 2 ml solution 1 (Luminol, a cyclic diacylhydrazide) and 2 ml solution 2 (hydrogen peroxide) for 1 minute at room temperature. The membranes were blotted with paper tissue to remove excess solution, and then placed in saran wrap before exposure to photographic film (Biomax-ML; Kodak, UK) in dark room. Exposure time varied from a few seconds to a few minutes according to the strength of the signal. The film was manually developed using commercial developing (Kodak GBX developing solution), stopping and fixing solutions. The film was then washed several times with water and left to dry in the oven.

II.6.6 Striping and re-probing of the membranes

The method used for membrane stripping was similar to that described by Dyer *et al.* (2002). PVDF membranes used for western blotting were reprobed with other antibodies. To do so, membranes were washed three times, 10 minutes each in stripping buffer containing 137 mM NaCl and 20 mM glycine, pH 2.5 with HCl at room temperature. The membranes were rinsed for 10 minutes in large volumes of PBS and then blocked using relevant blocking solution. Immunoblotting was proceeded as aforementioned.

II.7 Transport studies

II.7.1 Glucose uptake into BLMV

- Uptake Buffer:

    - 300 mM Mannitol

    - 20 mM Hepes/ Tris, pH 7.4

    - 0.1 mM $MgSO_4$

    - 1 mM D-[U-^{14}C]-glucose

- Stop Solution:

    - 300 mM Mannitol

    - 20 mM Hepes/ Tris, pH 7.4

    - 0.1 mM $MgSO_4$

The method of Shirazi-Beechey *et al.* (1988) was employed to study the transport of [U-^{14}C]-α-D-glucose. The method was based on the rapid filtration stop technique.

Prior to the experiment, BLM vesicles were thawed and aliquoted into a test tube already maintained on ice (100 µg protein per tube). The uptake was initiated by the addition of 100 µl of incubation buffer to the test tube. Incubation was carried out at 38°C for appropriate lengths of time (3 sec- 30 min) and stopped by the addition of 1 ml ice cold stop solution. 900 µl was taken from the reaction mixture and filtered, under vacuum, through a 22 µm-pore cellulose acetate/ nitrate filter (GSTF02500; Millipore, UK) pre-wetted with ddH$_2$O. After washing 5 times with 1 ml ice cold stop solution, the filter paper was placed in scintillation vials and 4 ml of scintillation liquid (Optiphase; Perkin Elmer, UK) was added. Radioactivity was counted using scintillation counter (LS6500 Beckam Coulter, USA).

To correct for the non-specific binding of the radiolabelled substrate to the filter and/ or membrane vesicles, radioactivity present in zero time vesicles blank was subtracted (uptake

at zero time was measured by adding the ice cold stop solution to the vesicles before adding the incubation buffer).

50 µl of unfiltered-stopped reaction mixture was randomly selected and taken for scintillation counting. Glucose uptake was expressed in pmoles. (mg protein)$^{-1}$. min^{-1}.

Inhibition of D-glucose uptake was determined by addition of 50 µM cytochalasin-B into uptake buffer as described by Pinches *et al.* (1993).

II.7.2 Butyrate uptake into BLMV

a) Time course

Similar principle described in section II.7.1 was applied for butyrate uptake. 100 µg of the membrane vesicles protein preloaded with appropriate buffers was diluted in 100 µl of incubation buffer containing 150 mM K-gluconate, 20 mM Mes/ tris, pH 5.5 and 1 mM [U-^{14}C]-butyrate. Stop solution consisted of 100 mM mannitol, 100 mM K-gluconate and 20 mM Hepes/ tris, pH 7.4.

b) Effect of pH

Uptake buffers of varying pH values were used. For pH values of 5.5; 6.0 and 6.5, uptake buffer consisted of 150 mM K-gluconate, 20 mM Mes/ tris and 1 mM [U-^{14}C]-butyrate. For pH values of 7.0; 7.5 and 8.0, uptake buffer consisted of 150 mM K-gluconate, 20 mM Hepes/ tris and 1 mM [U-^{14}C]-butyrate. All steps were carried out as described before.

c) Kinetics

The uptake buffers of varying sodium-butyrate (NaBt) concentrations ranging from 1 mM to 50 mM were used. Osmolarity was maintained by adjusting the K-gluconate

concentration. A Michaelis-Menten curve was drawn and values for K_m and V_{max} were calculated by linear regression analysis of Hanes plot.

d) Substrate specificity

100 µg of BLM vesicles were incubated in the uptake buffer containing 10 mM potassium salts of indicated organic anions. All steps were carried out as described before and stopped using ice-cold stop solution. The radioactivity was then measured using a scintillation counter.

e) Valinomycin-induced membrane potential

The initial rate of $[U-^{14}C]$-butyrate uptake into equine BLMV was measured to examine the effect of K^+/ valinomycin-induced membrane potential. To accomplish the experiment, 100 µg of BLM vesicles protein was pre-incubated with the potassium ionophore (Valinomycin) at the concentration of 10 µg.mg^{-1} protein for 30 min on ice. The reaction was started by the addition of 100 µl uptake buffer and stopped by adding ice-cold stop solution as described before.

f) Inhibition

Prior to the assay, BLM vesicles were pre-incubated with specific inhibitors for 30 minutes on ice. The inhibitors and their concentrations are indicated in the figure legends of the result section. The uptake was started by the addition of 100 µl uptake buffer and carried out as described before.

g) Chloride and $[^{14}C]$-butyrate uptake

BLMV preloaded 150 mM KHCO$_3$ and 20 mM Hepes/tris, pH 7.4 were incubated in medium containing 20 mM Mes/ tris (pH 5.5) and either 130 mM of K-gluconate, 20 mM KCl or 150 mM of K-gluconate.

II.8 Preparation of total RNA

II.8.1 RNeasy kit

Total RNA was isolated from the horse colon (30 mg of mucosal scraping) using the RNeasy kit (Qiagen, UK) according to the manufacturer's instructions. Briefly, frozen tissue was pulverised in liquid nitrogen and immediately 30 mg of disrupted tissue was transferred into a clean centrifuge tube. After adding 600 µl of lysis buffer RTL, the homogenisation step was carried out using a polytron (Ystral Scientific Instruments, UK) at setting 6 for 2 minutes. The tissue lysate was centrifuged for 3 min at maximum speed using bench top centrifuge (Eppendorf, UK) then the supernatant was carefully transferred to a new centrifuge tube. 600 µl of 70% ethanol was added to the lysate and gently mixed by pipetting. The sample was applied to an RNeasy mini column (already placed in a 2 ml collection tube) and the tube was centrifuged for 15 seconds at 8000g, then the flow through was discarded. 700 µl of buffer RW1 was added to the tube, which was centrifuged for 15 sec at 8000g, and the flow through was discarded. The washing step was performed by transferring the RNeasy mini column to a new 2 ml collection tube and adding 500 µl of buffer RPE then centrifuged at 8000g for 2 min. Second wash was completed as previously stated. Finally, the elution was done using 40 µl of RNase-free water and centrifuged for 1 min at 8000g. RNA was stored at -70°C.

II.8.2 Quantification of RNA and DNA

The nucleic acids were quantified using a spectrophotometer (U-2000 Hitachi; Berks, UK). The principle is to measure the optical density (O.D.) at absorbance A260$_{nm}$. One O.D. unit is equivalent to 50 ng.ml^{-1} and 40 ng.ml^{-1} for double stranded DNA and single stranded RNA respectively. Briefly, 69 µl of ddH$_2$O was placed in a clean quartz cuvette, which was placed in the spectrophotometer, and the non-specific reading was auto-zeroed. Then 1 µl of cDNA/ RNA was added into the cuvette and mixed by pipetting. Finally the reading was taken.

cDNA and RNA concentration was measured as follow:

$$[cDNA]_{\mu g/\mu l} = \frac{50 \times A260 \times Dilution}{1000}$$

$$[RNA]_{\mu g/\mu l} = \frac{40 \times A260 \times Dilution}{1000}$$

A260/280 nm ration of 1.8-2.0 is a good indicator of low protein contaminated-pure cDNA or RNA preparation.

II.9 Northern blotting analysis

II.9.1 Preparation of the cDNA probe

The appropriate cDNA template was labelled by the use of HEXALABEL DNA labelling kit (Pharmacia, UK) according to the manufacturer's instructions and as previously described by Sambrook *et al.* (1989). Briefly, in a clean 1.5 ml eppendorf tube, 50 ng of purified DNA was mixed with 5 µl hexanucleotide in 5 x reaction buffer and made up to 20 µl total volume with deionised water. The tube was vortexed and quickly spin down in a microcentrifuge. The cDNA was denatured at 95^0C for 2 min and immediately placed on ice. After a brief centrifugation, the following components were added to the tube: 1.5 µl Mix C, 2.5 µl [α-^{32}P]-dCTP (final activity 25 µCi) and 0.5 µl Klenow fragment. The eppendorf tube was shaken, briefly spun down then incubated at 37°C for 30 min. 2 µl of dNTP mix was added to the tube and followed by a 5 min incubation at 37°C. In-incorporated nucleotides were removed using NICK column (Pharmacia, UK) as described by the manufacturer.

Briefly, excess liquid of the column was poured off then the column was rinsed twice with TEN buffer (50 mM tris/ HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). The radiolabelled cDNA was added onto the column followed by the first elution (400 μl TEN) which was discarded. The second elution (containing labelled cDNA) was preserved in a clean capped tube.

II.9.2 Preparation of the gel

The RNA samples were separated on 1% (w/v) gel by denaturing agarose gel electrophoresis. 0.4 g of agarose was melted in 34 ml of ddH$_2$O and boiled in the microwave. The gel was allowed to cool to 50°C before adding 4 ml of 10 x MOPS (0.4 M MOPS, 100 mM sodium acetate, 10 mM EDTA, pH 7.0 with NaOH) and 2.16 ml of 40% (w/v) formaldehyde. An 8-well comb was inserted and the gel was poured into a horizontal casting tray (Hoefer, UK) in a fume hood. When the gel was set, it was transferred into electrophoresis rig and equilibrated using 1 x MOPS running buffer.

The volume required for RNA to be loaded was determined and mixed with an equal volume of denaturing solution (100 μl ddH$_2$O, 30 μl of 40% (w/v) formaldehyde and 10 μl of 10x MOPS). The RNA mixture was heated at 55°C for 15 min then cooled on ice for 2 min. the RNA sample dye was prepared by mixing RNA dye solution (50% (v/v) glycerol, 1 mM EDTA, 0.4 % (w/v) bromophenol blue, 0.4 % (w/v) xylene cyanol) with ethidium bromide (5 mg/ml) at the ratio 1:1. 10 % (v/v) RNA sample dye was added to the RNA sample prior to loading onto the gel. The RNA samples were loaded alongside RNA marker (Promega) treated similarly to the samples described above. The gel was electrophoresed at 80 mV until the dye front reached the end of the gel.

II.9.3 transfer of the RNA

The RNA was transferred onto a nylon membrane (Duralo-UV, Strategene, UK) under high salt concentration. A clean sponge was placed in a pool of transfer buffer and soaked in 10 x SSC (3 M NaCl, 0.3 M sodium acetate, pH 7.0). A sheet of Whatman 3 mm

filter paper (size of the sponge) was placed on the sponge. Subsequently, the gel was centred above the filter paper. Four strips of plastic wrap were placed under the gel so that the exposed area is just smaller than the gel to avoid wicking. The appropriate nylon membrane (size of the gel) was cut and placed onto the gel avoiding air bubbles. A further 3 pieces of Whatman 3 mm paper filter were placed on the nylon membrane followed by a stack of dry paper towels. A glass plate was placed on the paper towels and a weight was added to hold everything in place. RNA transfer was proceeded overnight. The nylon membrane was uncovered and placed on a dry sheet of Whatman 3 mm filter paper. The RNA was fixed to the nylon membrane by UV cross-linking (UV-Stratalinker-2400, Strategene, UK). Equal loading of RNA was confirmed by soaking the nylon membrane in a methylene blue-based dye (0.02% (w/v) methylene blue, 0.3 M sodium acetate, pH 5.5) as previously described by Herrin and Schmidt (1988). The membrane was distained by a solution containing 0.2 x SSC and 1% (w/v) SDS for 30 min using rocking platform.

II.9.4 Hybridisation of the RNA

Hybridisation of the nylon membrane was carried out in the hybridisation tube using a rotating hybridisation oven (mini 10, Hybaid, UK). A nylon membrane was placed in the tube then 5 ml of hybridisation solution (5 x SSC, 3x Denhardt's solution, 25 mM Mes pH 6.5, 0.2 % (w/v) SDS, 10 % (w/v) dextran sulphate, 2.5 mM sodium pyrophosphate pH 6.5, 0.01% (v/v) antifoam B (Sigma, UK), 40-50% (v/v) formamide) was added to the tube which was incubated for 2 hours at 42°C. The desired volume of radiolabelled cDNA probe was pipetted into the hybridisation tube and the incubation continued with rotation overnight at 42°C.

II.9.5 Post- hybridisation washes

The hybridisation solution containing radiolabelled probe was disposed and the membrane was washed twice; first wash with 5 x SSC containing 5% (w/v) SDS and 0.25% (w/v) sarkosyl at 42°C for 15 min, the second wash with 0.1 x SSC containing 0.1% (w/v) SDS for 2 x 15 min at 55°C. Excess liquid was blotted by placing the membrane on a sheet of

Whatman 3 mm filter paper and then wrapped in Saran wrap. The membrane was marked with Trakertape (Amersham Ltd, UK) and subjected to autography (section II.9.6).

II.9.6 Autoradiography

The wrapped membrane was placed in an X-ray cassette (Sigma, UK) and exposed to the photographic film of Kodak BioMax MS-1 (Anachem, UK) with film on top. The intensifying screens (Anachem, UK) were placed over the film to enhance the intensity of the signal obtained. The cassette was stored at -80°C for an appropriate length of time before the film was developed as described in section II.6.5.

II.9.7 Striping and re-probing of nylon membrane

Nylon membranes used for northern blotting were re-analysed. To do so the original hybridisation probe was removed by washing in a boiling striping solution containing 0.1% (v/v) SDS and 0.1 x SSC 3 times 5 min each. The membrane was re-probed as described in section II.9.4.

II.10 PCR and RT-PCR

II.10.1 First strand cDNA synthesis

The RNA was reverse transcribed to complementary DNA (cDNA) using the Suprescript II RT protocol (Strategene, UK) according to the manufacturer's instructions. In a 0.5 ml microfuge tube, 0.5 µl of 500 ng/ ml random primers (Promega, UK) was mixed with 5 µg RNA and made up to 11.4 µl total volume with ddH$_2$O. The tube's content was mixed and placed in a heating block at 70°C for 10 min then transferred immediately onto ice for 1 min. To the tube, 4 µl of 5 x first strand reaction buffer, 2 µl of 0.1 mM DTT, 1 µl of 40

mM dNTP mix and 0.6 µl of 40 U/µl RNasin (Promega) were added. The tube content was mixed and incubated for 10 min at 25°C then for 2 min at 42°C. After the addition of 1 µl of Supersrcipt II, the tube was heated at 42°C for 60 min then brought up to 70°C for 15 min followed by incubation at 37°C for 2 min. Finally, 2 µl of 1 U/ µl RNase H was added and the tube was incubated at 37°C for 20 min.

Later, purification steps were performed using a Qiaquick spin column (Qiagen, UK).

II.10.2 Purification of first strand cDNA

First strand cDNA product was purified from residual primers, nucleotides, polymerases and salts using QIAquick PCR purification kit (Qiagen, UK) according to the manufacturer's instructions. Briefly, 5 volumes of buffer BP were added to 1 volume of PCR sample. The sample was applied onto QIAquick column and centrifuged for 1 minute at 10,000g. The flow through was discarded and a washing step was performed with 750 µl buffer PE followed by centrifugation at 10.000g for 1 min. The flow through was discarded again and an additional centrifugation was done at 10,000g for 1 min. Finally, the cDNA was eluted with 50 µl buffer EB (10mM Tris/HCl, pH 8.5) and stored at -20°C for latter use.

II.10.3 High fidelity PCR

All steps were carried out in a PCR workstation. Prior to the experiment, the ultra violet light was lit to inactivate any primers or RNA that may contaminate the reaction.

Amplification of cDNA was undertaken in an automated thermal cycler (GeneAmp PCR system 2400, Applied Biosystem, UK) and using *Pfu* DNA polymerase (Promega, UK). In a 0.5 ml microfuge tube, the following components were mixed in a 50 µl reaction: 5 µl of 10x reaction buffer, 5 µl of 8 mM dNTP mix, 10 mM forward primer, 10 mM reverse primer, 2.5U/µl *Pfu* DNA polymerase and 1 µg cDNA. Water was added to give a final volume of 50µl.

The amplification programme was started by 5 min at 94°C to activate the *Pfu*. Then followed by 30 cycles of: 20 sec denaturing step at 94°C, 20 sec annealing step at 50°C and 1 min extension step at 72°C. This was followed by a final extension phase at 72°C for 10 min. The temperature was then reduced to 4°C and the resulting cDNA amplicons were analysed by agarose gel electrophoresis as described in section II.10.4.

Reaction mix for standard PCR:

	Vol (µl)
10 x reaction buffer	5
8 mM dNTP mix	5
10 µM forward primer	1
10 µM reverse primer	1
Pfu (2.5U/µl)	1
DNA template	x (1µg)
ddH$_2$O	37-x
Total	50

Programme for standard PCR:

94°C for 15 min

94°C for 20 sec (denaturing)

50°C for 20 sec (annealing) 30 cycles

72°C for 1 min (extension)

72°C for 10 min (final extension)

II.10.4 Agarose gel electrophoresis of DNA

DNA samples were electrophoresed on 1% (w/v) agarose. 0.4 g agarose was dissolved in 40 ml 1 x TTE buffer (3 mM tris, 3 mM tris, 0.1 mM EDTA) by heating for 3 min in a microwave. Ethidium bromide was added to a final concentration of 0.1% (v/v). The gel mix was poured onto a flat casting rig (Hoefer Scientific Instruments, Staffs, UK) and allowed to set. DNA samples were mixed with 1% (v/v) DNA loading buffer (Promega , UK) and electrophoresed alongside DNA ladder (Promega, UK) in 1 x TTE at 100 volts. A UV illumination was used to visualise the DNA bands and photographs were taken with a Polaroid camera.

II.10.5 Extraction of DNA bands

DNA bands were purified from agarose gel using QIAquick gel extraction kit (Qiagen, UK) following the manufacturer's recommendations. Briefly, DNA bands were excised from agarose gel using a sharp scalpel under UV light and transferred to a clean 1.5 ml eppendorf tube. 3 volumes of buffer QG was added to the gel which was incubated at 50^0C for 10 min. After the gel was completely dissolved, 1 volume of isopropanol was added to the sample. The tube content was applied to a QIAquick spin column and centrifuged for 1 min at 13,000g. A washing step was performed followed by elution of the DNA with 50 µl of 10 mM tris/ HCl, pH 8.5. The DNA concentration was measured by agarose gel electrophoresis.

II.10.6 Rapid amplification of cDNA ends (RACE)

a) 5' RACE

The 5' RACE was performed to get the 5' end of the anion exchanger 2 (AE2) transcript of the horse colon. RACE cDNA amplification kit (RACE-READY cDNA kit, Invitrogen) was used. Manufacturer's instructions were followed to perform the procedure. Briefly, first

strand cDNA was synthesised using Superscript II reverse transcriptase (Strategene, UK), 5 μg of horse colon RNA and AE2 antisense-specific primer (GSP1A) 5′ - TGTAGGGGGAGATGGGCTGGT-3′. Following cDNA synthesis, purification phase was carried out using QIAquick kit (QIAGEN, UK). Homopolymeric tail was added to the 3′ end of the cDNA with the use of terminal deoxnucleotidyl transferase (TdT). The cDNA was then amplified by PCR using AE2 specific primer (GSP2A) 5′-ACGTCCTCAAACCGGTGACT-3′ and the kit-supplied anchor primer 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′. The PCR cycling programme was: 94°C, 5 sec followed by 94°C, 5 sec; 55°C, 20 sec; 72°C, 60 sec for 25 cycles. The products were analysed by agarose gel electrophoresis (section II.10.4), gel purified (section II.10.5), cloned into pGEM-T vector (section II.11) and sequenced commercially.

b) 3′ RACE

The 3′ RACE was performed to capture the 3′ end of the equine AE2 transcript. In this procedure 5 μg of equine colonic AE2 RNA and 20 pmol of oligo(dT)-containing adaptor primer 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTT-3′ were mixed and heated for 10 min at 70°C. The mixture was immediately snap-chilled on ice for 1 min. The following components were added to the tube: 2 μl of 0.1 mM DDT, 1 μl of 10 mM each dNTP (dGTP, dCTP, dATP, dTTP), 24 units RNasin and 200 units superscript II. Incubation was performed for 1 hour at 42°C to reverse-transcribe the mRNA. The superscript II was inactivated and the template RNA was degraded by an incubation at 70°C for 15 min and addition of RNase H respectively. The cDNA was amplified by PCR using an abridged universal amplification primer (AUAP) 5′-GGCCACGCGTCGACTAGTAC-3′, AE2 sense-specific primer (GSP) 5′-ATCACCACGCTGATCATCTCC-3′ and Pfu DNA polymerase. The cycling parameters were: 94°C, 5 sec followed by 94°C, 20 sec; 55°C, 20 sec; 72°C, 60 sec for 25 cycles. The products were analysed by agarose gel electrophoresis (section II.10.4), gel purified (section II.10.5), cloned into pGEM-T vector (section II.11) and sequenced commercially.

II.11 Cloning and sequencing

II.11.1 Preparation of the competent cells

a) Preparation of LB plates

LB plates were made by adding 15 g.l^{-1} agar to one litre of LB medium (10 g.l^{-1} bactotryptone, 5 g.l^{-1} bacto-yeast extract and 5 g.l^{-1} sodium chloride). The mixture was autoclaved and allowed to cool to 50°C before adding the ampicillin to a final concentration of 100 μg.ml^{-1}. 30 – 35 ml of medium was poured into 85 mm Petri dishes and let to solidify.

b) Ligation of the cDNA into bacterial plasmid

Before the ligation of the cDNA took place, the concentration of the insert was optimised using an insert: vector molar ratio of 3:1. The following formula was applied:

$$\frac{ng \ of \ Vector \ \times \ Kb \ size \ of \ insert}{Kb \ size \ of \ the \ vector} \times \frac{3}{1} \equiv ng \ insert$$

The ligation was set up by mixing the following components in a 0.5 ml microfuge tube: 2 x ligation buffer, 50 ng pGEM-T vector, the appropriate volume of insert cDNA and 3 units of T$_4$ DNA polymerase. The incubation was carried out overnight at 4°C.

II.11.2 Transformation of the competent cells

Competent E. coli JM109 cells (Promega, UK) were thawed on ice bath before being used. The tube containing the ligation reaction was centrifuged, and then 2 µl of each ligation reaction was collected from the bottom of the tube and placed in a sterile 1.5 ml microfuge tube. Carefully 50 µl of JM109 cells was transferred into the tube which was gently flicked and incubated on ice for 20 minutes. The tube was subjected to heat shock treatment at 42°C for 75 seconds followed by 4 minutes incubation on ice. 950 µl of SOC medium (2.0 g.l^{-1} bacto-tryptone, 0.5 g.l^{-1} bacto-yeast extract, 1 ml of 1M NaCl, 0.25 ml of 1M KCl, 1 ml of 2M Mg^{+2} and 1 ml of 2M glucose) was added to the cells and incubated under slow shaking at 37°C for 1.5 hour.

To select for plasmid-vector containing the Lac Z gene (coding for β-galactosidase), the plates were pre-spread with 100 µl of 100 mM isopropyl-β-d-thiogalacto-pyranoside (IPTG) and 50 µl of 50 mg.ml^{-1} X-Gal. IPTG is a non metabolised inducer of the Lac operon and is needed to switch on the expression of Lac Z. 100 µl of transformed cells were plated out and incubated overnight at 37°C. Blue/ white screening was an indicator of the transformation efficiency. Recombinant cells were acknowledged by their white colour whereas non-recombinant cells were blue.

II.11.3 PCR-based colony screening

After an overnight incubation many white colonies were picked from the plates using clean pipette tips and resuspended in 20 µl LB media in microfuge tubes. The tubes were incubated for 2 hours at 37°C. A PCR-based strategy was then employed to identify the presence of the relevant cDNA product in the selected colonies. The same sense and antisense primers used for high fidelity PCR (section II.10.3) were utilised in a 50 µl reaction mix containing: 1 x reaction buffer, 800 µM dNTPs, 1 µM synthetic primers, 5 µl colony mix and 1 unit of *Pfu* DNA polymerase (Promega, UK). The PCR programme was started by heating the samples at 94°C for 5 min before amplification step. The following temperature cycle was applied: 94°C, 20 sec (denaturing); 50°C, 20 sec (annealing) and 72°C, 60 sec (extension) for 25 cycles. A final extension step was performed at 72°C for 10 min. The

product was subjected to agarose gel electrophoresis analysis (section II.10.4). The presence of band with size corresponding to the cDNA of interest indicated successful transformation.

II.11.4 plasmids miniprep

Colonies identified as having been successfully transformed were incubated overnight at 37°C in 5 ml LB media containing 100 μg.ml^{-1} ampicillin (Sigma, UK). This step was performed to yield large amounts of cDNA. The bacterial cells were centrifuged at 3000 x g for 10 min (Sorvall, HS3000 rotor). The resulting pellet was further processed to isolate the plasmid using the commercially available QIAprep spin miniprep kit (Qiagen, UK). Briefly, the bacterial cells containing plasmid-DNA were pelleted and lysed under alkaline condition by the addition of buffer P_1 containing RNase A and buffers P_2 and N_3. The mixture was centrifuged at 10,000 x g for 10 min and the supernatant was applied on a QIAprep spin column. This procedure allowed the plasmid to be retained on the silica-gel membrane. Washing steps were performed with the use of buffers PB and PE followed by elution of the product using buffer EB (10 mM Tris/ HCl, pH 8.5).

RESULTS

CHAPTER III

Plasma membrane vesicles: Origin and Purity

Membrane vesicles: Origin and Purity

III.1 Introduction

Studying the transport mechanism of the short chain fatty acids (SCFA) across the equine colonic basolateral plasma membrane was one of the major goals of this study. This work was approached by primarily isolating membrane vesicles from the basolateral pole of equine large intestinal cells using differential centrifugation and sucrose density gradient techniques. Membrane vesicles provide an excellent model to study membrane phenomena especially solute transport at the luminal and anti-luminal membrane surfaces.

Such studies depend on the isolation procedure, which determine the purity and origin of the vesicles. In the present investigation, basolateral membrane vesicles were isolated from equine colonocytes, then they were characterised in term of purity and the presence of any potential contamination by other organelle and luminal membranes. The isolation procedure relied on the methods described by Wiener *et al.* (1989) and Pinches *et al.* (1993) with introduction of some modifications. The vesicles were subsequently used to elucidate the mechanism of butyrate transport. Even though it was easy to obtain and in abundance horse colonic tissue from the local abattoir, the purification method of BLMV faced some difficulties, taking in consideration the huge amount of mucus present in the colon and the time consuming of the technique. Therefore a more rapid and reproducible method was developed for the isolation of basolateral membranes from horse colon. Previous studies carried out in our laboratory successfully isolated BLMV from human and pig colon to study the mechanism of glucose transport (Pinches *et al.* 1993).

III.2 Histological examination of the equine colonic tissue

The horse's large intestine account about 25 % of the total length of the gut which represent more than 60 % of the horse's digestive tract capacity. The large intestine comprised the caecum, ventral colon (left and right), dorsal colon (left and right),

transverse colon, small colon and rectum (Hintz, 1994). Previous studies showed that the large intestine has a great anatomic and physiological diversity between its various regions, which leads to variations in the function. Despite differences in function between colonic regions, morphological and structural properties remain similar (Dawn Grant and Specian, 2000).

The histological examination of the horse colon was performed to perceive the overall structure and cellular organisation of the large intestine. It has been shown that the colonic epithelium was not disposed to cell shedding and that epithelial cells in all regions on the large intestine remained undamaged after rapid blood loss (Pinches *et al.* 1992). The epithelium lining the large intestine was shown to be folded to form a number of invaginations called crypts. On these crypts lie epithelial cells, which regenerate in the lower part of the crypt and migrate in an upward direction (Marshman *et al.* 2002).

The epithelium of the colon consists mainly of three types of cells: columnar absorptive cells (colonocytes), the mucus-secreting goblet cells and the peptide-producing enteroendocrine cells. The colonocytes have microvilli, which are less prominent than those of the enterocytes. The colonic crypts are intruded by the lamina propria, which serves as a support. The lamina propria contains blood vessels, lymphatics and nerve cells. Underneath lamina propria lay the muscularis mucosa, the submucosa and the muscularis externa. The submucosa holds loose connective tissue with blood vessels, lymphatics and nerves. The muscularis externa is composed of circular and longitudinal muscle cells.

Figure III.1a is a longitudinal cross-section through the left ventral colon showing different layers of the colon. All parts described above are very noticeable. Figure III.1b showed the surface of the epithelium with different cell-type lining on the epithelium wall, predominantly absorptive cells and goblet cells, attached the basal membrane and lamina propria. Under high power (x 400), the goblet cells are distinguished due to clear zone in the cytoplasm corresponding to mucus-containing droplets (figure III.1c). The histological examination is essential; it gives evidence about the nature of the tissue used for the preparation of basolateral membrane vesicles.
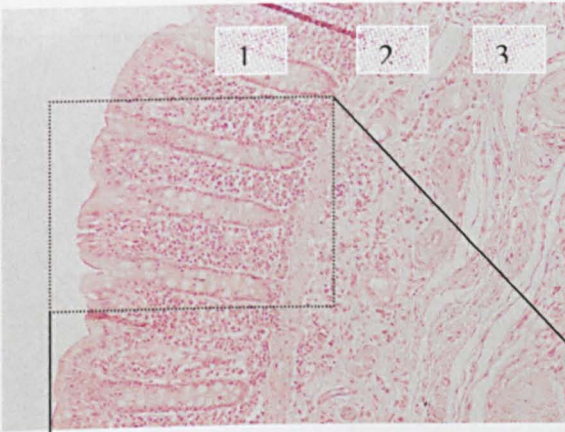
a

b
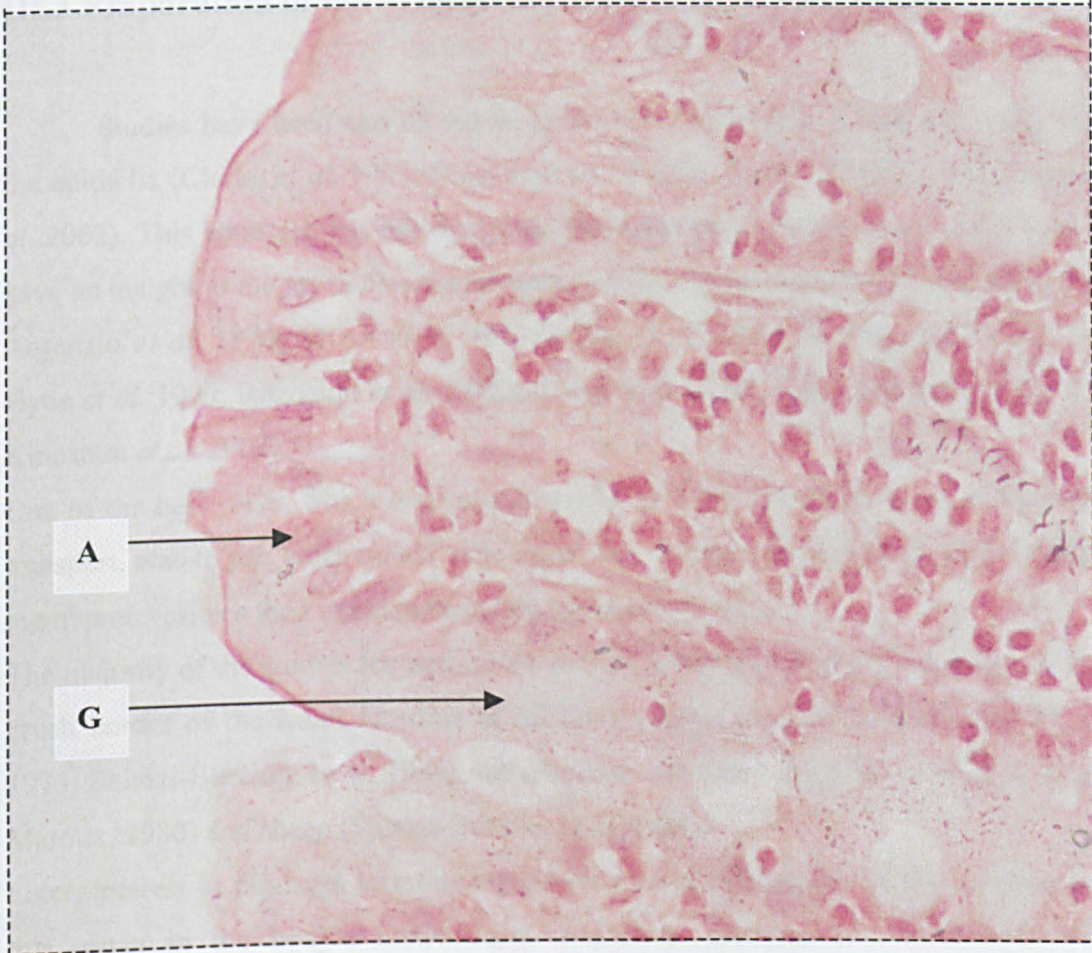
c

Figure III.1: *Histological examination of the equine ventral colon. 1) Mucosa, 2) Muscularis mucosa, 3) Sub-mucosa,(see figure a). A) absorptive cells, G) goblet cells (see figure c). LP) lamina propria (see figure b). Hematoxylin and eosin a) x100, b) x 200 and c) x 400 magnification.*

III.3 Preparation of horse large intestinal membrane vesicles

Studies have been carried out to assess the mechanism of ions and water across the epithelia (Clarke *et al.* 1992; Rajendran and Binder, 2000; Liedtke, 1989; Masyuk *et al.* 2002). This approach has allowed to delineate the trans-cellular routes of solutes and gave an insight to the molecular characteristic of the carrier proteins involved (Lee, 2000; Argenzio *et al.* 1974; Merot *et al.* 1997; Harig *et al.* 1996; Sterling and Casey, 1999; Hyde *et al.* 1999; Ritzhaupt *et al.* 1998a; Tyagi *et al.* 2000; Rajendran and Binder, 1994; Kinoshita *et al.* 2002).

One of the best ways, which was proven useful, to study the mechanism of electrolytes transport, was to use biochemical techniques for the isolation and purification of plasma membrane vesicles then to characterise the transport activity.

The majority of the studies focused on the plasma membrane vesicles originated from the brush border of the small intestine of different species such as human (Schmitz *et al.* 1973; Shirazi-Beechey *et al.* 1990), rat (Brasitus and Keresztes 1984), rabbit (Colas and Maroux, 1980) and sheep (Shirazi-Beechet *et al.* 1988).

Later, interest in the large intestine has become more evident due to the contribution of this organ in the body's homeostasis. Therefore, attempts to isolate luminal and basolateral membranes were achieved successfully using as a starting materials rabbit colon (Gustin and Goodman. 1981; Wiener *et al.* 1989), human colon (Tyagi *et al.* 2002), rat colon (Brasitus and Keresztes, 1984) and pig colon (Pinches *et al.* 1993; Ritzhaupt *et al.* 1998a).

Further than being time consuming, isolation of membrane vesicles from basolateral domain was challenging. The difficulty is due to the considerable amounts of mucus and a noticeable heterogeneity of cell types (Kaye *et al.* 1973). The main cell types found in the colonic epithelium include absorptive cells, goblet cells, few enteroendocrine cells and other immature epithelial cells.

Several approaches to isolate basolateral plasma membrane vesicles were endeavored. Jackson *et al.* (1977) was the first to isolate basolateral membranes from scraped colonic

mucosa of rat. Since then, other methods have been reported. These methods used different strategies and with time led to the improvement of the quantity and quality of the product. Furthermore, the complexity of the purification step was minimised as well as the time consumed.

In the present study, the isolation technique described by Pinches *et al.* (1993) based on the method reported by Wiener *et al.* (1989) was applied with some modifications. Scrapings of horse colonic tissue were used in this study. These modifications include: 1) Re-suspension of pellet (P_1) in the first buffer, homogenisation and centrifugation, then gathering of supernatants S_1 and S_2 which were further processed (see figure II.1). This step was performed because considerable amounts of proteins were lost in P_1 fraction. 2) The use of varying speeds of centrifugation to optimise the isolation yield.

III.3.1 Isolation of basolateral membrane vesicles (BLMV)

The isolation of plasma membrane from the basolateral pole of horse colonocytes was carried out in two stages. The first one was the fractionation of cellular organelles and membranes using differential centrifugation. The second stage of the purification scheme included density gradient centrifugation using a continuous sucrose gradient (25 - 55 %).

III.3.2 Differential Centrifugation

The technique of differential centrifugation was employed to prepare basolateral membranes. After disruption and homogenisation of the colonic tissue, the homogenate was centrifuged at a high rate of speed.
The process of differential centrifugation was based on the fact that organelles have differences in shape, size and density; therefore separation of an organelle from the homogenate was achieved artificially by putting the homogenate in a variable speed and rotating it at varying rate of speed. This created a force which pulled particles that would

normally stay in solution down and form a pellet at the bottom of the tube. Afterwards, the supernatant obtained by low speed centrifugation was directly applied onto a continuous sucrose gradient.

III.3.3 Sucrose Gradient centrifugation

800 µl of solution was loaded on top of a continuous sucrose gradient (25–55 %). The tubes were centrifuged at 100,000 x g for 60 minutes. A discrete band on top of the gradient was colleted using Pasteur pipette and further processed. Enzyme assays showed that this band was highly enriched in basolateral membrane marker $Na^+/K^+/ATPase$. Moreover, western blotting analysis exhibited a strong cross-reaction between the vesicles's protein and the $Na^+/K^+/ATPase$ antibody, and a negligible contamination with villin, a classical luminal membranes marker.

Density gradient centrifugation was previously used to isolate both luminal and basolateral membranes. This method was successfully utilised by Pinches *et al.* (1993) to isolate basolateral membrane vesicles from human and pig colon.

The density gradient profile was established for cellular fractionation by many authors who used either sucrose gradient (Ikuma *et al.* 2003; Brasitus and Keresztes, 1984) or percoll gradient (Gustin and Goodman, 1981; Inui *et al.* 1981). Mircheff and Wright, (1976) used sorbitol density gradient to isolate intestinal plasma membranes and so did Dyer *et al.* (1990) to study the transport mechanism of glycyl-L-proline in rabbit enterocytes basolateral membrane vesicles.

Substituting sucrose with sorbitol gradient did not give any promising results. Hence, a modification of the procedure described by Pinches *et al.* (1993) was followed for the isolation of colonic basolateral membrane vesicles from horse.

III.3.4 Protein Estimation

The determination of the protein concentration in the final membrane fraction is a pivotal stage for subsequent experiments.

A rapid reproducible method described by Bradford, (1976) was referred to, to estimate the quantity of the protein in the BLMV. The principle of the assay was based on the capacity of protein in the membrane fraction to bind coomassie blue. The absorbance was measured using spectrophotometer at a wave length of 595 nm against control tubes. Pig gamma globulin was used as standard. After the absorbance was read, the calibration curve was drawn and the protein quantity was calculated.

Protein determination was essential, since it made the analysis of the results more accurate when comparing the strength of the signals in western blot analysis. It was advised to use determined amounts of protein during transport studies of butyrate. It has been shown that 100 µg of protein of membrane vesicles represented the optimal protein concentration for transport measurement for glucose (Dyer *et al.* 1990; Pinches *et al.* 1993) and for butyrate (Ritzhaupt *et al.* 1998). Thus, a concentration of 100 µg per assay was performed to characterise the mechanism of butyrate uptake in the BLMV.

III.4 Characterisation and assessment of basolateral membrane vesicles

Two methods were adopted to verify the origin of BLMV isolated from equine colon. Both methods relied on the concept of enrichment and/ or dis-enrichment of marker proteins of the cellular organelles. The first method used immunodetection as a tool to assess the purity and origin of BLMV. The antibody raised against $Na^+/K^+/ATPase$ was shown to localise the protein to the basolateral plasma membranes of all animal cells (Mobasheri *et al.* 2000). The potential expression of the sodium pump was investigated. The presence of GLUT2, which was shown to be specifically expressed on the basolateral membranes (Pinches *et al.* 1993), in equine colonic BLMV was also investigated. Antibody to MCT1, which was confirmed to be on the luminal membrane of human and pig colonic cells (Ritzhaupt *et al.* 1998b), was used to screen BLMV for

potential presence of luminal membranes. Villin is restricted to luminal membrane of the small and large intestine (Grone *et al.* 1986). Antibody to villin was used to determine the potential presence of this protein in BLMV.

The second approach which is the enzyme assays has enabled us to draw quantitative conclusions about the distribution of enzyme activities in the basolateral membranes. Cysteine-sensitive alkaline phosphatase has been used as a luminal membranes marker in the colon (Brasitus and Keresztes, 1984). The dis-enrichment of this protein in the equine BLMV over the original homogenate is an indicative of the purity of the BLMV. Other marker enzymes have been assayed in this study such as $Na^+/K^+/ATPase$ (marker for the basolateral membranes), succinate dehydrogenase (marker for the mitochondria), Tris-resistant α-glucosidase (marker for the endoplasmic reticulum), α-mannosidase (marker for the Golgi apparatus).

III.4.1 Immunodetection

a) Villin

Villin is a protein, which is expressed almost exclusively in the microvilli of the brush-border of the small and large intestine (Bretscher & Weber, 1979). Expression of villin in intestinal epithelial cells is consistently maintained in their corresponding carcinoma (Pringault *et al.* 1986). It is a reliable marker for colonic luminal membranes. BLMV and its original homogenate isolated by differential centrifugation and sucrose gradient techniques were separated on 8 % SDS-PAGE and electro-transferred into PVDF membrane. The purity and origin of the BLMV were confirmed by western blot using a monoclonal antibody to mouse villin at a concentration of 1:1000. The blot was then incubated with horseradish conjugated secondary antibody (goat anti-mouse) and bands were visualised by enhanced chemiluminescence (ECL). As shown in figure III.2, the expected size band of 95 kDa was predominantly detected in the original homogenate

but not in the basolateral membranes. The abundance of villin in the colonic BLMV (lane b) was highly dis-enriched compared to the original homogenate (lane a). The enrichment of villin protein in the original homogenate, as analysed by densitometry, was 20 times that in the final membrane fraction.

a   b

95 kDa ➝
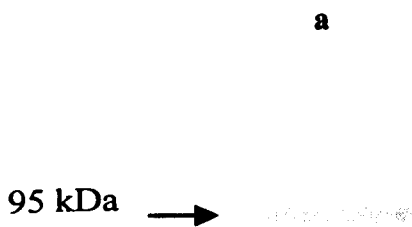
Figure III.2 Immunodetection of Villin: *Samples from equine colonic homogenate and BLMV (20 µg of protein per lane) were separated on an 8 % SDS-PAGE and electrotransferred to PVDF membrane. The blot was immunostained with villin antibody and detected with peroxidase-linked rabbit anti-mouse secondary antibody as described in Methods (section II.6.4). Lane a, equine colonic homogenate; lane b, equine colonic BLMV .*

b) MCT1

It has been shown that butyrate as well as L-lactate transporter, MCT1, is located on the luminal membranes isolated from the human and pig colonocytes. Western blot analysis indicated that the abundance of MCT1 protein was 25-30 fold enhanced in the LMV compared to the original homogenate (Ritzhaupt *et al.* 1998b). Immunoblot analysis of the BLMV prepared from the horse large intestine was performed in the presence of LMV isolated from the same species to establish in which membrane domaine MCT1 protein is expressed. A polyclonal antibody that had been raised against the COOH-terminal end of human MCT1 was used. The sequence of the human MCT1

exhibits high homology the equine MCT1 (see chapter V). As shown in figure III.3 (lane c), the antibody to MCT1 reacted with a band an approximately 45 kDa in the LMV. In contrast no signal was seen in the BLMV. These results confirm a negligible contamination of the equine colonic BLMV by membranes originated from the luminal domain.
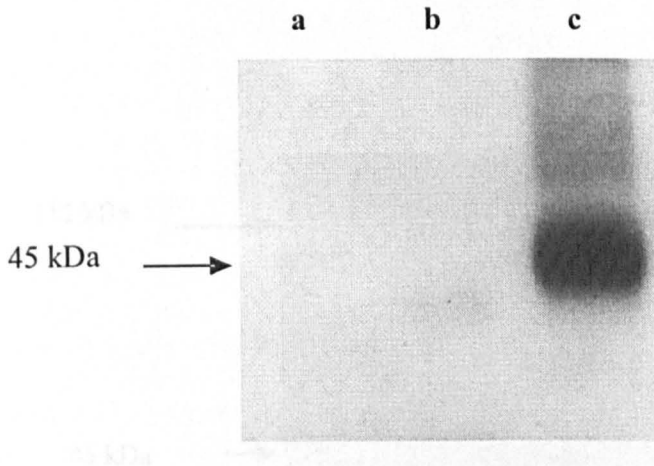
Figure III.3 Immunodetection of MCT1: *Luminal and basolateral membranes were prepared from equine colon as aforementioned (section II.3.1; II.3.2). Samples from equine colonic homogenate and BLMV (20 µg of protein per lane) were separated on an 8 % SDS-PAGE, electrotransferred to PVDF membrane, immunoblotted for MCT1 antibody and detected with peroxidase-conjugated swine anti-rabbit secondary antibody as described in Methods (section II.6.4). Lane a, equine colonic homogenate; lane b, equine colonic BLMV; lane c, equine colonic LMV.*

c) Na$^+$/K$^+$/ATPase

The sodium pump or Na$^+$/K$^+$/ATPase is predominantly located in the basolateral membranes of polarised epithelial cells (Mobasheri *et al.* 2000). It is considered as a putative membrane marker of small and large intestine (Dyer *et al.* 1990; Pinches *et al.* 1993). Therefore the determination of the Na$^+$/K$^+$/ATPase abundance in isolated basolateral membranes is considered of value to examine the purity and origin of the

fraction of interest. The BLMV isolated from equine colonocytes were incubated in a buffer containing a polyclonal antibody against the $Na^+/K^+/ATPase$. Figure III.4 showed strong cross-reaction of the antibody with both α and β subunits of the $Na^+/K^+/ATPase$, 112 kDa and 45 kDa respectively in the BLMV originated from the equine colon. Densitometry revealed that the band containing basolateral membranes was enriched 10-fold in $Na^+/K^+/ATPase$ over the homogenate.
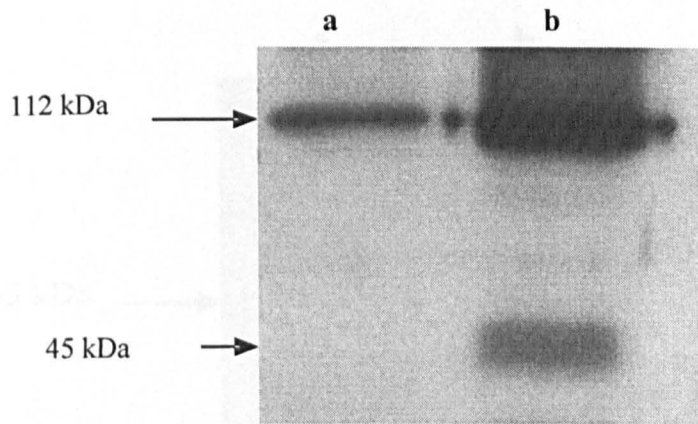
Figure III.4 Immunodetection of the $Na^+/K^+/ATPase$: *Samples from equine colonic homogenate and BLMV (20 µg of protein per lane) were separted on an 8 % SDS-PAGE and electrotransferred to PVDF membrane. The blot was immunostained with $Na^+/K^+/ATPase$ antibody and detected with peroxidase-linked swine anti-rabbit secondary antibody as described in Methods (section II.6.4). Lane a, equine colonic homogenate; lane b, equine colonic BLMV.*

d) GLUT2

It is well documented that glucose exits into the bloodstream by a member of the facilitative glucose transporter family, GLUT2, across the basolateral membranes of small and large intestinal mucosal cells (Kellet, 2001; Shirazi-Beechey, 1996 and Pinches *et al.* 1993). The presence of GLUT2 isoform in the colonic basolateral membranes was

further investigated by western blot analysis using the antibody raised against the equine intestinal GLUT2. As shown in figure III.5, the BLM fraction prepared in the isolation process from horse colonocytes exhibited a cross reaction with the antibody at a molecular weight of 55 kDa. The abundance of GLUT2 protein, as measured by scanning densitometry, demonstrated a 9-fold enrichment in the horse colonic BLMV over the levels determined in the homogenate.
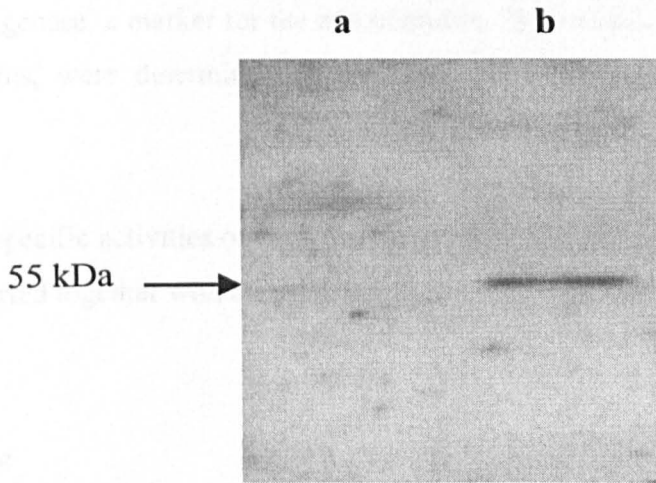
Figure III.5 Immunodetection of the sodium-independent glucose transporter (GLUT2): *Samples from equine colonic BLMV and the respective homogenate (20 µg of protein per lane) were separated on an 8 % SDS-PAGE and electrotransferred to PVDF membrane. The blot was immunostained with GLUT2 antibody and detected with peroxidase-linked swine anti-rabbit secondary antibody as described in Methods (section II.6.4). Lane a, equine colonic homogenate; lane b, equine colonic BLMV.*

III.4.2 Enzyme Assays

The potential of BLMV contamination by luminal membranes and other intracellular organelle membranes was also assessed by determining the levels of the activity of appropriate marker enzymes.

The activity of: 1) The basolateral membrane marker enzyme, $Na^+/K^+/ATPase$, 2) Cysteine-sensitive alkaline phosphatase, a marker for the ileal and colonic luminal membranes, 3) Tris-resistant α-glucosidase, a marker for the endoplasmic reticulum, 4) Succinate dehydrogenase, a marker for the mitochondria, 5) α-mannosidase, a marker for the Golgi apparatus, were determined in the final BLM fraction and its respective homogenate.

In table III.1, the specific activities of each marker enzyme in the final membrane vesicle fraction were reported together with the enrichment and recoveries.

a) $Na^+/K^+/ATPase$

The specific activity of $Na^+/K^+/ATPase$ was expressed as the quantity of inorganic phosphate (P_i) liberated. BLMV were treated with saponin to permeabilise the vesicles.

There was a 15-fold increase in specific activity of $Na^+/K^+/ATPase$ in BLMV compared to homogenate with recovery of activity of 21%. This result was evaluated with the data generated by Wiener *et al.* (1989) and Pinches *et al.* (1993) when isolating basolateral membrane vesicles from rabbit and human colon respectively. They obtained 34 – 12.8 fold enrichment and 16 – 29 % recovery for $Na^+/K^+/ATPase$.

b) Cysteine-Sensitive Alkaline Phosphatase

The purity of BLMV was further assessed by the activity of the luminal marker cysteine-sensitive alkaline phosphatase. The enrichment factor for this enzyme was only

0.28 fold and recovery of 1 % (see table III.1). The alkaline phosphatase activity in the basolateral membranes of the colon opposed to the high activity in the luminal membrane from the colon (Pinches *et al.* 1993), the small intestine (Colas & Maroux. 1980) and the kidney cortex (Boumendil-Podevin & Podevin. 1983).

Table III.1 distribution of marker enzyme activities in the basolateral membrane vesicles of the equine colon

Enzyme	Specific activity nmoles.(mg protein)$^{-1}$. min^{-1}	Enrichment [1]	Recovery (%) [2]
Na$^+$/K$^+$/ATPase	3.29 ± 7.2	15 ± 2	21
Cys-sensitive Alkaline Phosphatase	0.81± 0.3	0.28 ± 0.04	1.05
Tris-resistant α-Glucosidase	8.84 ± 2.5	1.35 ± 0.5	0.8
α-Mannosidase	1.26 ± 0.3	1.36 ± 0.2	1.51
Succinate Dehydrogenase	0.38 ± 0.1	0.91 ± 0.4	0.75

Basolateral membrane vesicles from horse colon were prepared as described in the methods section (II.3.2). Values represent mean ± S.E.M of 4 experiments carried out in triplicate.

[1] Enrichment is the ratio of the specific activity of the BLMV fraction to that of the original homogenate.

[2] Recovery is the total enzyme activity in the BLMV fraction as a percentage of the total enzyme activity in the original homogenate.

c) Other organelle markers

The purity of the BLMV isolated from equine colon was also tested for any possible contamination by other organelle membranes. The specific activities of marker enzymes summarised in table III.1 showed enrichment factors of 1.35, 1.36 and 0.38 and recoveries of 0.8, 1.51 and 0.75 for tris-resistant α-glucosidase (marker of the Endoplasmic Reticulum), α-mannosidase (marker of the Golgi apparatus) and succinate dehydrogenase (marker of the mitochondrial membranes) respectively. BLMV isolated from equine large intestine revealed a substantial enrichment in the classical marker of the basolateral membranes, $Na^+/K^+/ATPase$, and negligible levels of contamination by luminal and intracellular organelle membranes, indicating that the final vesicle fraction originate from the basolateral domain the colonocyte plasma membrane, and was devoid of contamination by any luminal or organelle membranes.

The enzyme assay data, together with the western blotting results strongly supported the proposition that this fraction contained basolateral plasma membranes.

III.5 Sodium independent glucose uptake

It is well documented that glucose enters the intestinal absorptive cells via sodium-dependent glucose transporter, SGLT1, (Shirazi-Beechey, 1995) and exit into the blood via facilitative sodium independent glucose transporter, GLUT2 (Brown, 2000). It has been reported that GLUT2 mediates basolateral fructose transport as well (Kellett, 2001). A study performed in our laboratory showed that GLUT2 was expressed in the basolateral plasma membranes of human and pig colon and was able of transporting glucose (Pinches *et al.* 1993). This observation was confirmed by Noguchi *et al.* (2000). It was revealed that the fungal metabolite, Cytochalasin B, inhibits the Na^+-independent glucose transporter (GLUT2) (Axelrod and Pilch, 1983). Cytochalasin B was also employed to characterise the mechanism of glucose transport in the BLMV, isolated from

pig colonocytes. The data indicated that GLUT2 was present on these membranes (Pinches *et al*. 1993).

Therefore, in an attempt to determine the origin of the final membrane fraction and its ability to transport glucose, BLMV isolated from equine colon were tested for the uptake of D-glucose in the presence and absence of 50 µM of Cytochalasin B. Uptake experiments were performed as described in the methods (Section II.7.1). Figure III.6 showed the time course of D-glucose uptake into colonic BLMV. In the absence of Cytochalasin B, glucose uptake was a saturable process exhibiting characteristics of a carrier mediated transport. The simultaneous addition of Cytochalasin B significantly decreased the rate of glucose uptake in the BLMV.

These findings, together with the data of western blotting analysis and enzymes assays prove that the fraction of interest effectively originate from the basolateral membrane of equine colonocytes.
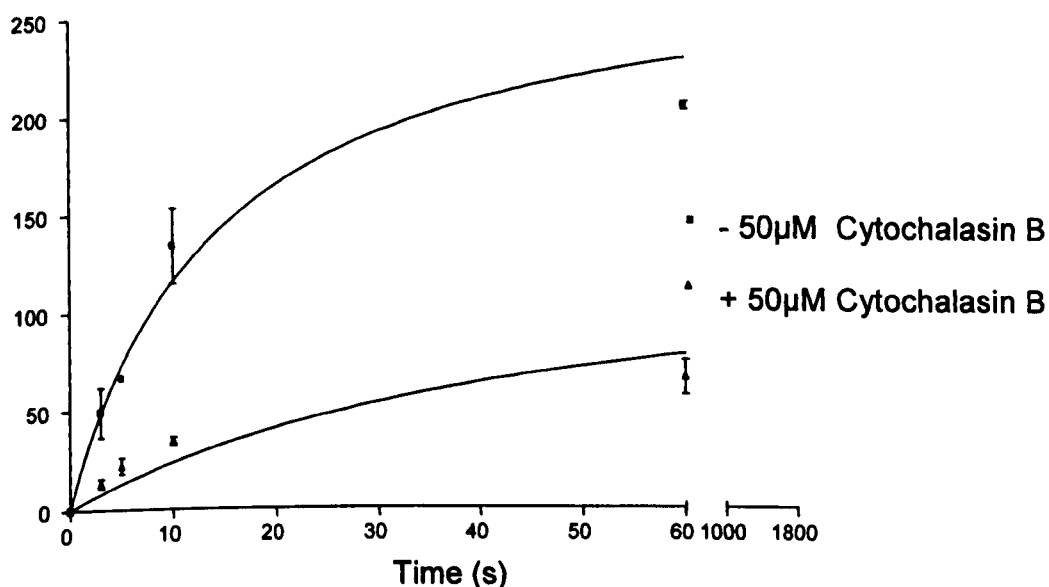
Figure III.6 Time course of the uptake of D-glucose by horse colonic BLMV. *D-[^{14}C]-glucose uptake was measured at 38°C by incubating the BLMV (100 µg protein) in a reaction medium containing 300 mM mannitol, 0.1 mM MgSO$_4$, 20 M Hepes/ Tris (pH 7.5) and 1 mM D-[^{14}C]-glucose in the presence (■)or absence (▲) of 50 µM cytochalasin B.*
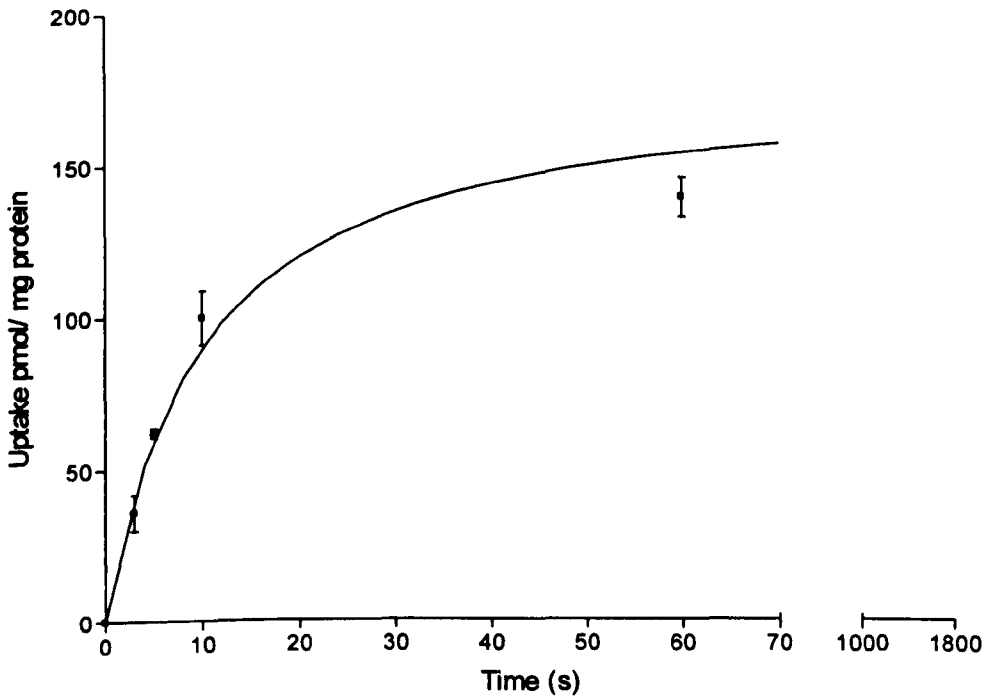
Figure III.7 Mean time course of D-glucose uptake by horse colonic BLMV. *Carrier mediated component of transport was obtained by subtracting uptake in the presence of cytochalasin B from total uptake. (Similar uptake condition was used as mentioned in figure III.6)* .

III.6 Discussion

There has been an increased recognition of the importance of the large intestine in the body's homeostasis. It is becoming evident that the large intestine is an important site for salvaging the nutrients which are not absorbed in the small intestine. Transport of solutes and substrates is one of the major biological processes in the large intestine. Studying the mechanism of solute transport *in vivo* is challenging and hampered by several factors. Interestingly, the trans-epithelial transport process of electrolytes in the colon using purified plasma membrane vesicles has become an attractive methodology.

Therefore, experiments with membrane vesicles isolated from the basolateral domain of colonocytes have helped to delineate membrane transport mechanism involved in colonic ions and substrates transport. This approach has been described as convenient and controllable; moreover the mechanism of substrates transport can be studied in greater detail.

A number of methods have been employed to study the uptake mechanism of short chain fatty acids such as *in vivo* perfusion, Ussing chamber technique and membrane vesicles (Sellin, 1994). In the present thesis, the transport mechanism of butyrate into isolated BLMV from equine colon was investigated.

A variety of methods have been reported for isolating brush border membranes vesicles (BBMV) from a number of tissues including kidney cortex (Boumendil-Povedin and Povedin, 1983; Molitoris and Hoilien, 1987) and small intestine. Rat small intestine has been used (Mircheff and Wright, 1976; Shirazi *et al.* 1981) for BBMV isolation. BBMV were prepared from rabbit small intestinal (Colas and Maroux, 1980), human small intestine (Shirazi-Beechey *et al.* 1990), mouse small intestine and equine small intestine (Dyer *et al.*2002).

An increasing recognition of the importance of the colon has led to a concomitant increase in the number of studies examining substrates transport in this organ. Since then more work on isolated luminal membrane vesicles (LMV) have been performed. In an attempt to investigate the mechanism of ion movement across the apical membrane of the colon, Gustin and Goodman (1981) were the first to isolate luminal membrane from rabbit descending colon. The transport in LMV was extensively studied by Harig *et al.* (1996) and Ritzhaupt *et al.* (1998).

Several different experimental approaches have been described to isolate basolateral membrane vesicles (BLMV) from the colon. The methods described by Biber *et al.* (1983) and Brasitus and Keresztes (1983) were laborious and complicated due to the lack of suitable membrane markers. However, other analytical isolations of BLMV from rat, rabbit, pig and human were developed using either percoll or sucrose density gradients (Wiener *et al.* 1989; Reynolds *et al.* 1993 and Pinches *et al.* 1993).

In the present work, the method of Pinches *et al.* (1993) based on the technique described by Wiener *et al.* (1989) was used with some modifications. One of the modifications introduced in the isolation method was the re-suspension of pellet (P_1) in the first buffer, homogenisation and centrifugation, then gathering of supernatants S_1 and S_2 which were further processed. This step was performed because considerable amounts of proteins were lost in P_1 fraction. The second modification was the use of varying speeds of centrifugation to optimise the isolation yield.

In the present study the purity of isolated equine colonic BLMV was extensively characterised by measuring the abundance and activities of putative marker enzymes. Several enzyme activities classically associated with luminal, basolateral and other organelle membranes were determined. The specific activity ratio of the $Na^+/K^+/ATPase$, a marker of basolateral plasma membranes (Schultz, 1984), was enriched 15-fold over the original homogenate which means that 21% of the total activity was recovered. The enrichment and recovery parameters of the $Na^+/K^+/ATPase$ activity in this study were comparable to those obtained by Pinches *et al.* (1993). This seems logical if we consider the procedures used in the preparation of the BLMV. Cysteine-sensitive alkaline phosphatase activity has long been known to be concentrated on the luminal plasma membranes (Brasitus and Keresztes, 1984). Controversially, Biber *et al.* (1983) and Pinches *et al.* (1993) failed to detect alkaline phosphatase in guinea pig, human and pig colon respectively by immunohistochemistry. Measurement for cysteine-sensitive alkaline phosphatase in equine colonic BLMV exhibited a negligible level of this enzyme with enrichment factor of 0.28 compared to the original homogenate and recovery of 1%. The activities of cysteine-sensitive alkaline phosphatase and $Na^+/K^+/ATPase$ alongside with other enzyme activities (succinate dehydrogenase, α-mannosidase, tris-resistant α-glucosidase) in the equine colonic BLMV were summarised in table III.1. The distribution of these two enzymes in the BLMV prepared by different procedure and reported by other investigators is listed in table III.2. These workers isolated BLMV by a combination of differential centrifugation and sucrose density gradient techniques, similar principle to that described here. The authors agreed that the $Na^+/K^+/ATPase$ activity was highly enriched in the BLMV, this was associated with a trivial

contamination by cysteine-sensitive alkaline phosphatase (Tyagi *et al.* 2000; Pinches *et al.* 1993; Wiener *et al.* 1989; Biber *et al.* 1983).

The abundance of the marker proteins, specifically located on the luminal or basolateral membranes of small and large intestine, was further assessed using suitable antibodies to characterise the basolateral membrane vesicles prepared from horse large intestine. The protein components of the BLMV were separated on SDS-PAGE, and then electro-transferred to PVDF membranes. The potential of the proteins to interact with antibodies to MCT1, GLUT2, $Na^+/K^+/ATPase$ and Villin was assessed. Antibody to $Na^+/K^+/ATPase$ detected specific bands at molecular weights of 112 KDa and 45 KDa corresponding to the α and β subunits of the protein respectively. Immunolocalisation of the $Na^+/K^+/ATPase$ protein to the BLMV is consistent with the suggested physiological role of this pump in maintaining Na^+ and K^+ gradients across the plasma membrane (Pacha, 2000). As shown in figure III.4, the protein was enriched 10-fold in the BLMV over the original homogenate. This finding was also reported by Pinches *et al.* (1993) who succeed to detect the $Na^+/K^+/ATPase$ protein in the BLMV from human and pig colon. Using immunohistochemistry, Scheiner-Bobis *et al.* (2002) showed that $Na^+/K^+/ATPase$ was restricted to the basolateral membrane of rat colonic epithelial cells.

Antibody to the GLUT2 protein, the predominant facilitative Na^+-independent glucose transporter in the small and large intestine (Thorens, 1996), identified a band with molecular weight of 55 KDa in the BLMV. The presence of this protein determined the aptitude of the membrane vesicles to function as glucose transporter, hence the suitability of the vesicles to study butyrate uptake. It has been reported that GLUT2 was able to transport glucose, mannose, galactose and fructose in xenopus oocytes injected protein (Thomson and Wild, 1997).

In this study, we have used a monoclonal villin antibody to examine the potential presence of the corresponding protein in the BLMV. Villin, which was shown to be a major component of microvilli at the apical surface of intestinal epithelial cells, is a calcium-regulated actin-binding protein (West *et al.* 1988). Villin expression was seen in

renal tumor cells (Grone *et al.* 1986). A similar observation was reported by Pringault *et al.* (1986) in intestinal cells. Therefore, it could serve as a marker of tumor cell differentiation in the intestine and the kidney. The villin antibody failed to detect any antigen in the BLMV proteins.

Expression of MCT1 and its ability to transport short chain fatty acids was extensively studied in our laboratory. It was shown to be located on the luminal membranes of human and pig colon, and has the potential to transport L-lactate as well as butyrate (Ritzhaupt *et al.* 1998a; 1998b). Immunohistochemical and in-situ hybridisation studies revealed a significant decrease in MCT1 expression in human colon during transition from normality to malignancy (Lambert *et al.* 2002). In the study reported in the present thesis, expression of MCT1 in the BLMV was determined. Membrane vesicles from luminal domain (LMV) of the equine colon were used alongside as a positive control. Western blotting analysis revealed a strong cross-reaction of the MCT1 antibody with protein components of the LMV, whereas no band was detected in the BLMV.

The aforementioned procedure of BLMV isolation from equine colonocytes is rapid (5 hours) and reproducible. The membrane vesicles, which were characterised by a range of methods, are highly enriched in basolateral membranes with slight contamination by luminal and other organelle membranes. This finding strongly prove that the fraction of interest originate from the basolateral domain of the colonocytes.

In conclusion, the equine colonic BLMV were of high purity and provided a suitable model to study the mechanism of butyrate transport.

Table III.2 Comparison of basolateral membrane isolation procedure from the colon of different species

| Species (BLMV) | Cysteine-sensitive Alkaline Phosphatase | | Na$^+$/K$^+$/ATPase | | Reference |
	Enrichment[1] (Fold)	Recovery[2] (%)	Enrichment[1] (Fold)	Recovery[2] (%)	
Human colon	0.97	0.51	10.3	11.8	Taygi *et al.* 2000
Human colon	-	-	12.8	29	Pinches *et al.* 1993
Rabbit colon	8	3.3	34	16.7	Wiener *et al.* 1989
Pig colon	3.5	4.5	10	13	Pinches *et al.* 1993
Guinea pig colon	1.5	-	12	12.5	Biber *et al.* 1983
Horse colon	0.28	1.05	15	21	Nedjadi

[1]Enrichment is the ratio of the specific activity of the enzyme in the BLMV fraction over the specific activity in the homogenate.

[2]Recovery is expressed as the percentage of total enzyme activity in the BLMV fraction of the total activity present in the homogenate.

CHAPTER IV

Characterisation of butyrate transport across the equine colonic BLMV

Characterisation of butyrate transport across the equine colonic BLMV

IV.1 Introduction

Short chain fatty acids (acetate, propionate and butyrate) are the major end-products from anaerobic breakdown of dietary fibers in the large intestine of mammals including human and horse (Engelhardt *et al.* 1998). They are the main anions in the colonic content (Cummings, 1984), and are absorbed across the colonic mucosa (Bugaut, 1987). Differences in SCFA production and absorption along the large intestinal segments have been reported (Hume *et al.* 1993). In animals with a voluminous large intestine, such as horse, up to 80% of the energy requirements derive from SCFA especially butyrate (Bergman, 1990). The energy contribution of SCFA accounts for about 60 – 70% in human (Scheppach *et al.* 1994). This illustrates the importance of these substrates for the overall energy metabolism and hence the body performance in some animals.

There is evidence regarding the important role of SCFA in the regulatory functions of the colon as well as prevention of ulcerative colitis. Butyrate is known to influence the proliferation and differentiation of normal colonic mucosa (Schappach, 1994) and protect against colon cancer (Schappach *et al.* 1997). These alterations result from the effect of butyrate on the expression of genes regulating these functions (Kruh *et al.* 1994). The trophic effects of SCFA on the mucosa of the colon were reported by Frankel *et al.* (1994). Furthermore, Choshniak and Mualem, (1997) revealed that SCFA provide a driving force for concomitant water and electrolyte absorption in the colon.

These earlier observations and others have led to the consensus on the importance of SCFA in the body homeostasis. Despite that, only recently more work shed light on how SCFA are absorbed through the intestinal membranes. A number of studies were performed to identify the mechanism by which SCFA move across the colonic luminal membranes.

From the literature, it is well known that the transport of SCFA occur throughout the length of the large intestine. Some authors have suggested nonionic diffusion of protonated SCFA across the colon. This view is proposed by the fact that at the colonic pH the predominant species of SCFA (pKa 4.8) are in the ionised form as a result of SCFA link to the hydrogen ions originating either from the luminal Na^+-H^+ exchanger or from the metabolism of CO_2 and H_2O and formation of HCO_3^- and H^+. Afterward, SCFAH are absorbed by passive diffusion (Sellin and DeSoigne, 1990; Ruppin *et al.* 1980; Charney *et al.* 1998). The second mechanism proposed for SCFA transport on the luminal membranes of the colon involved a carrier mediated process (SCFA-anion exchanger). The following models have been proposed: SCFA-H^+ symporter, SCFA-OH^- exchange or SCFA-HCO_3^- exchanger (Mascolo *et al.* 1991; Titus and Ahearn, 1992; Ritzhaupt *et al.* 1998; Harig *et al.* 1996).

Although the movement of SCFA across the luminal membranes of the large intestine has been studied, the mechanism by which these compounds are transported across the basolateral domain of the colonocytes is unclear. Therefore, this study was undertaken using isolated purified and well characterised horse colonic basolateral membrane vesicles (BLMV), to identify the mechanism and the properties of the transporter responsible for moving butyrate and its analogues from the inside of the colonocytes out into the bloodstream.

BLMV is a good model to study SCFA transport across polarised epithelia. BLM vesicles have been used to study the transport of butyrate, lactate and acetate in human colon, rat colon, rat jejunum and ovine parotid gland (Tyagi *et al.* 2002; Reynolds *et al.* 1993; Cheeseman *et al.* 1994; Nguyen and Beechey, 1999). The present study was therefore performed to characterise the mechanism of SCFA transport in the BLMV from equine colon.

IV.2 Effect of Bicarbonate on butyrate uptake

To determine the effect of bicarbonate anions on butyrate uptake across the BLMV, the vesicles were loaded with Hepes/ Tris (pH 7.5) and either $KHCO_3$, mannitol or K-gluconate. The vesicles were incubated in medium that contained 1mM [^{14}C]-butyrate and K-gluconate, pH 7.5 or 5.5. As shown in figure IV.1, in the absence of a pH gradient, the presence of an outward-directed bicarbonate gradient significantly stimulated [^{14}C]-butyrate uptake compared to that found in mannitol and gluconate loaded vesicles. Imposition of a pH gradient substantially increased the rate of butyrate uptake in the condition where bicarbonate is present. These experiments suggest that butyrate uptake could be attributed to a carrier mediated process and not to passive diffusion.
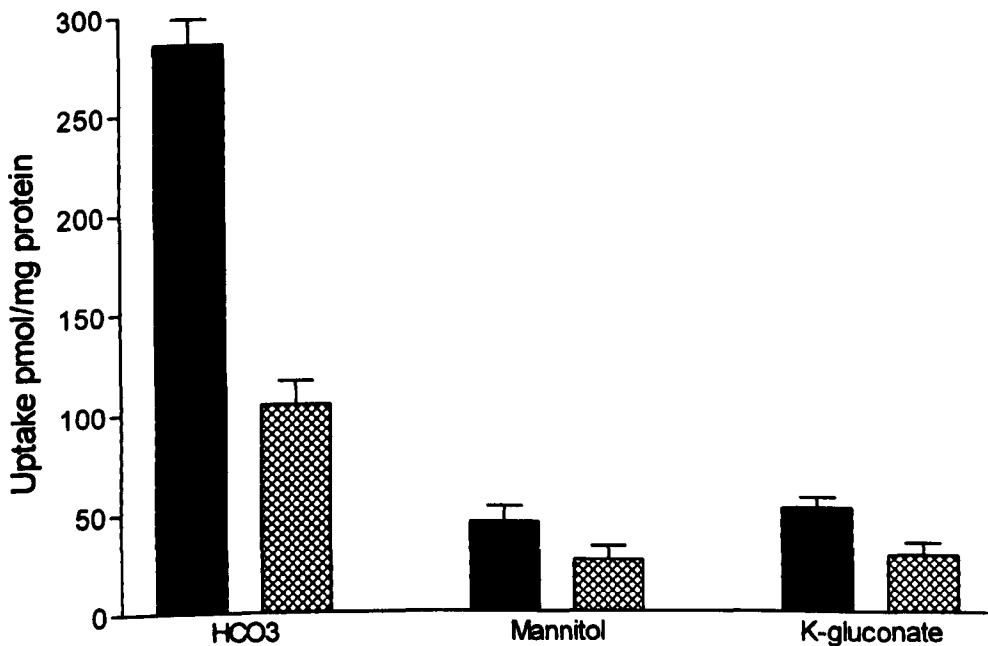
Figure IV.1. Effect of HCO_3 and pH gradients on butyrate uptake. BLMVs were preloaded with 20 mM Hepes/Tris (pH 7.5) and either 150 mM $KHCO_3$ or 150 mM K-gluconate or 300 mM mannitol. The vesicles (100 μg protein per assay) were incubated at 38°C for 3 seconds by dilution in the incubation medium containing 150 mM K-gluconate, 1 mM [^{14}C]-butyrate and either 20 mM Hepes/ Tris pH 7.5 ▨ 20 mM Mes/Tris pH 5.5 ■ Values are presented as the means ± S.E.M. for three separate experiments.

IV.3 Effect of pH of varying extra-vesicular pH on butyrate uptake

Reynolds *et al.* (1993), performed an experiment to study the mechanism of butyrate transport using BLMV isolated from rat colon, and showed that bicarbonate stimulated-butyrate uptake was significantly higher while a pH gradient was imposed (pH_{in}7.5, pH_{out} 6.0) compared to that in the absence of pH gradient (pH_{in} 7.5, pH_{out} 7.5). In addition, Tyagi *et al.* (2002) have demonstrated that the activity of butyrate-bicarbonate exchange in human colonic BLMV was markedly stimulated in the presence of an outward directed pH gradient (pH_{in} 8.2, pH_{out} 6.5). In order to elucidate whether bicarbonate stimulated butyrate uptake, in BLMV from equine colonocytes, is stimulated by changes in the extra-vesicular pH, butyrate uptake was performed in the presence of different pH gradients in the extra-vesicular media. The equine colonic BLMV, preloaded with a buffer containing K-HCO_3, pH 7.5, were incubated in an iso-osmolar uptake media consisting of K-gluconate and either Hepes/ Tris (pH 7.0, 7.5, 8.0) or Mes/ Tris (pH 5.5, 6.0, 6.5), 1 mM [U-^{14}C]-butyrate. Experiments were carried out as described in the methods section (II.7.2). As shown in figure IV.2, the initial rate of butyrate uptake was significantly higher at low pH with an optimal uptake at pH 5.5, corresponding to an uptake level of 280 pmol. (mg protein)$^{-1}$. There was a 10-fold enhancement of the HCO_3^--stimulated butyrate uptake at pH 5.5 compared to pH 8.0. This observation suggests that the enhancement of the butyrate uptake in the presence of both HCO_3 and pH gradients is either pH-activated due to the acidic extra-vesicular environment or pH-driven (H-butyrate symporter).

To examine these two possibilities, butyrate uptake was performed using BLMV loaded with either HCO_3 or gluconate or mannitol, pH 7.5 and incubated in media containing an iso-osmolar concentration of gluconate buffered at pH 5.5. In the absence of a HCO_3^- gradient, butyrate uptake was lower compared to that in its presence, even though a pH gradient was imposed in all conditions. This finding indicated that the HCO_3^--stimulated butyrate uptake is a pH-activated process resulting from the accumulation of a high proton concentration in the extra-vesicular space.
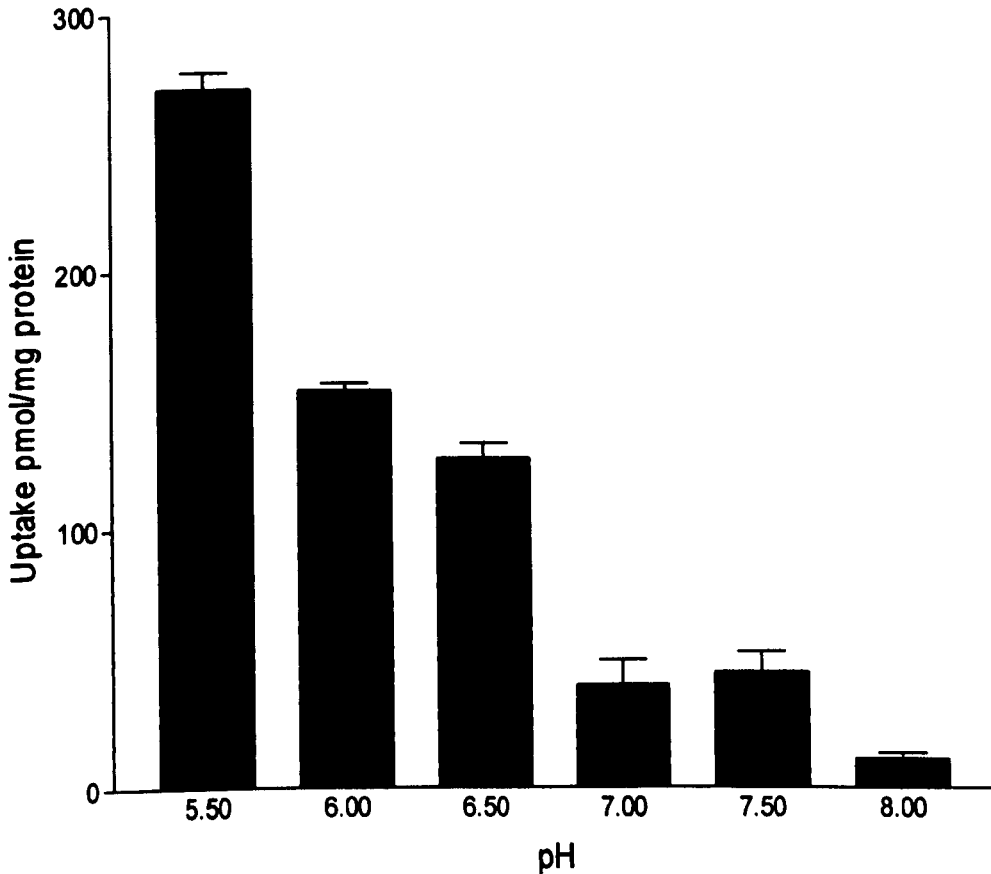
Figure IV.2. Effect of extravesicular pH on HCO_3^--stimulated butyrate uptake. BLMVs were preloaded with 150 mM $KHCO_3$ and 20 mM Hepes/Tris (pH 7.5). The vesicles (100 μg protein per assay) were incubated at 38°C for 3 seconds by dilution in the incubation medium containing 150 mM K-gluconate, 1 mM [^{14}C]-butyrate and 20 mM Hepes/Tris for pH gradients ranging 7.0, 7.5, 8.0 or Mes/Tris for pH gradients ranging 5.5, 6.0, 6.5. Values are presented as the means ± S.E.M. for three separate experiments.

IV.4 Time course of butyrate uptake

Equine colonic BLMV were loaded with either 150 mM potassium-bicarbonate ($K\text{-}HCO_3$) or 300 mM mannitol buffered at pH 7.5. The vesicles were incubated at 38°C for the indicated time periods in the incubation media containing 150 mM K-gluconate and 1 mM radiolabelled butyrate buffered at pH 5.5. As seen in figure IV.3a, IV.3b, the amount of butyrate taken up into the vesicles was increasing with time and reached an equilibrium level that was approximately 700 $pmol.mg^{-1}$ protein. This pattern of transport correlates with the presence of a carrier mediated mechanism in the BLMV. In contrast to what had been seen in the luminal membrane vesicles, no overshoot was observed. The uptake of butyrate was linear up to 3 seconds (figure IV.3c); therefore an uptake period of 3 sec was employed as an initial rate in the subsequent experiments.

Figure IV.3. Time course of butyrate uptake into equine colonic BLMV

a)

b)
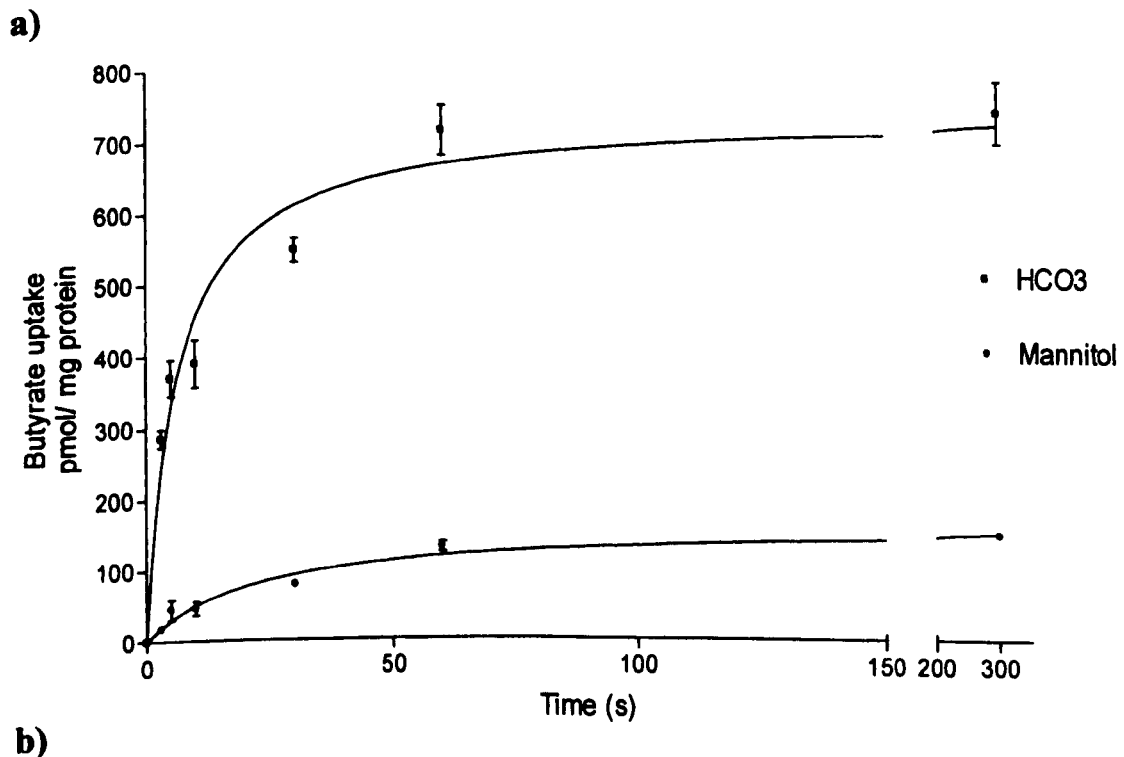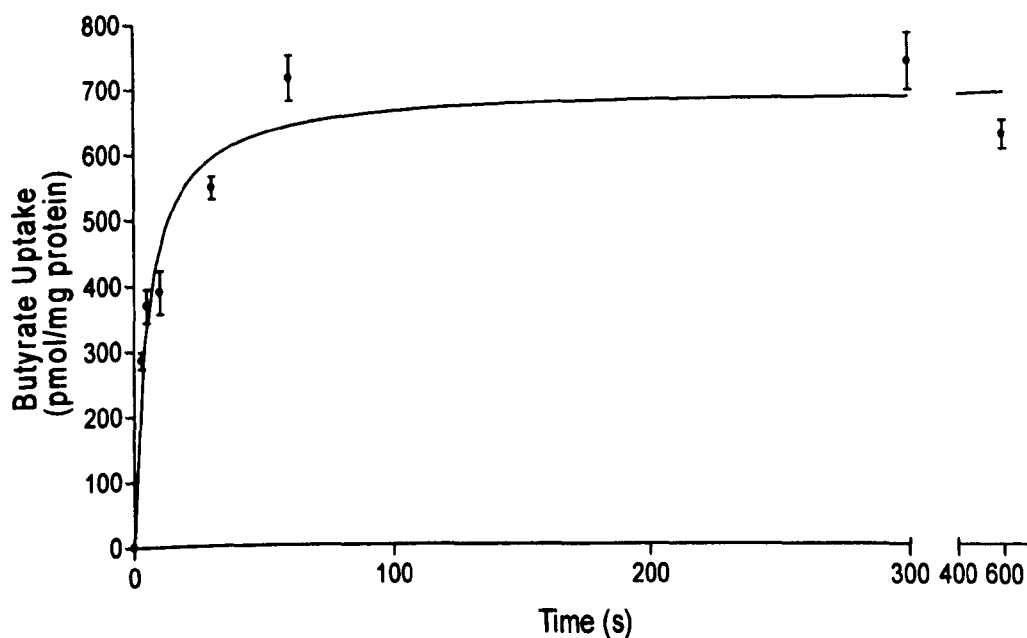
c)
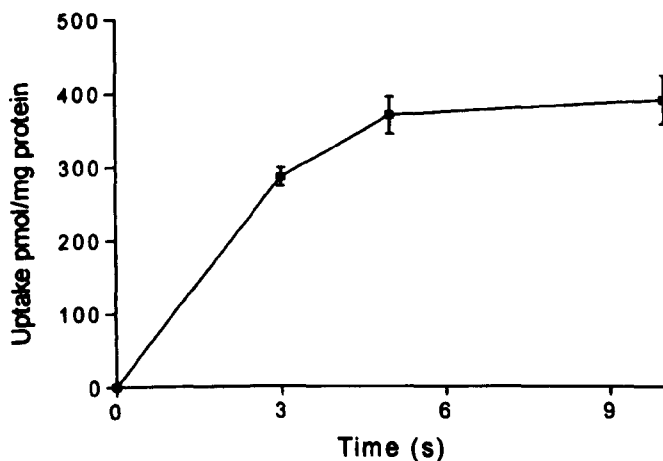
Figure a, b) Time course of butyrate uptake. BLMV were loaded with 20 mM Hepes/ Tris (pH 7.5) and either 150 mM of KHCO$_3$ or 300 mM mannitol. The Uptake was measured at 38°C by incubating the vesicles (100 μg protein per assay) in reaction solution containing 150 mM K-gluconate, 20 M Mes/ Tris (pH 5.5) and 1 mM [^{14}C]-butyrate. Figure c) linearity of butyrate transport (same condition was used). The assays were carried out in triplicate. Values are presented as means± S.E.M for three experiments.

IV.5 Kinetics

To assess the kinetics of butyrate uptake, the effect of varying butyrate concentrations on the initial rates of the uptake was investigated. The BLMV were loaded with K-HCO$_3$ and incubated in reaction media containing K-gluconate and increasing concentrations of butyrate (1 – 50 mM). The uptake was measured in the presence of an inward-directed pH gradient (pH$_{in}$ 7.5, pH$_{out}$ 5.5) as described in the methods section.

The results presented in figure IV.4 show that the increase in the butyrate concentration was associated with an increased rate of [^{14}C]-butyrate uptake. The HCO$_3^-$-stimulated butyrate uptake was a saturable process, which is a characteristic feature of a carrier-mediated anion exchanger in these membranes. Linear regression analysis of Hanes plot demonstrate an apparent Michaelis-Menten constant (K_m) of 12.2 mM and maximum velocity (V_{max}) of 3.05 nmol/ mg protein/ 3 se. This K$_m$ appeared to be comparable to the K$_m$ value of 17.5 ± 4.5 mM previously described by Tyagi *et al.* (2002). The present result indicated that bicarbonate-stimulated butyrate uptake across the equine colonic BLMV occur through a carrier mediated process.

Figure IV.4. Concentration dependency of butyrate uptake into equine colonic BLMV.

a)

b)
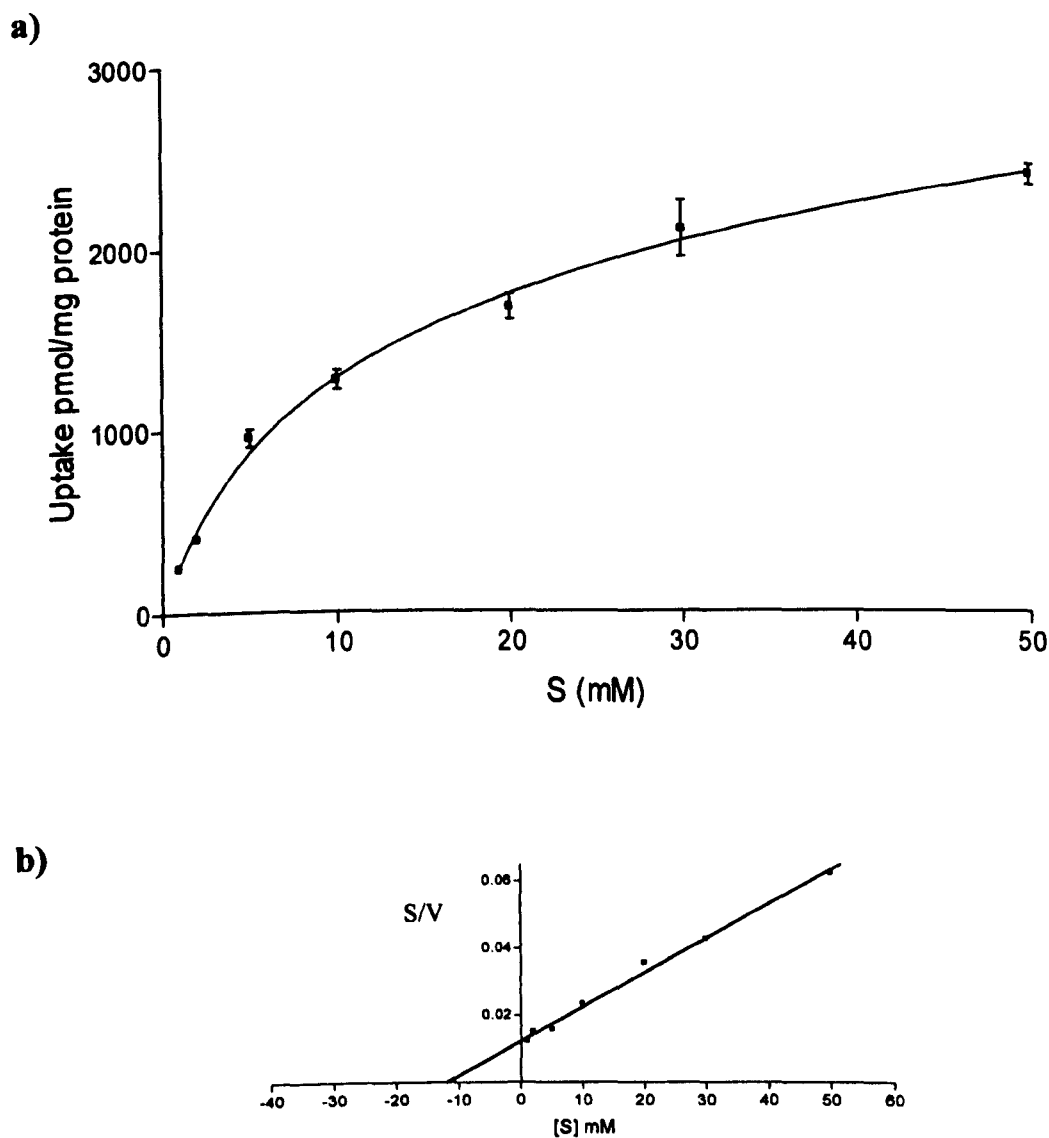
Figure a) Michealis-Menten curve. The initial rate (3 sec) of [^{14}C]-butyrate uptake by BLMV was determined with an increasing butyrate concentration over the range 1-50 mM at 38°C. The vesicles (100 μg protein per assay) were preloaded with 150 mM KHCO$_3$ and 20 mM Hepes/ Tris (pH 7.5) and diluted into incubation media containing 150 mM K-gluconate, 20 mM Mes/ Tris (pH 5.5), 1 mM [^{14}C]-butyrate and varying concentrations of sodium butyrate. Isosmolarity were maintained by adjusting K-gluconate concentrations. Figure b) Hanes plot of linear regression analysis was used to calculate K$_m$ and V$_{max}$. Values are presented as means ± S.E.M for three experiments.

IV.6 Effect of membrane potential

To examine the effects of changing membrane potential generated by K^+/ valinomycin on butyrate uptake, the BLM vesicles were loaded with either 150 mM $KHCO_3$, 150 mM K-gluconate or 300 mM mannitol. Prior to the experiment, the vesicles were pre-incubated with the potassium ionophore, valinomycin, at the concentration of 10 μg. mg^{-1} protein for 30 min on ice. The data presented in table IV.1 reveal that changes in membrane potential failed to alter butyrate uptake. These results indicate that bicarbonate-dependent butyrate uptake is an electroneutral process.

Buffer conditions	[^{14}C]-Butyrate uptake pmol/mg protein/3se	
	+ Val	- Val
150mM $KHCO_3$ in / K-gluconate out $[K^+]_{in} = [K^+]_{out}$	274.4 ± 22	301 ± 17
150mM K-gluconate in/ Mannitol out $[K^+]_{in} > [K^+]_{out}$	47 ± 9	46.6 ± 5
300 Mannitol in/ 150mM K-gluconate out $[K^+]_{in} < [K^+]_{out}$	51 ± 11	49 ± 3

Table IV.1. Effect of Valinomycin-induced membrane potential on butyrate uptake. Equine BLM vesicles (100 μg protein per assay) were pre-incubated with valinomycin (10 μg/ mg protein) for 30 minutes at 4°C prior to the uptake. BLMV were loaded with 20 mM Hepes/ Tris (pH 7.5) and either 150 mM $KHCO_3$ or 150 K-gluconate or 150 mM mannitol, and incubated in media containing 20 mM Mes/ Tris (pH 5.5), 1 mM [^{14}C]-butyrate and either 150 mM K-gluconate in first and third condition or 150 mM mannitol in second condition. Control vesicles were kept on ice for 30 min prior to the uptake. The reaction was terminated by the addition of 1 ml of ice-cold stop solution after 3 seconds. Values are presented as mean ± S.E.M for three experiments.

IV.7 Effect of butyrate analogues

Another series of experiments were designed to determine the specificity of the butyrate/ HCO_3^- exchanger. The effects of 20 mM monocarboxylates (formate, acetate, propionate, butyrate, pyruvate and oxalate) in the incubation media on the initial rate of $[^{14}C]$-butyrate uptake was investigated. As shown in figure IV.5, addition of 20 mM potassium salt of indicated organic anions resulted in substantial inhibition of the HCO_3/ pH-stimulated butyrate uptake. Acetate and butyrate reduced the rate of $[^{14}C]$-butyrate uptake by 75% whereas formate, propionate, pyruvate and oxalate gave 45 – 60% decrease. These results agree to a certain extent with what has been published so far (Reynolds *et al.* 1993; Tyagi *et al.* 2002). These findings suggest that the protein involved in the exchange process of butyrate with bicarbonate has a broad range of specificity.
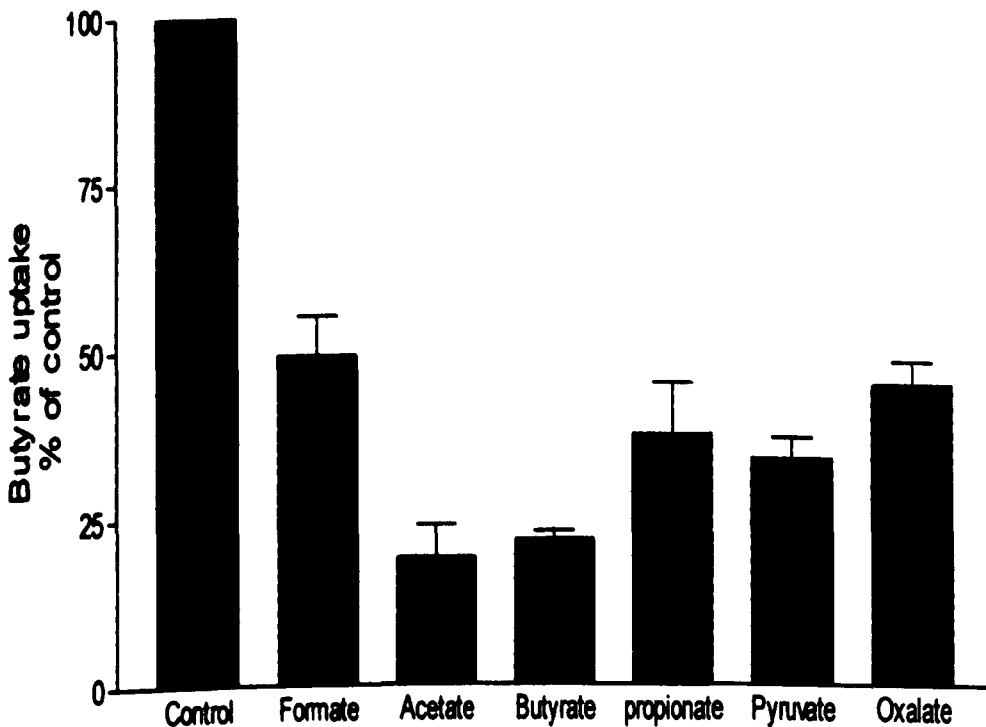
Figure IV.5. Effect of organic anions on bicarbonate-stimulated butyrate uptake. Equine colonic BLMV were preloaded with 20 mM Hepes/ Tris (pH 7.5) and 150 mM KHCO₃. The vesicles (100 µg protein per assay) were incubated in medium containing 20 mM Mes/ Tris (pH 5.5), 1mM $[^{14}C]$-butyrate,

130 mM K-gluconate and 20 mM potassium salts of indicated anions (propionate, butyrate, acetate, formate, pyruvate and oxalate). The reaction was terminated by the addition of 1 ml of ice-cold stop solution after 3 seconds. Uptake was expressed as a percentage of control. Values are presented as means ± S.E.M for three experiments.

IV.8 Effect of inorganic anions

To further assess the aptitude of the HCO_3^-/ pH-stimulated butyrate exchanger to transport sulfate, nitrate and chloride, equine colonic BLMV were incubated in the presence of 20 mM of indicated anions. Figure IV.6 showed that SO_4^{2-} and NO_3^- had no effect on the rate of butyrate uptake, whereas chloride resulted in a significant inhibition of the HCO_3^-/ pH-stimulated butyrate uptake. This leads to the possible inter-dependence of butyrate and HCO_3^-/ Cl^- exchange. Two possibilities are postulated; either butyrate and chloride are both substrates of the same HCO_3^-/ Cl^- protein, known to be widely expressed on the basolateral plasma membrane of most epithelial cells (Alper et al. 2002, Rossmann et al. 2001) especially in the intestinal cell (Alper et al. 1999). The second possibility is that the HCO_3^-/ Cl^- exchanger makes a HCO_3^- gradient available for the butyrate/ HCO_3^- exchanger, therefore increasing the extra-vesicular chloride concentration should in turn inhibit HCO_3^--stimulated butyrate uptake by reducing intracellular bicarbonate.
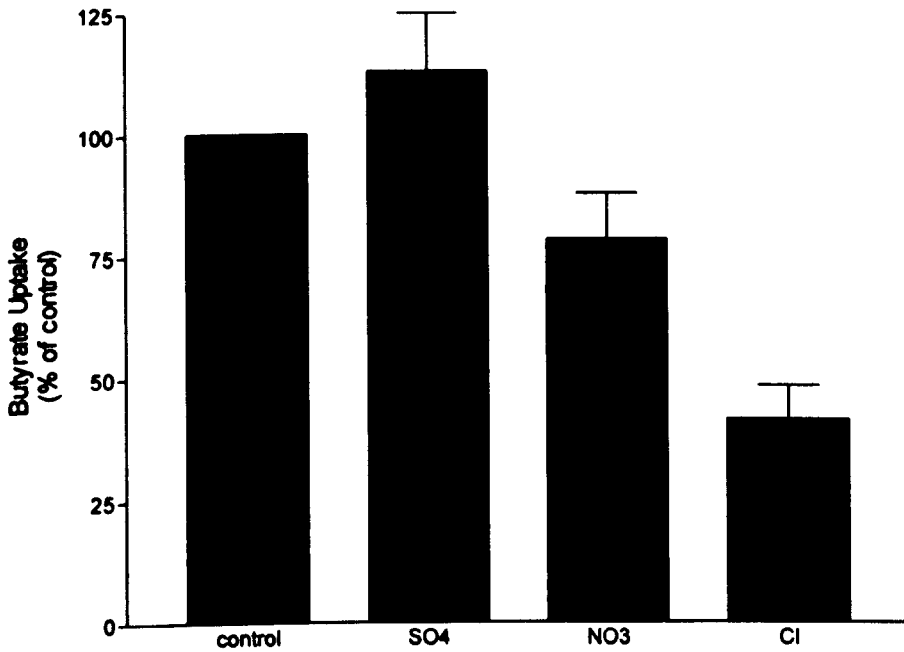
Figure IV.6. Effect of inorganic anion on butyrate uptake. The horse colonic BLMV were preloaded with 20 mM hepes/ Tris (pH 7.5) and 150 mM $KHCO_3$. Uptake was measured at 38°C for 3 seconds by incubating membrane vesicles (100 μg protein per assay) in medium containing 20 mM Mes/ Tris (pH 5.5), 1mM [^{14}C]-butyrate, 130 mM K-gluconate and 20 mM potassium salts of Cl^-, SO_4^{2-}, NO_3^-. The reaction was terminated by the addition of 1 ml of ice-cold stop solution after 3 seconds. Uptake was expressed as a percentage of control. Values are presented as means ± S.E.M for three separate experiments.

Figure IV.7. Schematic representation for the transport mechanism of butyrate in BLMV isolated from horse colon. HCO_3^- gradient is dissipated because HCO_3^- is used by both butyrate/ HCO_3^- exchanger and Cl^-/ HCO_3^- exchanger.

IV.9 Effect of transport inhibitors

Inorganic and organic/ anion exchange proteins are expressed on most if not all epithelial cells. These exchangers behave differently towards several specific transport inhibitors. The anion exchanger, which mediates sulfate transport was shown to be totally inhibited by 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS) and its homologue 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS). DIDS and SITS are known to be inhibitors of the chloride/ bicarbonate anion exchanger family (AE), including the erythrocyte band 3 (Cabantchik and Rothstein, 1972, 1974; Casey and Reithmeier, 1998), the colonic Cl^-/ HCO_3^- (AE2) in rat (Ikuma et al. 2003) and renal basolateral Cl^-/ HCO_3^- exchangers (Eladari et al. 1998).

In the present study we investigated the effect of DIDS and SITS and other specific transport inhibitors on butyrate transport across equine colonic BLMV. Prior to the experiment, the equine colonic BLMV were incubated on ice for 30 minutes with 0.5 mM DIDS, SITS, phloretin and 1 mM amiloride, pCMB and 4CHC. Control vesicles (100 µg

protein per assay) were also maintained on ice for 30 min. The initial rate (3 sec) of [^{14}C]-butyrate uptake was measured. As shown in figure IV.8, 0.5 mM of stilbene derivatives (DIDS and SITS) substantially inhibited the HCO_3^-/ pH-stimulated butyrate uptake. These results suggest that the transporter is sensitive to these inhibitors, therefore butyrate transport may be mediated by the same transport process as chloride (see figure IV.7). Lack of inhibition of butyrate uptake on the LMV by SITS and DIDS, even at high concentrations, have been reported previously (Mascolo *et al.* 1991; Harig *et al.* 1996 and Ritzhaupt *et al.* 1998a) indicating the distinct inhibitory properties of butyrate transport across the luminal and basolateral membranes.
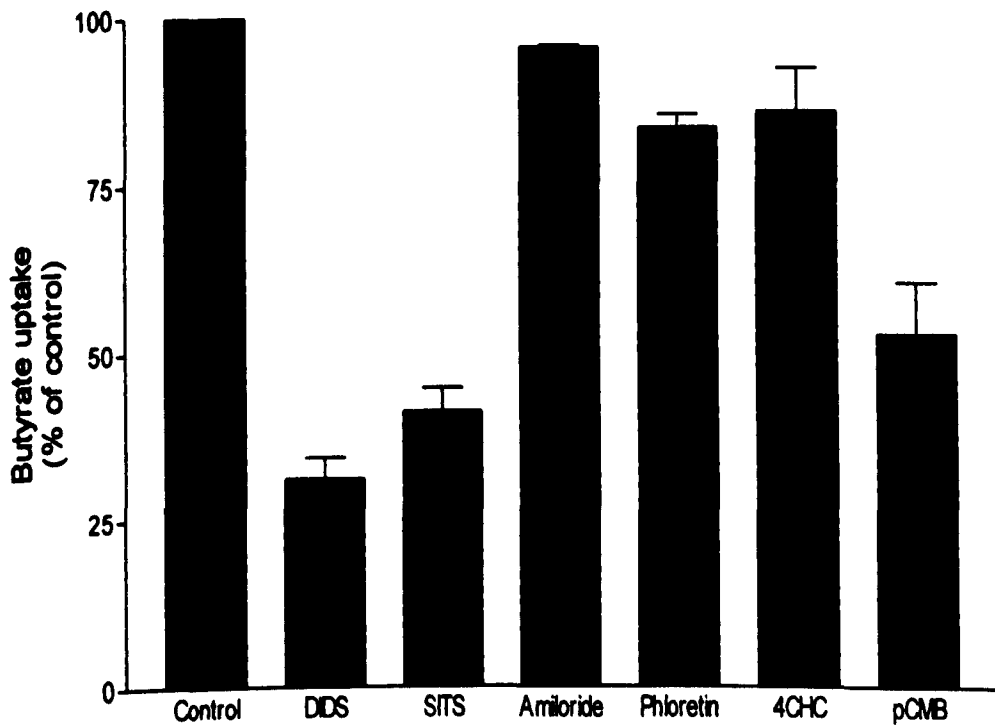
Figure IV.8. Effect of transport inhibitors on bicarbonate-stimulated butyrate uptake. Prior to the experiment, BLMV of equine colon were preloaded with 150 mM $KHCO_3$ and 20 mM Hepes/ Tris (pH 7.5) and incubated for 30 minutes at 4°C with the anion inhibitors; SIDS, SITS and Phloretin were added to the vesicles to give a final concentration of 0.5 mM, Amiloride, pCMB and 4CHC were added at a final concentration of 1 mM. uptake was measured for 3 sec by incubating the vesicles (100 µg of protein per assay) in media containing 20 mM Mes/ Tris (pH 5.5), 1mM [^{14}C]-butyrate and 150 mM K-gluconate.

After 3 seconds, the reaction was stopped by the addition of 1 ml of ice-cold stop solution. Values are presented as means ± S.E.M for four experiments.

It was revealed that many transport proteins reacted vis-à-vis to mercury compounds, which are modifiers of SH-groups (Poole and Halestrap, 1993). p-Chloromercuribenzoate pCMB), a member of the mercurials, inhibited the rate of $[^{14}C]$-butyrate uptake by up to 40%.

The presence of amiloride, a potent inhibitor of the electro-neutral Na^+-H^+ exchanger (Tyagi et al. 2000), in the incubation buffer did not influence butyrate transport. Opposite to what had been seen regarding the transport of butyrate on the LMV, 0.5 mM phloretin and 1 mM α-cyano-4-hydroxycinnamic acid (4-CHC) did not alter butyrate uptake in the presence of an inward-directed pH gradient and an outward-directed HCO_3^- gradient. It was reported that both substrates inhibit the MCT1 carrier (Cuff et al. 2002). These findings suggest that the protein involved in the transport of butyrate on the basolateral plasma membranes is distinct and different from MCT1 and may belong to the anion exchangers family (AE).

IV.10 Discussion

Dietary plant fibre is fermented in the mammalian large bowel by resident colonic microflora to monocarboxylates, commonly referred to as short chain fatty acids mainly acetate, propionate and butyrate (Cummings, 1984). Differences in SCFA production was

noticed between species. Substantial amounts are produced in herbivores especially those having voluminous fermentation chamber such as equine and to a lesser extent in omnivores (Engelhardt *et al*. 1998). In the colon, SCFA, especially butyrate, is the dominant source of fuel for epithelial cells, it may account for up to 70 – 80% of energy requirement (Bergman, 1990; Bugaut, 1987). One of the major biological effects of butyrate is to promote proliferation and growth of normal colonic mucosa (Frankel *et al*. 1994; Boosalis *et al*. 2001). Moreover, butyrate was pointed as a factor preventing the development of colorectal cancer and may be used as a potential therapeutic agent (Milovic *et al*. 2000; Scheppach *et al*. 1997; Wachtershauser and Stein, 2000).

It is well documented that SCFA are produced in the large intestine, and then absorbed throughout its length with different proportions from the caecum to the rectum (Bugaut, 1987). A number of studies have been performed to study the mechanism of trans-cellular absorption of SCFA. In the luminal membrane of the colon several different experimental approaches have been employed to assess SCFA transport. These experiments were performed either *in vivo* using *in vivo* perfusions (Ruppin *et al*. 1980; Fitch and Fleming, 1999), or *in vitro*, under short-circuit condition (Ussing chamber technique) (Charney *et al*. 1998; Choshniak and Mualem, 1997; Sellin and DeSoigne, 1990). The third approach employed uptake into membrane vesicles as a model to study SCFA transport across epithelia. The studies using the latter model conclude that butyrate is transported via a carrier-mediated anion exchange process (Mascolo *et al*. 1991; Harig *et al*. 1996; Ritzhaupt *et al*. 1998a). Whereas the first model has shown rapid absorption of SCFA which appears to be concentration dependent, the former model considers passive diffusion of protonated SCFA the main route for the transport (Sellin, 1994).

The only work published so far regarding the transport mechanism of butyrate across the colonic basolateral plasma membranes came from Reynolds *et al*. (1993) and Tyagi *et al*. (2002). Both investigators used BLMV as a system and their results were consistent with the presence of a carrier-mediated anion exchange process.

The present study was performed to give further insight on the mechanism and the properties of the exchanger responsible for butyrate transport. Membrane vesicles isolated from equine colonic basolateral domain were used to perform this study.

Evidence of the existence of SCFA in the portal vein, as well as the peripheral venous blood and the extensive metabolic use of SCFA by the liver have been shown (Cummings *et al.* 1987). This finding suggested the presence of a transport process in the basolateral membrane of the colonocytes. The present study provides compelling evidence of the existence of transport for SCFA across the basolateral plasma membranes of equine colon.

The studies carried out on human and pig LMV, showed that butyrate is transported in exchange with an anion (butyrate/ HCO_3^-, butyrate/OH^-) and was DIDS insensitive (Mascolo *et al.* 1991; Harig *et al.* 1996; Ritzhaupt *et al.* 1998a). Previous studies have demonstrated that butyrate transport on the colonic BLMV was enhanced in the presence of an inward-directed pH gradient (Reynolds *et al.* 1993; Tyagi *et al.* 2002). The exchange process found in the present study is specific for butyrate/ bicarbonate exchange and the rate of butyrate transport was enhanced in the presence of an inward-directed pH gradient. The carrier-mediated butyrate transport did not represent an electrogenic but rather an electro-neutral process, because changes in trans-membrane potential with K^+ and valinomycin did not alter the [^{14}C]-butyrate uptake (see table IV.1).

Like most anion transporters (Vanghan-Jones and Spitzer, 2002), Butyrate/ HCO_3^- exchanger is regulated in part by pH (Reynolds *et al.* 1993; Harig *et al.* 1996; Tyagi *et al.* 2002). In the absence of a bicarbonate gradient, an inward-directed pH gradient (pH_{in} > pH_{out}) produced a modest rate of butyrate uptake, whereas the presence of an outward-directed HCO_3^- gradient simultaneously with imposition of pH gradient markedly enhanced [^{14}C]-butyrate uptake. The data presented in figures IV.1 indicated that the increased butyrate uptake by a pH gradient is due to the high proton concentration and not due to butyrate/ H^+ cotransporter. The only explanation for the pH sensitivity is that butyrate is transported by a butyrate/ HCO_3^- exchange protein which have a proton sensor to which protons can bind. This is probably followed by conformational changes of the

protein therefore leading to an enhanced rate of transport. It is known that pH is a permissive regulator of ion transporters (Vanghan-Jones and Spitzer, 2002). The pH sensitivity is a complex mechanism; it involves an auto-inhibitory domain and pH sensor (Pucéat, 1999).
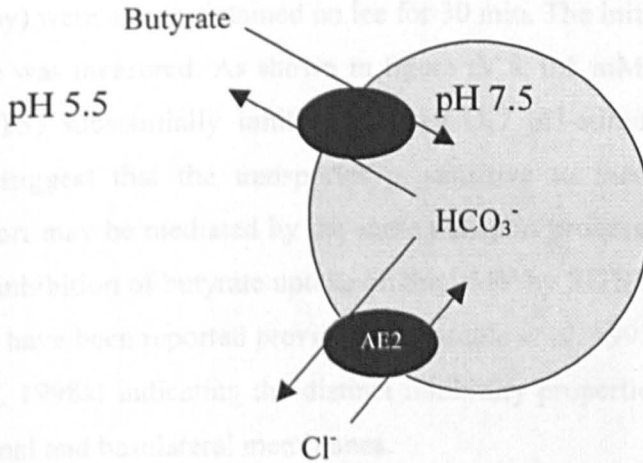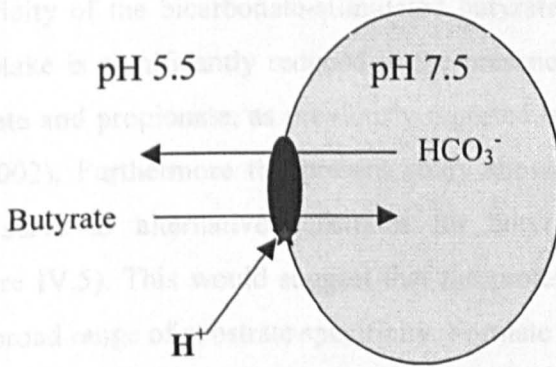

Figure IV.9. Schematic representation for the transport mechanism of butyrate in BLMV isolated from horse colon.

This finding is consistent with results of previous studies on butyrate uptake in BLMV (Reynolds et al. 1993; Tyagi et al. 2002). These authors have reported that Butyrate/ HCO_3^- exchange as the only mechanism of butyrate transport across the colonic basolateral membranes of human and rat.

Further evidence for a carrier-mediated butyrate transport process comes from the kinetics analyses. In the presence of increasing concentrations of butyrate in the extra-vesicular media, the HCO_3^--stimulated butyrate uptake demonstrates the presence of a saturable transport process. The K_m value for external butyrate is 12.2 mM with

maximum velocity (V_{max}) of 3.05 nmol/ mg protein/ 3 se. This K_m value campared well with the K_m of 17.5 mM for butyrate uptake in human colon (Tyagi *et al.* 2002). In contrast, it appears significantly higher than K_m for butyrate exchange in LMV (Harig *et al.* 1993; Hume *et al.* 1993). The K_m value presented in this study appears also to be different from K_m for butyrate transport in basolateral membrane of rat colon (Reynolds *et al.* 1993), from lactate transport in the basolateral membrane in rat jejunum (Cheeseman *et al.* 1994) and from butyrate transport in the basolateral membrane of ovine parotid gland (Nguyen and Beechey, 1999).

Substrate specificity of the bicarbonate-stimulated butyrate exchange has demonstrated that butyrate uptake is significantly reduced in the presence of 20 mM of its structural analogues; acetate and propionate, as previously reported by Reynolds *et al.* (1993) and Tyagi *et al.* (2002). Furthermore the present study showed that formate, oxalate and pyruvate may serve as alternative substrates for butyrate in the butyrate/ HCO_3^- exchanger (figure IV.5). This would suggest that the protein involved in SCFA/ HCO_3^- transport has a broad range of substrate specificity. Formate was shown to be taken up via a carrier-mediated process in exchange for chloride (Soleimani and Bizal, 1996) and recycled by nonionic diffusion in the kidney proximal tubule. Oxalate, which was revealed to be transported via a carrier-mediated process on the brush-border and basolateral membrane of rabbit ileum (oxalate/ HCO_3^- or oxalate/ Cl^-) (Knickelbein *et al.* 1986), reduced the rate of [^{14}C]-butyrate uptake in the equine colonic BLMV. On the other hand oxalate was reported to have no effect on the butyrate transport in the human colonic BLMV (Tyagi *et al.* 2002).

The erythrocyte anion exchanger band 3 (AE1) (Casey and Reithmeier, 1998) alongside with the AE2 and AE3 have been shown to catalyse the electro-neutral, sodium-independent Cl^-/HCO_3^- exchange (Kopito, 1990). This anion exchange process is specifically inhibited by very low concentrations of DIDS and SITS (Cabantchik and Rothstein, 1973). The anion exchanger isoform 2 (AE2) is expressed in the basolateral membranes of the colon (Ikuma et al. 2003; Alper *et al.* 1999) and in the basolateral membranes of the kidney (Eladari *et al.* 1998) but it was also reported to be expressed in

the apical membrane of the small intestine (Chow *et al.* 1992) and even the Golgi complex (Holappa *et al.* 2001). In the work presented in this thesis we found that AE2 but not AE1 nor AE3 is expressed in the basolateral membranes (see chapter VI).

At a concentration of 0.5 mM, the stilbene derivatives significantly inhibited the butyrate/ HCO_3^- exchanger. This result leads to the assumption of a possible role of AE2 in the transport of butyrate across the basolateral membranes. Recently, Yabuuchi *et al.* (1998) found that anion exchanger AE2 was functionally involved in the transport of organic monocarboxylates (benzoic acid, butyric acid and propionic acid) in the intestine.

In contrast to the present findings in the BLMV, DIDS and SITS inhibitors failed to inhibit MCT1, the butyrate transporter in the LMV (Ritzhaupt *et al.* 1998a). This result again supports the idea that there are two distinct anion exchange processes for butyrate transport in the BLMV and LMV.

Butyrate transport was also inhibited by pCMB, a specific inhibitor of monocarboxylate transport (Poole and Halestrap, 1993), indicating the involvement of SH-functional groups in the transport of butyrate in the basolateral membranes. Although butyrate uptake in both BLMV and LMV is stimulated by HCO_3^-, the properties of both anion exchangers differ in term of sensitivity towards potential inhibitors. 4CHC has been shown to inhibit L-lactate transport in erythrocytes (Halestrap and Price, 1999). Further studies have shown that 4CHC abolished the L-lactate uptake in rat jejunum (Cheeseman *et al.* 1994) and butyrate transport in the LMV (Ritzhaupt *et al.* 1998a; Cuff *et al.* 2002). The effect of 4CHC on butyrate transport was investigated. As shown in figure IV.8, 1 mM of 4CHC failed to inhibit butyrate transport in the equine colonic BLMV. Phloretin also failed to influence the rate of butyrate uptake. Phloretin is a potent inhibitor of monocarboxylate transporters MCT1 (Ritzhaupt *et al.* 1998a) and MCT2 (Broer *et al.* 1999).

In summary, the results presented so far show that the major route of butyrate transport across the basolateral membrane of the colonocytes is via a carrier-mediated butyrate/ HCO_3^- exchanger and not by passive diffusion. Inhibition studies revealed that the candidate transporter is distinct and separate from the previously characterised SCFA/ anion exchanger in the colonic luminal membranes. These studies do not exclude the possibility of involvement of anion exchanger type 2 (AE2) in the transport process. This possibility is explored in chapter VI.

CHAPTER V

Characterisation of butyrate transport across

the equine colonic LMV

Characterisation of butyrate transport across the equine colonic LMV

V.1 Origin and purity of the membrane vesicles

V.1.1 Introduction

Studying the trans-cellular movement of the short chain fatty acids (SCFA) across the equine colonic plasma membranes (luminal and basolateral) is the objective of this study. Characterisation of butyrate transport across the basolateral plasma membrane was delineated in detail in chapter four. This chapter gives more consideration to the mechanism of butyrate transport on the luminal membrane vesicles isolated from the colon of the same species. Studying the mechanism of short chain fatty acids transport in the LMV was approached in similar context as BLMV. The study was approached by: 1) isolating membrane vesicles from the luminal domain of the equine colonocytes, 2) assessing of the purity and origin of the faction of interest and then 3) characterising the mechanism of butyrate transport studies using radiolabelled butyrate.

The isolation method used relied on the method described by Ritzhaupt *et al.* (1998a) who successfully isolated the LMV using scrapings from human and pig colon as starting material. This method is highly reproducible and not time consuming.

Again few modifications have been introduced to Ritzhaupt's method: 1) homogenisation was performed at setting 5 of the polytron for 3 minutes, 2) the homogenate was filtered through double layer of nylon gauze to remove mucus, 3) centrifugation steps were carried out with modified time length.

To study the mechanism of solutes transport (Mascolo *et al.* 1991; Ritzhaupt *et al.* 1998a; Tyagi *et al.* 2000; Rajendran and Binder, 1994; Kinoshita *et al.* 2002), purified luminal plasma membrane vesicles were used to characterise the transport of butyrate.

The isolation of plasma membrane from the luminal pole of horse colonocytes was carried out in two stages, using cation precipitation and differential centrifugation techniques. In the original procedure for membrane purification, Harig *et al.* (1990) used $CaCl_2$ to precipitate the membranes; however this cation was substituted with $MgCl_2$ to avoid intrinsic activation of phospholipase A, therefore preventing lipid decomposition (Hauser *et al.* 1980). Mg^{+2} was employed to aggregate contaminating membranes, since its effect is likely to be correlated to the electrostatic interactions between the cation and the negatively charged glycocalyx (Schmitz *et al.* 1973). Differential centrifugation leads to the removal of the cation-bound membranes.

After isolation was accomplished, the concentration of the protein in the final luminal membrane vesicles and other sub-cellular fraction was determined by their ability to bind coomassie blue using the method described by Bradford, (1976). Pig gamma globulin was used as standard. 100 µg of protein of membrane vesicles represented the optimal protein concentration for transport studies as previously reported (Dyer *et al.* 1990; Pinches *et al.* 1993; Ritzhaupt *et al.* 1998). Thus, a concentration of 100 µg per assay was used to characterise the mechanism of butyrate uptake in the LMV.

Additional experiments were set up to characterise the luminal membrane vesicles in terms of purity and origin (as reported in section V.1.2). Next, the properties of butyrate transport in the well characterised equine colonic LMV were examined using the rapid filtration stop technique as described by Shirazi-Beechey *et al.* (1988)

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V.1.2 Immuno-detection of marker protein

Immunoblotting studies using antibodies to specific proteins characteristic of luminal and basolateral plasma membranes were performed to assess the purity and membrane origin of the LMV. Antibodies to villin, a protein expressed almost exclusively in the microvilli of the brush-border of the small and large intestine (Bretscher & Weber, 1979), MCT1, located on the luminal membranes isolated from the human and pig colonocytes (Ritzhaupt *et al.* 1998) and GLUT2, located on the intestinal basolateral plasma membranes (Shirazi-Beechey, 1996 and Pinches *et al.* 1993) were used as probes for western blots.

Samples were separated on SDS-PAGE and electro-transferred as described in the methods section.

a) Villin

The protein villin is associated with the microvilli in the brush-border of the small and large intestine (Bretscher & Weber, 1979). Therefore, it is a reliable marker for colonic luminal membranes. Western blot analysis was performed using a monoclonal antibody to mouse villin at a concentration of 1:1000. The blot was then incubated with horseradish conjugated secondary antibody (goat anti-mouse) and bands were visualised by enhanced chemiluminescence (ECL). As shown in figure V.1, the antibody was able to react with a protein with expected size of 95 kDa in the luminal membrane vesicles.
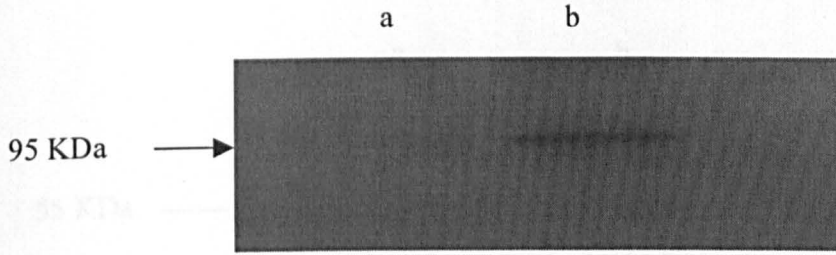
Figure V.1 Immunodetection of Villin: *Samples from equine colonic homogenate and LMV (20 µg of protein per lane) were separated on an 8 % SDS-PAGE and electrotransferred to PVDF membrane. The blot was immunostained with villin antibody and detected with peroxidase-linked rabbit anti-mouse secondary antibody as described in Methods section. Lane a, equine colonic homogenate; lane b, equine colonic LMV.*

b) GLUT2

GLUT2 is a member of the facilitative glucose transporter family. It mediates the basolateral exit of the glucose as well as fructose from the cell to the blood stream (Thorens *et al.* 1990; Bird *et al.* 1996). The presence of GLUT2 isoform in the colonic luminal membranes was further investigated to determine the potential contamination of LMV with the basolateral membranes. As shown in figure V.2, western blotting analysis using the antibody raised against the equine intestinal GLUT2 failed to detect the 55 KDa protein on the LMV fraction prepared in the isolation process from horse colonocytes.

a   b

55 KDa ⟶
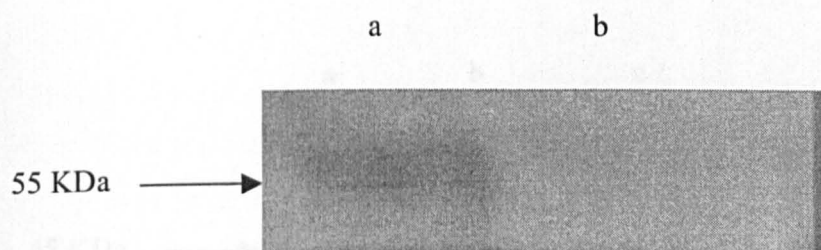
Figure V.2 Immunodetection of the GLUT2: *Samples from equine colonic LMV and the respective homogenate (20 μg of protein per lane) were separated on an 8 % SDS-PAGE and electrotransferred to PVDF membrane. The blot was immunostained with GLUT2 antibody and detected with peroxidase-linked swine anti-rabbit secondary antibody as described in Methods section. Lane a, equine colonic homogenate; lane b, equine colonic LMV.*

c) MCT1

In order to characterise the transport activity of the monocarboxylate transporter 1, expression and abundance of the corresponding MCT1 protein in the LMV isolated from equine colon was measured. A polyclonal antibody that had been raised against the COOH-terminal end of human MCT1 was used. There are significant sequence homology between MCT1 C-termini of human and equine (see below). As shown in figure V.3, the MCT1 antibody recognised a specific protein with an apparent molecular mass of 45 KDa in the LMV. This finding is consistent with that reported by Ritzhaupt *et al.* 1998b).
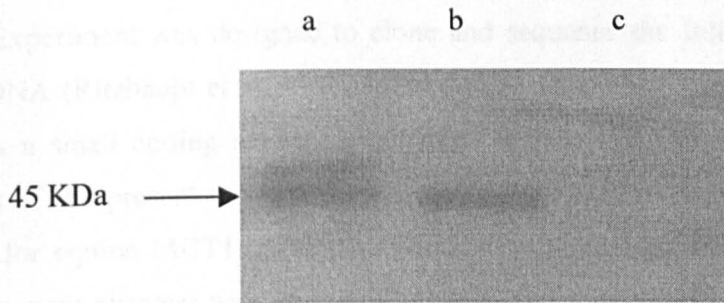
Figure V.3 Immunodetection of MCT1: *Luminal and basolateral membranes were prepared from equine colon as aforementioned (methods section). Samples from equine colonic homogenate and LMV and BLMV (20 µg of protein per lane) were separated on an 8 % SDS-PAGE, electrotransferred to PVDF membrane, immunoblotted for MCT1 antibody and detected with peroxidase-conjugated swine anti-rabbit secondary antibody. Lane a, equine colonic homogenate; lane b, equine colonic LMV; lane c, equine colonic BLMV.*

It has been shown in our laboratory that, in the oocytes of xenopus laevis microinjected with MCT1 cRNA, the transport of butyrate was higher compared with water injected oocytes. Experiment was designed to clone and sequence the full length human colonic MCT1 cDNA (Ritzhaupt et al. 1998b). The MCT1 protein is encoded by 3.3 Kb mRNA which has a small coding sequence and huge untranslated regions (UTR). One of the objectives of the present work was to determine the nucleotide sequence of the cDNA encoding for equine MCT1. A similar protocol as to that used for the cloning of AE2 cDNA (see next chapter) was employed to clone and sequence the equine MCT1 cDNA. This would allow us to determine the amino acid sequence including the C-terminus region.

The full equine MCT1 nucleotide sequence was aligned alongside the sequences from human (figure V.4) and the degree of nucleotide homology and amino acid similarity were compared as summarised in table V.1. The horse nucleotide sequence has high degree of homology with the human (75.7 %). Comparison of amino acid sequences shows 87 % similarity between horse and human.

	Human	
	Nucleotide (%)	Amino acid (%)
Horse	75.7	87

Table V.1 *Comparison of the equine MCT1 nucleotide and amino acid sequences with human colonic MCT1 sequence.*

```
                    1                                                                                                    100
MCT1 Horse     ATGCCACCGGCGGTTGGAGGTCCAGTTGGATACACTCCCCCAGATGGAGGCTGGGGGTGGGCAGTGGTAGTTGGAGCTTTCATTTCCATTGGTTTCTCTT
MCT1 Human     ATGCCACCAGCAGTTGGAGGTCCAGTTGGATACACCCCCCCAGATGGAGGCTGGGGCTGGGCAGTGGTAATTGGAGCTTTCATTTCCATCGGCTTCTCTT
Consensus      ATGCCACC GC GTTGGAGGTCCAGTTGGATACAC CCCCCAGATGGAGGCTGGGG TGGGCAGTGGTA TTGGAGCTTTCATTTCCAT GG TTCTCTT
                    101                                                                                                  200
MCT1 Horse     ATGCATTTCCAAAATCAATTACTGTGTTTTTCAAAGAAATCGAAAGTATATTCAATGCCACCACCAGTGAAGTGTCATGGATATCCTCCATCATGTTGGC
MCT1 Human     ATGCATTTCCCAAATCAATTACTGTCTTCTTCAAAGAGATTGAAGGTATATTCCATGCCACCACCAGCGAAGTGTCATGGATATCCTCCATAATGTTGGC
Consensus      ATGCATTTCC AAATCAATTACTGT TT TTCAAAGA AT GAA GTATATTC ATGCCACCACCAG GAAGTGTCATGGATATCCTCCAT ATGTTGGC
                    201                                                                                                  300
MCT1 Horse     TGTCATGTATGGTGGAGGTCCTATCAGCAGTATCCTGGTGAATAAATATGGCAGTCGTCCAATCATGATTCTTGGCGGCTGCTTGTCAGGCAGTGGCCTG
MCT1 Human     TGTCATGTATGGTGGAGGTCCTATCAGCAGTATCCTGGTGAATAAATATGGAAGTCGTATAGTCATGATTGTTGGTGGCTGCTTGTCAGGCTGTGGCTTG
Consensus      TGTCATGTATGGTGGAGGTCCTATCAGCAGTATCCTGGTGAATAAATATGG AGTCGT  A TCATGATT TTGG GGCTGCTTGTCAGGC GTGGC TG
                    301                                                                                                  400
MCT1 Horse     ATTGCGGCTTCTTTCTGTAACACTGTGCAGGAACTTTACTTGTGTATCGGTGTCATTGGAGGTCTTGGGCTTGCCTTCAACTTGAATCCGGCTCTGACCA
MCT1 Human     ATTGCAGCTTCTTTCTGTAACACCGTACAGCAACTATACGTCTGTATTGGAGTCATTGGAGGTCTTGGGCTTGCCTTCAACTTGAATCCAGCTCTGACCA
Consensus      ATTGC GCTTCTTTCTGTAACAC GT CAG AACT TAC T TGTAT GG GTCATTGGAGGTCTTGGGCTTGCCTTCAACTTGAATCC GCTCTGACCA
                    401                                                                                                  500
MCT1 Horse     TGATTGGCAAGTATTTCTACAAGAGGCGACCCTTGGCAAATGGACTAGCCATGGCAGGCAGCCCTGTGTTCCTGTCTACCCTGGCCCCCCTCAATCAGGC
MCT1 Human     TGATTGGCAAGTATTTCTACAAGAGGCGACCATTGGCCAACGGACTGGCCATGGCAGGCAGCCCTGTGTTCCTCTGTACTCTGGCCCCCCTCAATCAGGT
Consensus      TGATTGGCAAGTATTTCTACAAGAGGCGACC TTGGC AA GGACT GCCATGGCAGGCAGCCCTGTGTTCCT T TAC CTGGCCCCCCTCAATCAGG
                    501                                                                                                  600
MCT1 Horse     TTTCTTCGGTATCTTTGGCTGGAGAGGAAGCTTCCTAATTCTTGGGGGCTTCCTATTAAACTGCTGTGTGGCTGGAGCCCTGATGCGGCCAATAGGGCCC
MCT1 Human     TTTCTTCGGTATCTTTGGATGGAGAGGAAGCTTTCTAATTCTTGGGGGCTTGCTACTAAACTGCTGTGTTGCTGGAGCCCTCATGCGACCAATCGGGCCC
Consensus      TTTCTTCGGTATCTTTGG TGGAGAGGAAGCTT CTAATTCTTGGGGGCTT CTA TAAACTGCTGTGT GCTGGAGCCCT ATGCG CCAAT GGGCCC
                    601                                                                                                  700
MCT1 Horse     AAGCCAACCAATGCAAAGAAAGAGAGGTCTAAAGAATCCCTTCAGGAAGCCGGAAAACCTGATGCACAAAAAGGGGCAGGTGATGCAAATACAGATCTTA
MCT1 Human     AAGCCAACCAAGGCAGGGAAAGATAAGTCTAAAGCATCCCTTGAGAAAGCTGGAAAATCTGGTGTGAAAAAAGATCTGCATGATGCAAATACAGATCTTA
Consensus      AAGCCAACCAA GCA  GAAAGA A GTCTAAAG ATCCCTT AG AAGC GGAAA CTG TG   AAAAG      TGATGCAAATACAGATCTTA
                    701                                                                                                  800
MCT1 Horse     TTGGAGGATACCCCAAGGAGGAGAAACAGTCAGTCTTCCAAACAATTAATAAATTCCTGGACTTATCCCTGTTCACACACAGAGGCTTCTTGCTATACCT
MCT1 Human     TTGGAAGACACCCTAAACAAGAGAAACGATCAGTCTTCCAAACAATTAATCAGTTCCTGGACTTAACCCTATTCACCCACAGAGGCTTTTTGCTATACCT
Consensus      TGGA GA ACCC AA  A GAGAAAC  TCAGTCTTCCAAACAATTAAT A TTCCTGGACTTA CCCT TTCAC CACAGAGGCTT TTGCTATACCT
                    801                                                                                                  900
MCT1 Horse     CTCTGGAAATGTGCTCATGTTTTTTGGACTATTTACTCCTTTAGTCTTTCTTAGTAATTATGGCAAGAGTCAGCATTACTCTAGTGAGAAGTCCGCCTTC
MCT1 Human     CTCTGGAAATGTGATCATGTTTTTTGGACTCTTTGCACCTTTGGTGTTTCTTAGTAGTTATGGGAAGAGTCAGCATTATTCTAGTGAGAAGTCTGCCTTC
Consensus      CTCTGGAAATGTG TCATGTTTTTTGGACT TTT C CCTTT GT TTTCTTAGTA TTATGG AAGAGTCAGCATTA TCTAGTGAGAAGTC GCCTTC
                    901                                                                                                  1000
MCT1 Horse     CTTCTTTCCATTCTGGCTTTTGTCGACATGGTAGCCAGACCTTCTATGGGACTTGTAGCCAACACAAAGTGGATAAGACCTCGAGTTCAGTATTTCTTTG
MCT1 Human     CTTCTTTCCATTCTGGCTTTTGTTGACATGGTAGCCCGACCATCTATGGGACTTGTAGCCAACACAAAGCCAATAAGACCTCGAATTCAGTATTTCTTTG
Consensus      CTTCTTTCCATTCTGGCTTTTGT GACATGGTAGCC GACC TCTATGGGACTTGTAGCCAACACAAAG   ATAAGACCTCGA TTCAGTATTTCTTTG
```

```
        1001                                                                                          1100
MCT1 Horse   CTGCTTCTATCGTTGCAAATGGAGTGTGTCATCTGCTAGCACCTTTATCCTCCAGCTATATTGGGTTCTGTGTCTATGCGGGATTCTTTGGATTTGCATT
MCT1 Human   CGGCTTCCGTTGTTGCAAATGGAGTGTGTCATATGCTAGCACCTTTATCCACTACCTATGTTGGATTCTGTGTCTATGCGGGATTCTTTGGATTTGCCTT
Consensus    C GCTTC  T GTTGCAAATGGAGTGTGTCAT TGCTAGCACCTTTATCC C A CTAT TTGG TTCTGTGTCTATGCGGGATTCTTTGGATTTGC TT
        1101                                                                                          1200
MCT1 Horse   TGGGTGGCTCAGCTCCGTATTGTTTGAAACACTGATGGACCTTGTTGGACCTCAGAGGTTCTCCAGCGCTGTGGGATTGGTGACCATTGTGGAATGCTGT
MCT1 Human   CGGGTGGCTCAGCTCCGTATTGTTTGAAACATTGATGGACCTTGTTGGACCCCAGAGGTTCTCCAGCGCTGTGGGATTGGTGACCATTGTGGAATGCTGT
Consensus    GGGTGGCTCAGCTCCGTATTGTTTGAAACA TGATGGACCTTGTTGGACC CAGAGGTTCTCCAGCGCTGTGGGATTGGTGACCATTGTGGAATGCTGT
        1201                                                                                          1300
MCT1 Horse   CCTGTCCTGCTGGGGCCACCACTTTTAGGTCGTCTCAATGACATATATGGAGACTACAAATACACATACTGGGCGTGTGGCATAATCCTAATCGTTGCGG
MCT1 Human   CCTGTCCTCCTGGGGCCACCACTTTTAGGTCGGCTCAATGACATGTATGGAGACTACAAATACACATACTGGGCATGTGGCGTCGTCCTAATTATTTCAG
Consensus    CCTGTCCT CTGGGGCCACCACTTTTAGGTCG CTCAATGACAT TATGGAGACTACAAATACACATACTGGGC TGTGGC T   TCCTAAT  TT C G
        1301                                                                                          1400
MCT1 Horse   GCATCTATCTCTTCATTGGCATGGGCATCAATTATCATCTTCTGGCAAAAGAACAGAAAGCAGAGAAGAAGCAGAGAAAGGAAAGTAAAGAGGTTGAGAC
MCT1 Human   GTATCTATCTCTTCATTGGCATGGGCATCAATTATCGACTTTTGGCAAAAGAACAGAAAGCA---AACGAGCAGAAAAAGGAAAGTAAAGAGGAAGAGAC
Consensus    G ATCTATCTCTTCATTGGCATGGGCATCAATTATC  CTT TGGCAAAAGAACAGAAAGCA   AA  AGCAGA AAAGGAAAGTAAAGAGG  GAGAC
        1401                                                                                          1500
MCT1 Horse   CGGTGTGGATGTTGCTGAGAAACCAAAGGAAGTTACCAATGCAGCAGGATCTCCAGAGCAGAAAGGCACAGAAGGAGACCCCAAAGAGGAG---AGTCCA
MCT1 Human   CAGTATAGATGTTGCTGGGAAGCCAAATGAAGTTACCAAAGCAGCAGAATCTCCGGACCAGAAAGACACAGAAGGAGGGCCCAAGGAGGAGGAAAGTCCA
Consensus    C GT T GATGTTGCTG GAA CCAAA GAAGTTACCAA GCAGCAG ATCTCC GA CAGAAAG CACAGAAGGAG  CCCAA GAGGAG   AGTCCA
        1501                                                                                          1600
MCT1 Horse   CTCTGAACCCATGGGGCTGAAGGGTAAATGGAGCAACTTGTGACCCAAGATATCGGAAAATGTTCTGCTGGCCTGTAATCTACCAGTGGTGCTCAATGCA
MCT1 Human   GTCTGAATCCATGGGGCTGAAGGGTAAATTGAGCAGTTCATGACCCAGGATATCTGAAAATATTCTACTGGCCTGTAATCTACCAGTGGTGCTCAATGCA
Consensus    TCTGAA CCATGGGGCTGAAGGGTAAAT GAGCA T  TGACCCA GATATC GAAAT TTCT CTGGCCTGTAATCTACCAGTGGTGCTCAATGCA
        1601
MCT1 Horse   AATAGCGGACATTCATATGGAAATCATACCAGGTGTTAGTTGATGGAATTTTTGTTTCACTCCTT
MCT1 Human   AATAGTAGACATTGTGTGGAAATCATACCAGTTGTTCATTGATGGGATTTTTGTTTGACTCCTT
Consensus    AATAG  GACATT  T TGGAAATCATACCAG TGTT  TTGATGG ATTTTTGTTT ACTCCTT
```

Figure V.4. *Alignment of the nucleotide coding sequence of the equine MCT1.*

This later was compared with the MCT1 sequence from human.

V.2 Transport studies

V.2.1 Introduction

Short chain fatty acids (acetate, propionate and butyrate) are of considerable interest due to their key role as fuel source for normal colonic epithelia (Scheppach *et al.* 1994). There has been evidence for the role of butyrate in maintaining mucosal health by regulating genes associated with cell proliferation, differentiation and apoptosis (Archer *et al.* 1998., Hague *et al.* 1997., Kim *et al.* 1994., Buda *et al.* 2002., Siavoshian *et al.* 2000).

Microbial produced short chain fatty acids (Ahmed *et al.* 2000., Pryde *et al.* 2002) are absorbed from the colonic lumen into the cell crossing a biological barrier (luminal membrane). Recent studies have shown that SCFA are readily absorbed giving evidence that: 1) the concentration of SCFA in the colon is more than the quantity excreted in the feces, 2) the detection of high levels of acetate, propionate and butyrate in the portal vein and the peripheral venous blood (Cummings *et al.* 1987., Hoverstad, 1986).

It was reported that SCFA transport across the luminal membrane of the colon occurs mainly via carrier-mediated mechanism, while evidence of simple diffusion of these anions through the luminal side of the colonocytes was also reported (Titus & Ahearn, 1992).

Studies utilising plasma membrane vesicle preparations from human, pig, and rat showed that butyrate is transported via an anion exchange process with intracellular bicarbonate or hydroxide (butyrate/ HCO_3^-, butyrate/ OH^-) (Mascolo *et al.* 1991., Harig *et al.* 1996., Ritzhaupt *et al.* 1998a). All authors revealed that: 1) butyrate uptake was enhanced by extravesicular acidic pH, 2) the classical anion exchange inhibitors (SITS and DIDS) had no effect on butyrate uptake and 3) the butyrate uptake was inhibited by C2- C5 SCFA indicating that the transporter mediating butyrate uptake is a highly SCFA-specific carrier.

Work from the laboratory has demonstrated that: 1) the protein that mediates butyrate transport belongs to the monocarboxylates transporter family and is MCT1, 2) MCT1 is expressed specifically on the luminal membrane of the colon, 2) butyrate uptake in MCT1 cRNA injected oocytes exhibits significant rates of butyrate uptake (Ritzhaupt *et al.* 1998b). This data was supported by the studies carried out by Cuff *et al.* (2002) and Stein *et al.* (2000) analysing butyrate uptake in colon-derived cell lines.

During the investigation of the mechanism of both butyrate and L-lactate transport in LMV isolated from human and pig colon, Ritzhaupt *et al.* (1998b) has proposed a model by which both substrates could be transported into the vesicles. This model is depicted schematically in figure V.5.
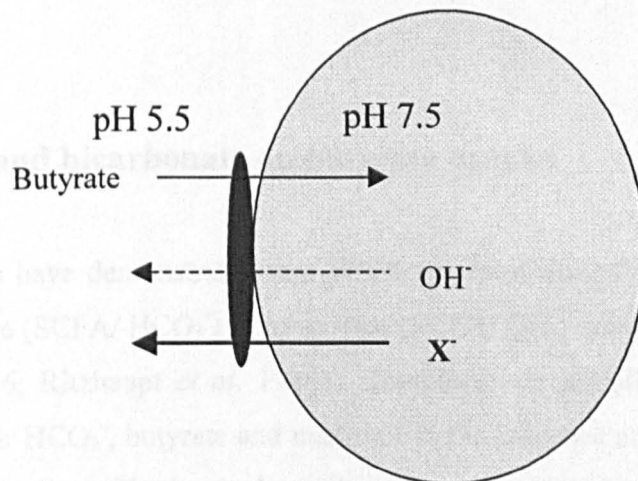
X^- = butyrate, bicarbonate, L-lactate

Figure V.5. *Schematic representation of butyrate transport into human and pig colonic LMV as proposed by Ritzhaupt et al. (1998)*

The model shows that butyrate is transported via a carrier mediated mechanism, either by H^+ - symporter or OH^- exchanger. However the presence of an internal anion (butyrate or bicarbonate) stimulates butyrate uptake and imposition of an inward-directed pH gradient further enhance the uptake (most supported model).

In the work reported in this thesis, butyrate transport was measured using isolated purified and well characterised horse colonic luminal membrane vesicles (LMV) and radiolabelled butyrate, to identify the mechanism and the properties of the transporter responsible for moving butyrate from the lumen of the colon into the colonocytes.

V.2.2 Effect of pH and bicarbonate on butyrate uptake

Previous studies have demonstrated that SCFA transport occurs via an exchange process with bicarbonate (SCFA/ HCO_3^-) or hydroxide (SCFA/ OH^-) anion (Mascolo *et al.* 1991; Harig *et al.* 1996; Ritzhaupt *et al.* 1998a). Therefore, we initially examined the effects of intra-vesicular HCO_3^-, butyrate and mannitol in the presence and absence of an outward directed OH^- gradient. The luminal membrane vesicles were loaded with Hepes/ Tris (pH 7.5) and either bicarbonate, butyrate or mannitol. The vesicles were incubated in medium that contained 1mM [^{14}C]-butyrate and Na-gluconate, pH of either 7.5 or 5.5. As shown in figure V.6, in the presence of an outward-directed directed HCO_3^- or butyrate gradient and presence of OH^- gradients (pH$_{in}$ > pH$_{out}$), the uptake of [^{14}C]-butyrate into equine colonic LMV was stimulated compared with the uptake observed in the absence of both anions (mannitol-loaded vesicles) and pH gradients. This data suggests that butyrate prefers intravesicular anion for the exchange process.
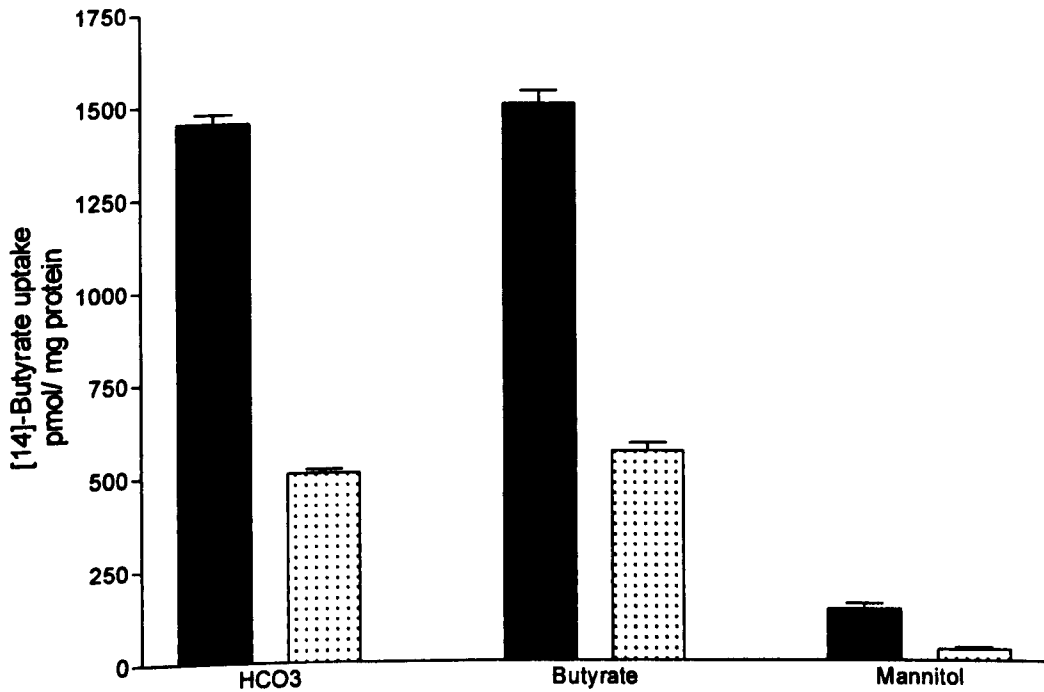
Figure V.6. Effect of substrates and pH gradients on butyrate uptake. LMVs were preloaded with 20 mM Hepes/Tris (pH 7.5) and either 300 mM mannitol or 100 mM mannitol and 100 mM of either NaHCO₃ or Na-Butyrate. Uptake was measured by incubating the vesicles (100 μg protein per assay) at 38°C for 5 seconds in the medium containing 100 mM mannitol, 100 mM Na-gluconate, 0.1 mM MgSO₄, 1 mM [¹⁴C]-butyrate and either 20 mM Mes/ Tris pH 5.5 ▇ or 20 mM Hepes/ Tris pH 7.5 ⬚ . Values are presented as the means ± S.E.M. for two separate experiments performed in triplicate.

V.2.3 Time course of butyrate uptake

Equine colonic LMV were loaded with sodium butyrate (Na-But) buffered at pH 7.5. The vesicles were incubated at 38°C for the indicated time periods in the incubation media containing Na-gluconate and 1 mM radio-labeled butyrate buffered at pH 5.5. The time course of the butyrate uptake is shown in figure V.7. The amount of butyrate taken

up into the vesicles produces an "overshoot" after 10 seconds time. The uptake reached an equilibrium level around approximately 30 min. This pattern of transport suggests the presence of a carrier mediated mechanism in the LMV. In contrast measuring butyrate uptake in the basolateral membrane vesicles no overshoot was observed (chapter four). The uptake of butyrate was linear up to 5 seconds (inset figure); therefore an uptake period of 5 sec was employed as an initial rate in the subsequent experiments.
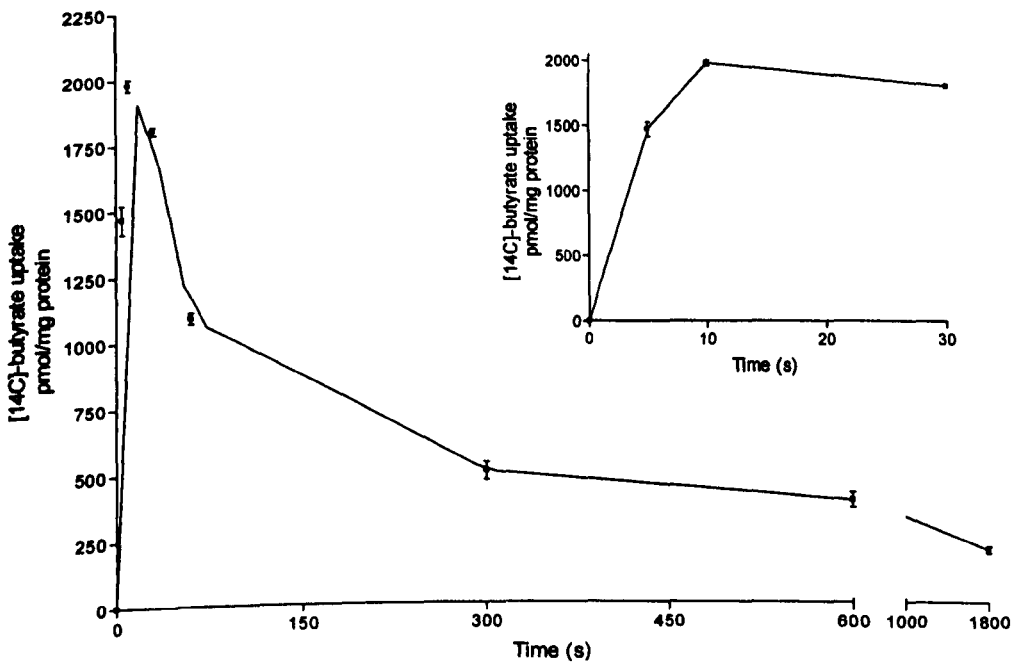
Figure V.7. Time course of butyrate uptake. LMVs were preloaded with 20 mM Hepes/Tris (pH 7.5), 100 mM mannitol and 100 mM Na-butyrate. Uptake was measured by incubating the vesicles (100 µg protein per assay) at 38°C for the indicated length of time in medium containing 100 mM mannitol, 100 mM Na-gluconate, 0.1 mM $MgSO_4$, 1 mM [^{14}C]-butyrate and 20 mM Mes/ Tris pH 5.5. Values are presented as the means ± S.E.M. for two separate experiments performed in triplicate. Inset shows the initial rate of butyrate uptake.

V.2.4 Effect of extra-vesicular pH on butyrate uptake

Previous experiments performed to study the mechanism of butyrate transport using LMV isolated from rat, human and pig colon, showed that butyrate uptake was significantly higher while pH gradient was imposed ($pH_{in} > pH_{out}$) compared to that in the absence of pH gradient ($pH_{in} = pH_{out}$) (Mascolo *et al.* 1991., Harig *et al.* 1996., Ritzhaupt *et al.* 1998a). In order to elucidate whether butyrate uptake in LMV, isolated from equine colonocytes, is stimulated by changes in the extra-vesicular medium pH, butyrate uptake was performed in the presence of different pH gradients. The equine colonic LMV, preloaded with a buffer containing Na-butyrate, pH 7.5, were incubated in an iso-osmolar uptake media consisting of Na-gluconate (gluconate is a membrane non-permeable anion (Shirazi-Beechey *et al.* 1990)) and either Mes/ Tris (pH 5.5, 6.0, 6.5) or Hepes/ Tris (pH 7.0, 7.5, 8.0), in addition to 1 mM [U-^{14}C]-butyrate. As shown in figure V.8, the initial rate of butyrate uptake was significantly higher at low pH with an optimal uptake at pH 5.5, corresponding to an uptake level of 1470 pmol. (mg protein)$^{-1}$.5 sec^{-1}. There was 5-fold enhancement of the HCO_3^--stimulated butyrate uptake at pH 5.5 compared to pH 8.0.
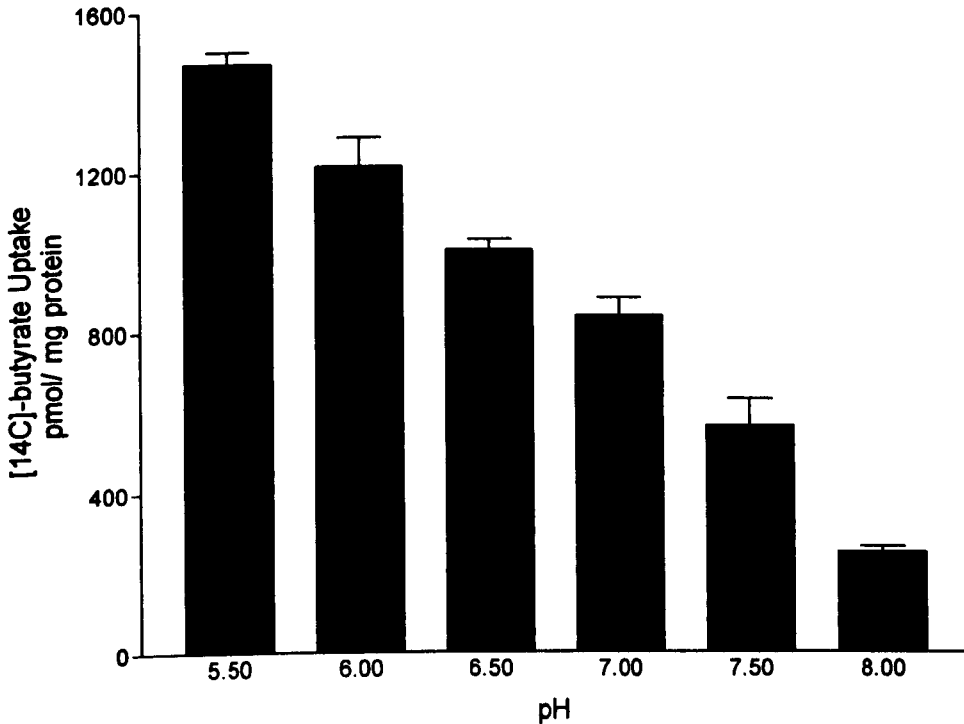
Figure V.8. Effect of various extravesicular pH on butyrate uptake. Equine colonic LMVs were preloaded with 100 mM mannitol, 100 mM Na-butyrate and 20 mM Hepes/ Tris (pH 7.5). The vesicles (100 µg protein per assay) were incubated at 38°C for 5 seconds by dilution in the incubation medium containing 100 mM mannitol, 100 mM Na-gluconate, 0.1 mM $MgSO_4$, 1 mM [^{14}C]-butyrate and 20 mM Hepes/Tris with pH ranges of 7.0, 7.5, 8.0 or Mes/Tris with pH ranges of 5.5, 6.0, 6.5. Values are presented as the means ± S.E.M. for two separate experiments performed in triplicate.

V.2.5 Kinetics

To assess the saturability of butyrate transport, the effect of varying butyrate concentrations on the initial rates of the uptake was investigated. The LMV were loaded with bicarbonate and incubated in reaction media containing gluconate and increasing concentrations of butyrate (0.5 – 50 mM). Bicarbonate-loaded vesicles were used since HCO_3^- gave similar uptake rates as butyrate (figure V.4) and to avoid any potential

alterations in the butyrate concentration of the incubation media by the vesicle suspension buffer. The uptake was measured in the presence of pH and HCO_3^- gradients.

The results presented in figure V.9 show that the increase in butyrate concentration was associated with an increased rate of $[^{14}C]$-butyrate uptake. The HCO_3-stimulated butyrate uptake was a saturable process, which is a characteristic feature of a carrier-mediated mechanism in these membranes. Linear regression analysis of Hanes plot demonstrate an apparent Michaelis-Menten constant (K_m) of 5.61 mM and maximum velocity (V_{max}) of 614.32 pmol/ mg protein/ se (figure V.10).
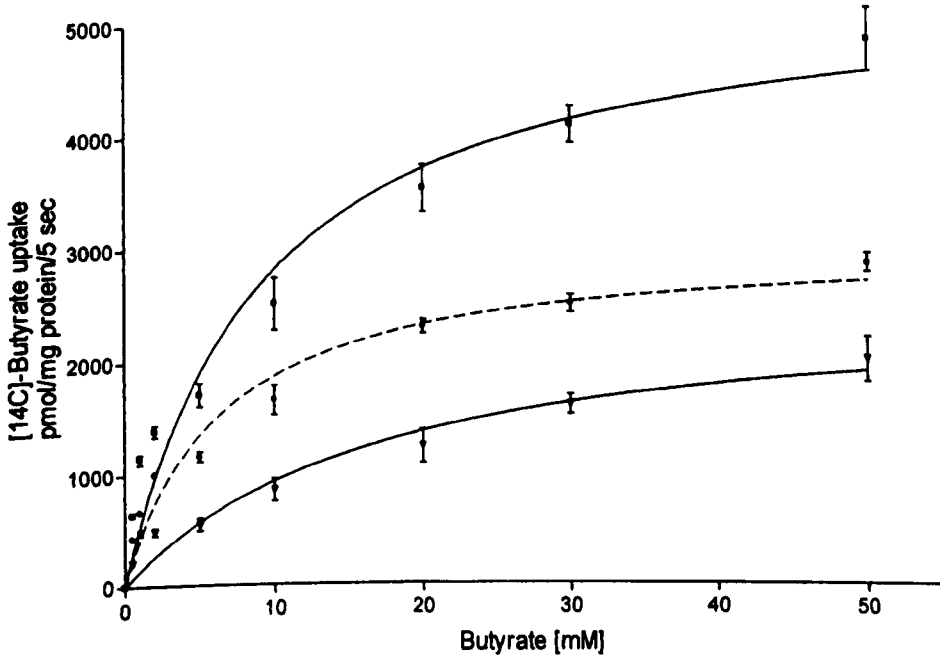
Figure V.9 Michealis-Menten curve. The initial rate (5 sec) of $[^{14}C]$-butyrate uptake by LMV was determined with an increasing butyrate concentration over the range 0.5-50 mM at 38°C. The vesicles (100 µg protein per assay) were preloaded with 100 mM mannitol, 100 mM $NaHCO_3$, 0.1 mM $MgSO_4$ and 20 mM Hepes/ Tris (pH 7.5) and diluted into incubation media containing 20 mM Mes/ Tris (pH 5.5) or Hepes/Tris (pH 7.5), 100 mM mannitol, 1 mM $[^{14}C]$-butyrate and varying concentrations of sodium butyrate and sodium gluconate. Carrier-mediated transport was determined by subtracting the uptake values in the presence of pH from those in the absence of pH gradient (dotted line). Values are presented as means ± S.E.M for two experiments.
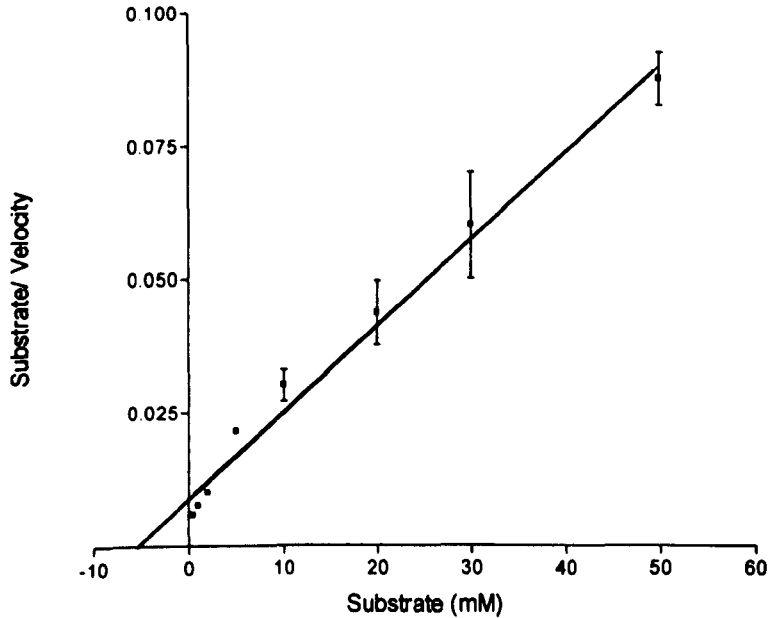
Figure V.10 Hanes plot of linear regression analysis. The graph was plotted to calculate K_m and V_{max}.

V.2.7 Effect of organic and inorganic anions on butyrate uptake

A series of experiments was designed to determine the specificity of the butyrate exchange process. The effects of 20 mM monocarboxylates (formate, acetate, propionate, pyruvate and lactate) in the incubation media on the initial rate of [^{14}C]-butyrate uptake was investigated. As shown if figure V.11, addition of 20 mM sodium salt of indicated organic anions resulted in varying inhibitions of the initial rate of butyrate uptake. Acetate, propionate, pyruvate and lactate reduced the rate of [^{14}C]-butyrate uptake by up to 50 % whereas the C1 monocarboxylate, formate, resulted in no inhibition of butyrate uptake. These results agree with what has been reported using human colonic LMV (Harig *et al.* 1996; Ritzhaupt *et al.* 1998a). These findings suggest that the protein involved in the

exchange process of butyrate with bicarbonate has a more limited range of specificity compared to the butyrate/ HCO_3^- exchanger on the basolateral membrane (section IV.7).

In another sets of experiments, the effect of inorganic anions (sulfate, nitrate and chloride) on butyrate uptake was assessed. Equine colonic LMV were incubated in the presence of 20 mM of indicated anions. Figure V.11 showed that the presence of SO_4^{-2}, NO_3^- and Cl^- had no effect on the rate of butyrate uptake.
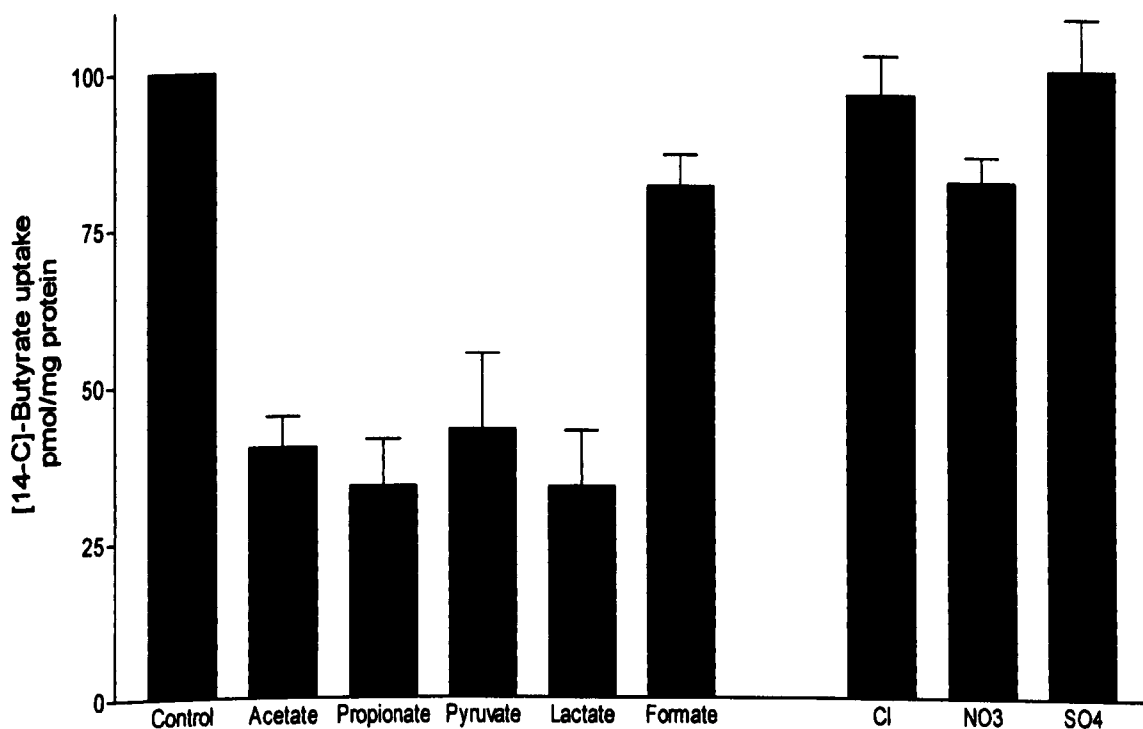
Figure V.11. Effect of organic and inorganic anions on butyrate uptake. Equine colonic LMV were preloaded with 20 mM Hepes/ Tris (pH 7.5) and 100 mM mannitol, 100 mM Na-butyrate. The vesicles (100 µg protein per assay) were incubated in medium containing 20 mM Mes/ Tris (pH 5.5), 0.1 mM $MgSO_4$, 1mM [^{14}C]-butyrate, 80 mM mannitol, 100 mM Na-gluconate and 20 mM sodium salts of indicated anions (acetate, propionate, pyruvate, lactate and formate, Cl^-, NO_3^- and 10 mM of SO_4^{-2}). The reaction was terminated by the addition of 1 ml of ice-cold stop solution after 5 seconds. Uptake was expressed as a percentage of control. Values are presented as means ± S.E.M for three experiments.

V.2.8 Effect of transport inhibitors

Anion exchange processes behave differently towards transport inhibitors. Therefore the potential inhibition of the colonic luminal butyrate/ anion exchanger was assessed. DIDS and SITS are known to be specific inhibitors for the chloride/ bicarbonate anion exchanger family (AE), including the erythrocyte band 3 (Cabantchik and Rothstein, 1972, 1974; Casey and Reithmeier, 1998), the colonic Cl^-/HCO_3^- in rat (Ikuma et al. 2003) and renal basolateral membrane Cl^-/HCO_3^- exchangers (Eladari et al. 1998).

In the present study we investigated the effect of DIDS and SITS and other inhibitors on butyrate transport across equine colonic LMV. Prior to the experiment, the equine colonic LMV were incubated on ice for 30 minutes with 0.5 mM DIDS, SITS, phloretin and 1 mM amiloride, pCMB and 4CHC. Control vesicles (100 μg protein per assay) were also maintained on ice for 30 min. The initial rate (5 sec) of [^{14}C]-butyrate uptake was measured. As shown in figure V.12, DIDS, SITS and the sodium-proton inhibitor (amiloride) did not alter butyrate uptake in the presence of butyrate and pH gradients. These results suggest that the transporter is insensitive to these inhibitors; therefore butyrate transport on the luminal membranes appears to be mediated by a dissimilar transport protein compared to baolateral membranes. Lack of inhibition of butyrate uptake on the LMV by SITS and DIDS, even at high concentrations, have been reported previously (Mascolo et al. 1991; Harig et al. 1993 and Ritzhaupt et al. 1998a) indicating the distinct inhibitory properties of butyrate transport across the luminal and basolateral membranes.

On the other hand, 1mM of pCMB (a member of the mercurials), α-cyano-4-hydroxycinnamic acid (4-CHC) and 0.5 mM phloretin inhibited the rate of [^{14}C]-butyrate

uptake by up to 50%. Previous work performed in our laboratory (Cuff *et al.* 2002) indicated that both phloretin and 4CHC inhibit the MCT1 carrier (Poole and Halestrap, 1993). These findings further support the idea that the protein involved in the transport of butyrate on the luminal membrane of the equine colon is distinct from that on basolateral plasma membranes.
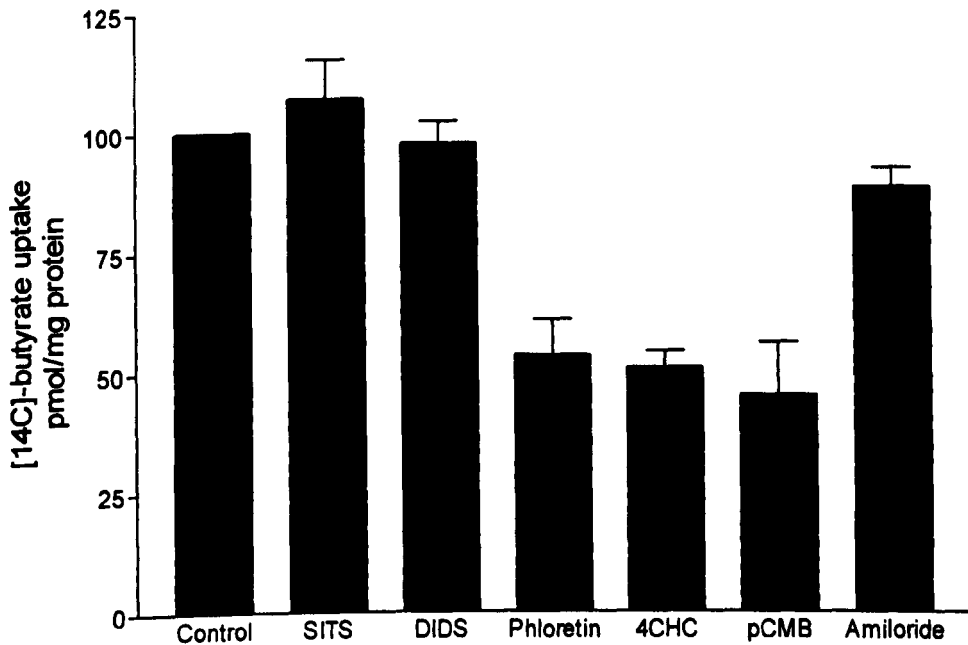
Figure V.12. Effect of transport inhibitors on butyrate uptake. Prior to the experiment, equine colonic LMV of were preloaded with 100 mM mannitol, 100 mM Na-butyrate and 20 mM Hepes/ Tris (pH 7.5) and incubated for 30 minutes at 4^0C with the anion inhibitors; SIDS, SITS and Phloretin were added to the vesicles to give a final concentration of 0.5 mM, Amiloride, pCMB and 4CHC were added at a final concentration of 1 mM. uptake was measured for 5 sec by incubating the vesicles (100 μg of protein per assay) in media containing 20 mM Mes/ Tris (pH 5.5), 1mM [^{14}C]-butyrate, 100 mM mannitol and 100 mM Na-gluconate. After 5 seconds, the reaction was stopped by the addition of 1 ml of ice-cold stop solution (see methods). Values are presented as means ± S.E.M for two experiments performed in triplicate.

V.3 Discussion

Short chain fatty acids (SCFA) mainly acetate, propionate and butyrate, are produced by fermentation of non-absorbed carbohydrates by resident microflora of the equine colon (Bugaut, 1987., Engelhardt *et al.* 1998). These SCFA or monocarboxylates, represent the majority of anions in the colonic lumen. They are absorbed throughout the length of the large intestine from the caecum to the rectum (Bugaut, 1987) and are metabolised by the colonocytes.

Increasing evidence regarding the significant role of SCFA, especially butyrate, in the physiology and pathophysiology of the colon has been reported (Cook & Sellin, 1998; Tappenden *et al.* 1998; Koruda *et al.* 1989). Short chain fatty acids have been shown to stimulate sodium absorption hence water assimilation as well. This effect might be the result of an increasing SCFA inside the cell resulting in escalating intracellular acidity therefore activation of Na^+/H^+ exchanger (Musch *et al.* 2001).

In an attempt to investigate the mechanism of butyrate transport across the apical membrane of the equine colon, a well defined method for the isolation of luminal membrane vesicles from the colon was used (Ritzhaupt *et al.* 1998) with some modifications (see section V.1.1). The isolation procedure of the LMV was carried out using Mg^{+2} precipitation and differential centrifugation techniques.

The purity of isolated equine colonic LMV was characterised by western blot analysis using antibodies for: a) villin, a major marker of microvilli at the apical surface of colonocytes, b) MCT1, which was shown to be located on the luminal membranes of human and pig colon, and c) GLUT2, the predominant facilitative Na^+-independent glucose transporter located on the basolateral membrane of the small and large intestine (Thorens, 1996). The results revealed immuno-localisation of villin and MCT1 at molecular weights of 95 KDa and 45 KDa respectively in the LMV fraction whereas, GLUT2 antibody failed to detect any protein. This data indicates that the fraction of interest originate from the luminal plasma membranes of the colon. These vesicles were used to characterise the mechanism of butyrate transport.

It has been proposed that SCFA across the membranes of the epithelium by non-ionic diffusion (Sehested *et al*. 1999; Engelhardt *et al*. 1993). This model showed that SCFA coupled to the H^+ originated from Na^+/H^+ exchanger. However, since 99 % of SCFA are ionised at the physiologic pH of the colon, the presence of a carrier-mediated process was emphasised (Mascolo *et al*. 1991; Harig *et al*. 1996).

Utilising LMV, our data have demonstrated the existence of a carrier mediated transport process (mainly butyrate/ HCO_3^- exchanger) which was markedly stimulated in the presence of an outward directed OH^- gradient. The existence of a minor part of nonionic diffusion in butyrate uptake cannot be neglected. The equine luminal butyrate/bicarbonate exchanger possesses some similar characteristic features to the rat, human and pig luminal butyrate/bicarbonate exchanger previously described (Mascolo *et al*. 1991; Ritzhaupt *et al*. 1998a; Harig *et al*. 1996).

Kinetic studies provide further evidence for a carrier-mediated butyrate transport process in the LMV. In the presence of increasing extravesicular concentrations of butyrate (0.5 – 50 mM), butyrate uptake is a saturable process exhibiting a K_m of 5.61 mM. This result indicates that the butyrate/ HCO_3^- transporter in the horse colon is a high affinity transporter. When compared to the K_m of butyrate transport in the BLMV (12.2 mM), it appears to be significantly lower. The K_m value of the butyrate/ HCO_3^- exchanger for butyrate on the luminal membranes showed a relatively high fluctuation between species. Mascolo *et al*. (1991) reported a K_m value of butyrate uptake in rat colon of 29.6 mM, whereas a significantly low K_m value (1.5 mM) for butyrate exchanger in the LMV isolated from human colon was revealed by Harig *et al*. (1996). Previously characterised butyrate exchanger in human colonic LMV exhibited a K_m value of 14.8 mM (Ritzhaupt *et al*. 1998a).

The present data provides compelling evidence of the existence of carrier mediated transport mechanism for SCFA across the luminal plasma membranes of equine colon.

Substrate specificity data of the bicarbonate-stimulated butyrate exchange have also demonstrated the existence of butyrate uptake process in the equine colonic LMV. The initial rate of butyrate uptake is significantly reduced by presence of 20 mM acetate and propionate, lactate and pyruvate. Furthermore the present study showed that formate had no effect on the butyrate transport in the human colonic LMV (figure V.11). This data compared well with the results previously reported (Mascolo et al. 1991; Harig et al. 1996; Ritzhaupt et al. 1998). This would suggest that there is strong competition of the SCFA/ HCO_3^- protein by SCFA substrates and indicates that the transport carrier is highly specific. Lactate has been shown to be transported across plasma membranes via several mechanisms either by lactate/ H^+ symporter (Poole & Halestrap, 1993) or via sodium-coupled cotransporter (Poole & Halestrap, 1993). In the colon the mechanism of L-lactate transport was revealed to be via a proton activated anion exchanger. Interestingly, L-lactate was shown to be transported in a similar manner to that of butyrate in LMV. The MCT1 cRNA-injected oocytes were demonstrated to transport butyrate as well as L-lactate (Ritzhaupt et al. 1998b).

The studies carried out on rat, human and pig LMV, showed that butyrate is transported in exchange with an anion (butyrate/ HCO_3^-, butyrate/ OH^-) and was DIDS insensitive (Mascolo et al. 1991; Harig et al. 1996; Ritzhaupt et al. 1998a).

It is well documented that the stilbene derivatives (SITS and DIDS) are specific inhibitors for the anion exchanger family. AE1, AE2 and AE3, which catalyse the electro-neutral, sodium-independent Cl^-/HCO_3^- exchange (Kopito, 1990), have been shown to be inhibited by very low concentrations of DIDS and SITS, 0.4 μM (Cabantchik and Rothstein, 1973; Janas et al. 1989).

In contrast to the results obtained with the BLMV, high concentration of the stilbene derivatives (0.5 mM) did not alter the initial rate of [^{14}C]-butyrate uptake in the equine colonic LMV. This result is concomitant with previous data reported by Mascolo et al. 1991; Harig et al. 1996; Ritzhaupt et al. 1998a. This finding leads to the assumption that

transport of butyrate across the luminal membranes is not mediated by an anion exchanger AE type protein.

1 mM pCMB inhibits butyrate transport by up to 50 % indicating the involvement of SH-functional groups in the transport of butyrate in the luminal membranes.

4CHC has been shown to inhibit L-lactate transport in erythrocytes (Halestrap and Deton, 1974). Further studies have shown that 4CHC abolished the L-lactate uptake in rat jejunum (Cheeseman *et al.* 1994) and butyrate transport in the LMV (Ritzhaupt *et al.* 1998; Cuff *et al.* 2002). The effect of 4CHC on butyrate transport into equine colonic LMV was investigated. As shown in figure V.12, 1 mM of 4CHC inhibit the initial rate of butyrate transport. Similarly, phloretin reduced the rate of butyrate uptake (50%). Phloretin is a potent inhibitor of monocarboxylate transporters MCT1 (Ritzhaupt *et al.* 1998) and MCT2 (Broer *et al.* 1999).

All together, these results again support the notion that there are two distinct anion exchange processes for butyrate transport in the BLMV and LMV.

In summary, these findings are consistent with results previously published on the mechanism of butyrate transport in the luminal membranes of human and pig colon (Ritzhaupt *et al.* 1998a). These authors have shown in subsequent work that the uptake of butyrate across the luminal pole of the colonocytes is mediated by MCT1 (Ritzhaupt *et al.* 1998b). Uptake of butyrate in the colonocyte luminal membranes of the horse exhibits similar characteristic to that reported in human and pig and the schematic representation of the mechanism of butyrate transport in the human and pig colonocyte luminal membranes (section V.2.1) is the best model to describe the mechanism of butyrate transport across the equine colonocyte luminal membranes as well. Considering that MCT1 is expressed on the luminal plasma membrane of the equine colon, we conclude that it is most likely that butyrate transport on the luminal membrane of the equine colon is mediated by MCT1.

CHAPTER VI

Identification of Basolateral Membrane AE2

VI.1. Introduction

In chapter IV, we showed that butyrate is transported across the equine colonic basolateral membranes via an electro-neutral anion exchange process (butyrate/bicarbonate exchanger). It was also shown that the butyrate/ HCO_3^- exchange process was inhibited by extravesicular chloride and by SITS and DIDS (0.5 mM). Therefore, further experiments were designed to assess the potential involvement of the anion exchange isoforms (AE) in the transport process of butyrate.

The anion exchanger isoform 2 (AE2) belong to the anion exchanger gene family, which involves two other well characterised members, AE1 and AE3 (Kopito, 1990). The AE family mediates the electro-neutral Cl^-/ HCO_3^- exchange that contribute to the regulation of intra-cellular pH (pH_i), chloride concentration and cell volume (Eladari et al. 1998; Aronson, 1989; Alper et al. 2002; Stewart et al. 2001). The prototype of this family is AE1 (band 3). Band 3 is one of the most thoroughly characterised plasma membrane proteins and is mainly linked to the erythrocytes even though it was found to be expressed in nonerythroid cells (Casey and Reithmeier, 1998; Aronson, 1989, Alper et al. 2002). The AE3 isoform has received special attention since it is expressed in the excitable tissues such as, heart (Puceat et al. 1995), brain (Kopito, 1990; Sterling and Casey, 1999) and retina (Alper et al. 2002). Previous studies have revealed that AE2 isoform is located on the basolateral plasma membrane of epithelial cells such as kidney (Castillo et al. 2000; Merot et al. 1997; Lindsey et al. 1990; Eladari et al. 1998; Lebeau et al. 2002), small intestine (Chow et al. 1992; Alper et al. 2001), liver (Garcia et al. 1998), epididymis (Jenson et al. 1999), colon (Rajendran et al. 2000; Ikuma et al. 2003) and stomach (Petrovic et al. 2002; Stuart-Tilley et al. 1994; Rossmann et al. 2001; Jons and Drenckhahn, 1998). Interestingly all members of AE family are inhibited by stilbene derivatives, SITS and DIDS at very low concentrations (i.e. 0.4 µM) (Aronson, 1989; Cabantchik and Rothstein, 1994).

In this chapter we set out to asses the expression of AE2 exchange at the RNA and protein levels in the equine colon. Accordingly, oligonucleotide primers were designed

from consensus nucleotide sequences of many species. 5′ and 3′ RACE were performed using specific primers to produce the 5′ and 3′ ends of the equine colonic AE2 sequence. The AE2 sequence was subsequently cloned, sequenced and analysed by commercial software (Vector NTI, Informax). The equine colonic AE2 nucleotide sequence was linearised (figure VI.4) and compared to those from other species (table VI.1).

VI.2. Detection of AE2 by PCR

Total RNA was extracted from equine colonic tissue, and then first strand cDNA was synthesized. Specific primers for AE1, AE2 and AE3 were used to amplify the fragments of interest. As shown in figure VI.1, the AE2 transcript of an expected size of 550 bp is detected in the equine colon. The PCR products of expected size of 458 and 490 for AE1 and bAE3 respectively could not be detected in the equine colon.
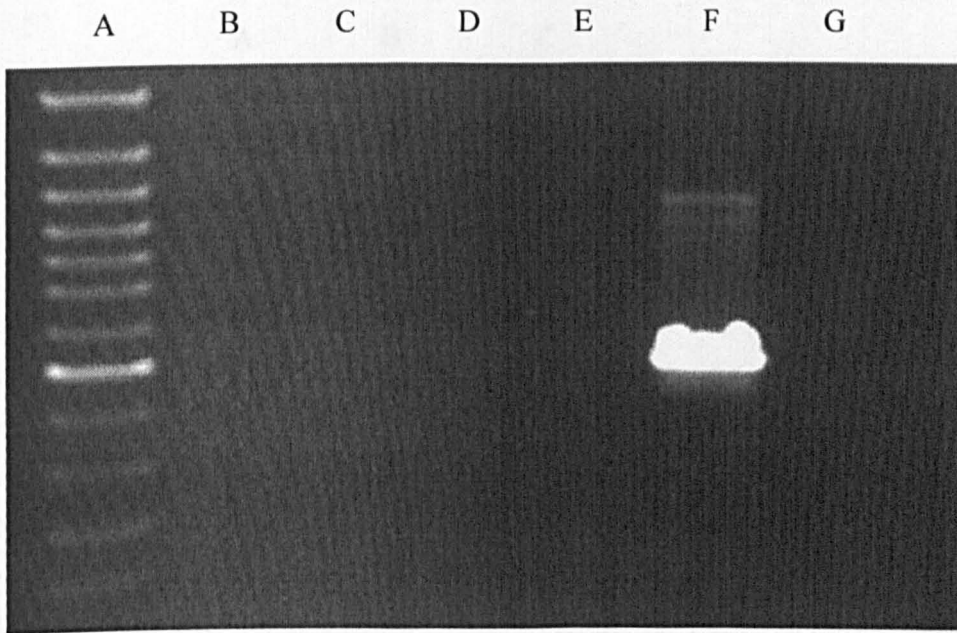
Figure VI.1. PCR analysis of the expression of AE1, AE2 and AE3 in the horse colon. Lane A: DNA ladder, lane B: AE1, lane C: control (- Taq polymerase), lane D: bAE3, lane E: control (- Taq polymerase), lane F: AE2, lane G: control (- Taq polymerase).

VI.3. Detection of AE2 by northern blot analysis

The next step was carried out to confirm the expression of the AE2 transcript and determine its size. Northern blot analysis was performed on mRNA isolated from equine colonic epithelial cells. The 550 bp product was used as a radiolabelled cDNA probe. mRNA transcript of 4.2 Kb (Rossmann *et al.* 2001; Chow *et al.* 1992) were observed (figure VI.2). This result indicates that AE2 isoform mRNA is expressed in colonocytes of the equine colon.
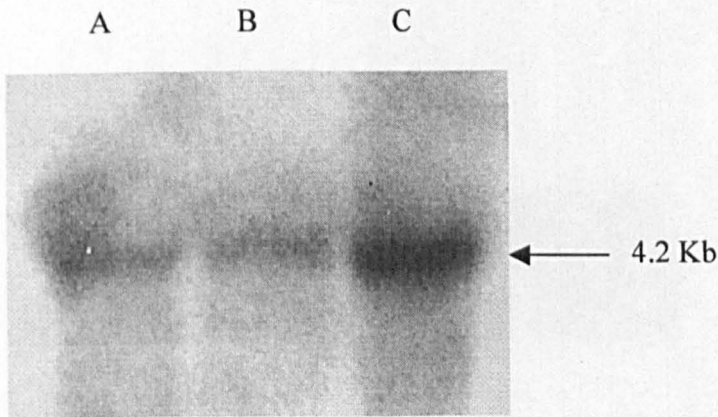
Figure VI.2. Northern blot analysis of AE2 using RNA isolated from equine colon. Experiment was carried out as previously described in Methods section. Lane A and B: 5 µg RNA, lane C: 10 µg RNA.

VI.4. Immunodetection of AE2

In order to examine the expression of AE2 at the protein level in the basolateral plasma membrane of equine colon, sample of BLMV (100 µg of protein per lane) was separated by SDS-PAGE and assessed by western blot analysis for AE2 using AE2 antibody (Alpha Diagnostic International). As shown in figure VI.3, AE2 antibody cross-reacted with a single band with molecular weight of 160 KDa. It has been previously demonstrated that AE2 protein is expressed in the basolateral plasma membranes of epithelial cells having a molecular weight ranging from 145 – 185 KDa (Kopito, 1990; Alper *et al*. 1999; Chow *et al*. 1992). AE2 protein is enriched in the BLMV fraction compared to the corresponding cellular homogenate.
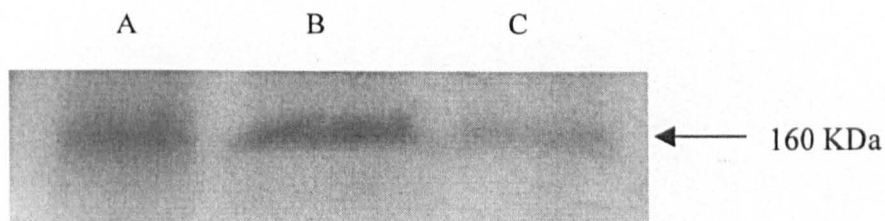
Figure VI.3. Western blot analysis of AE2 protein in equine colonic BLMV. 100 µg of sample was separated by SDS-PAGE and electro-transferred to PVDF membrane as described in Methods section. Lane A and B: BLMV, lanes C: Homogenate.

VI.5. Cloning and sequencing the anion exchanger isoform 2 (AE2)

The anion exchanger 2 mediates the electroneutral Cl^-/HCO_3^- in most tissues in many species (Rossmann *et al.* 2001). The polypeptide is encoded by the 4.2 Kb mRNA (figure VI.2) from the AE2 gene. Interestingly studies from several groups led to the identification of DNA sequence for AE2 in stomach and kidney (Alper *et al.* 2002., Kudrycki *et al.* 1990., Kopito *et al.* 1990).

In the present thesis the cDNA encoding the AE2 has been cloned from equine colonic tissue. To do so, total mRNA was isolated from equine colon and first strand cDNA was made. Then, oligonucleotide primers were designed from published consensus nucleotide sequences of many species. Six overlapping fragments of AE2 DNA were generated by PCR. These fragments were amplified, cloned and sequenced towards obtaining the entire transcript.

The full equine AE2 nucleotide sequence was aligned alongside the sequences from other species (figure VI.4) and the degree of homology was summarised in table VI.1 (p173).

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            1                                                                                                    100
AE2 Horse   ATGAGCAGCGCCCCCAGGCGCCCTGCCTCGGGCGCGGATTCTTTCCGCACTCCAGAGCCAGAGATCTTGGGCCCTGCAACAGCTGGGTTCCCCGAGCAGG
AE2 Human   ATGAGCAGCGCCCCTCGGCGCCCCGCCAAGGGCGCAGATTCTTTCTGTACGCCAGAGCCAGAGAGCTTGGGCCCTGGGACGCCTGGGTTCCCCGAGCAGG
AE2 Mouse   ATGAGCAGCGCCCCCAGGCGCCCCGCCTCGGGCGCAGATTCTTTGCACACGCCAGAGCCAGAGAGCCTGAGCCCCGGAACACCTGGGTTCCCCGAGCAAG
AE2 Rat     ATGAGCAGCGCCCCCAGGCGCCCCGCCTCGGGCGCAGATTCTTTGCACACGCCAGAGCCAGAGAGCCTGAGCCCCGGAACACCTGGGTTCCCTGAGCAAG
AE2 Rabbit  ATGAGCAGCGCCCCCCGGCGCCCCGCCTCGGGCGCAGATTCTTTCAGAACGCCAGAGCCAGAGAGCTTGGGCCCTGGGACACCTGGGTTCCCGGAACAGG
Consensus   ATGAGCAGCGCCCCCAGGCGCCCCGCCTCGGGCGCAGATTCTTTCCGCACGCCAGAGCCAGAGAGCTTGGGCCCTGGAACACCTGGGTTCCCCGAGCAGG
            101                                                                                                  200
AE2 Horse   AGGA---AGATGAACTTCACCGCACCCTGGGCGTGGAACGGTTTGAGGAGATCCTACAGGAGGCCGGGTCTCGAGGAGGGGAGGAGCCAGGCCGCAGCTA
AE2 Human   AGGA---AGACGAACTTCACCGCACCCTGGGCGTGGAGCGGTTTGAGGAGATCCTACAGGAGGCCGGGTCTCGTGGAGGGGAGGAGCCAGGCCGCAGCTA
AE2 Mouse   AGGA---AGATGAACTT---CGTACCTTAGGTGTGGAGCGGTTCGAGGAGATCCTCCAGGAGGCTGGATCCCGGGGGAGGAGAAGAGCCAGGACGCAGCTA
AE2 Rat     AGGAGGAAGATGAACTT---CGTACCTTAGGTGTGGAGCGGTTCGAGGAGATCCTCCAGGAAGCTGGATCCAGAGGAGGAGAAGAGCCAGGCCGCAGCTA
AE2 Rabbit  AGGA---GGATGAACTGCACCGCACCCTGGGCGTGGAGCGGTTTGAGAGAGATCCTACAGGAAGCCGGGTCTCGTGGAGGGGAGGAACCAGGCCGCAGCTA
Consensus   AGGA   AGATGAACTTCACCGCACCCTGGGCGTGGAGCGGTTTGAGGAGATCCTACAGGAGGCCGGGTCTCG GGAGGGGAGGAGCCAGGCCGCAGCTA
            201                                                                                                  300
AE2 Horse   TGGGGAAGAAGACTTTGAATACCACCGCCAGTCTTCCCACCATATCCATCACCCACTGTCCACCCACCTGCCGCCGGACACCCGCCGCCGCAAGACACCC
AE2 Human   TGGGGAGGAAGACTTTGAGTACCACCGCCAGTCCTCCCACCACATCCATCACCCACTGTCCACCCACCTGCCTCCGGATGCACGCCGCCGCAAGACACCC
AE2 Mouse   TGGAGAGGAAGACTTTGAATACCACCGCCAGTCCTCCCACCATATCCATCATCCACTATCCACCCACCTGCCTCCTGATGCCCGACGTCGCAAGACTCCC
AE2 Rat     TGGGGAGGAAGACTTTGAATACCACCGCCAGTCCTCCCACCATATCCATCATCCACTCTCCACCCACCTGCCTCCTGACGCCCGCCGTCGCAAGACTCCC
AE2 Rabbit  TGGGGAGGAAGACTTTGAATACCACCGGCAGTCCTCTCACCACATCCATCACCCGCTGTCCACCCACCTTCCACCGGATGCTCGCCGCCGCAAGACCCCT
Consensus   TGGGGAGGAAGACTTTGAATACCACCGCCAGTCCTCCCACCATATCCATCACCCACTGTCCACCCACCTGCCTCCGGATGCCCGCCGCCGCAAGAC CCC
            301                                                                                                  400
AE2 Horse   CAGGGCCCGGGACGGAAGTCTCGCAGGCGCCCTGGAGCCTCCCCAACGGGGGAGACCCCCACCATTGAGGAGGGGGAGGAAGATGAGGATGAGGCCAGTG
AE2 Human   CAGGGCCCAGGACGGAAGCCTCGAAGGCGCCCGGGAGCCTCCCCGACTGGAGAAACCCCGACCATTGAGGAGGGGGAGGAAGATGAGGATGAGGCCAGCG
AE2 Mouse   CAGGGCCCAGGACGGAAACCTCGGAGGCGCCCTGGTGCCTCTCCCACTGGAGAGACCCCCACTATTGAGGAAGGGGGAGGAAGATGAGGAAGAAGCTAGTG
AE2 Rat     CAGGGCCCAGGACGGAAACCTCGGAGGCGCCCTGGAGCCTCTCCCACTGGAGAGACCCCCACTATTGAGGAAGGGGGAGGAAGATGAGGATGAAGTCGGTG
AE2 Rabbit  CAGGGCCCAGGACGGAAGCCTCGCCGGCGCCCTGGAGCCTCCCCAACTGGAGCCACCCCCACCATTGAGGAGGGGGAGGAAGATGAGGAAGAGGCCAATG
Consensus   CAGGGCCCAGGACGGAAGCCTCG AGGCGCCCTGGAGCCTCCCC ACTGGAGAGACCCCCACCATTGAGGAGGGGGGAGGAAGATGAGGATGAGGCCAGTG
            401                                                                                                  500
AE2 Horse   AGGCTGAGGGAGCCCGGGCACCCACCCAAC---CATCCCCTGCGTCCACACCCTCTTCGGTGCAGTTCTTTCTCCAGGAGGATGATGGTGCCGACCGGAA
AE2 Human   AGGCTGAGGGGGGCCCGGGCTCTCACTCAGC---CGTCCCCTGTCTCCACACCCTCCTCGGTGCAGTTCTTTCTCCGAGAGGATGACAGTGCCGACCGGAA
AE2 Mouse   AAGCTGAAGGGGTTCAGAGCTCCCCCACAACAACCATCTCCTGCTACTACACCCTCTGCAGTTCAGTTCTTTCTCCAAGAGGATGAAGGTGCAGAAAGGAA
AE2 Rat     AAGCTGAAGGGGTTCAGAGCTCCCCCGCAACAACCATCTCCTGCCTCTTCACCCTCTGCAGTTCAGTTTTTTCTCCAAGAGGATGAAGGTACAGATAGGAA
AE2 Rabbit  AGGCTGAGGGGGGCCAGGGCACCCACTGAGC---CATCCCCCGCCTCCACTCCCTCTTCGGTACAGTTCTTTCTCCAGGAGGATGAAGGTGCGGAGCGGAA
Consensus   AGGCTGAGGGGGGCCAGGGCTCCCACCAAC     CATCCCCTGCCTCCACACCCTCTTCGGT CAGTTCTTTCTCCAAGAGGATGAAGGTGC GA CGGAA
            501                                                                                                  600
AE2 Horse   AGCAGAAAGGACCAGTCCGTCTCCCCCTCCACCACTGCCCCACCAGGAGGCAGCTCCCCGGGCCACCAAAGGGGCCCAGACTGGAGCCCTGGTGGAAGAG
AE2 Human   GGCAGAGAGGACCAGTCCATCTTCCCCTGCACCACTGCCCCACCAGGAGGCGACTCCTCGGGCCTCCAAAGGGGCCCAGGCTGGAACCCAGGTGGAGGAG
AE2 Mouse   GCCAGAGAGAACCAGCCCATCTCCCCCTACACAGACGCCCCACCAGGAGGCAGCTCCCCGGGCCAGCAAAGGGGCACAGACAGGAACTCTGGTGGAGGAG
AE2 Rat     GGCAGAGAGAACCAGCCCATCTCCCCCTACACAGACGCCCCACCAGGAAGCAGCTCCCCGGGCCAGCAAAGGGGCACAGACAGGAACTCTGGTGGAGGAG
AE2 Rabbit  GGCAGAGAGGACCAGCCCATCTCCTCCTACACTGCTGCCCCACCAAGAGGCAGCCCCTTGGGCCACCGAAGGGGCCCAGACGGGAGTCCCGGTGGAAGAG
Consensus   GGCAGAGAGGACCAGCCCATCTCCCCCTACAC GCTGCCCCACCAGGAGGCAGCTCCCCGGGCCACCAAAGGGGCCCAGAC GGAACCCTGGTGGAGGAG
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           601                                                                                            700
AE2 Horse  GTGA--------------TGGCAGTGGCCGGCGGCACAGCAGGAGGCGATGACGGGGGTGCCTCTGGGCGCCCCCTGACCAAAGCGCAGCCTGGGCACCGCA
AE2 Human  GCGGAGGCGGAGGCGGTGGCGGTGGCCAGTGGCACAGCAGGGGGTGACGACGGGGGTGCCTCGGGGCGCCCCCTGCCCAAAGCCCAGCCTGGGCACCGCA
AE2 Mouse  ATGG--------------TGGCGGTGGCCAGTGCGACAGCTGGAGGTGACGACGGAGGTGCTGCGGGGCGTCCCTTGACCAAAGCCCAGCCTGGACATCGAA
AE2 Rat    ATGG--------------TGGCCGTGGCCAGCGGCACGGCTGGAGGTGACGACGGAGGTGCTGCAGGGCGCCCCCTGACCAAAGCCCAGCCTGGACATCGAA
AE2 Rabbit GTGG--------------CAGTGGTGGCCAGTGGCACAGCAGGAGGGGATAACGGGGGTGCCTCCGGGCGCCCCCTGACCAAAGCCCAGCCCGGGCATCGCA
Consensus  TGGCGGTGGCCAGTGGCACAGCAGGAGGTGACGACGGGGGTGCCTC GGGCGCCCCCTGACCAAAGCCCAGCCTGGGCATCGCACAGCCTGGGCATCGCA
           701                                                                                            800
AE2 Horse  GCTACAACCTGCAGGAGAGGAGGCGCATTGGAAGCATGACTGGGGCTGAGCAGGCACTGCTGCCCCGGGTCCCCACGGATGAGAGCGAGGCCCAGACGCT
AE2 Human  GCTACAACCTTCAGGAGAGGAGGCGCATCGGGAGCATGACTGGGGCTGAGCAGGCACTGCTGCCCCGGGTCCCCACGGATGAGATTGAGGCCCAGACGCT
AE2 Mouse  GTTACAACCTTCAGGAGCGAAGACGAATTGGCAGTATGACAGGGGTGGAGCAGGCGCTGTTGCCTAGGGTCCCTACTGATGAAAGTGAGGCTCAGACACT
AE2 Rat    GTTACAACCTTCAGGAGAGAAGGCGAATTGGCAGTATGACAGGGGTGGAGCAGGCCCTGCTGCCCAGGGTCCCTACTGATGAGAGTGAGGCTCAGACGCT
AE2 Rabbit GCTACAACCTTCAGGAGAGGCGGCGCATTGGGAGCATGACCGGGGCCGAGCAGGCGCTACTGCCCCGCGTGCCCACAGATGAGAGCGAGGCGCAGACGCT
Consensus  GCTACAACCTTCAGGAGAGGAGGCGCATTGG AGCATGAC GGGGC GAGCAGGC CTGCTGCCCCGGGTCCCCAC GATGAGAGTGAGGC CAGACGCT
           801                                                                                            900
AE2 Horse  CGCCACGGCTGACCTAGACCTGATGAAGAGTCACCGGTTTGAGGATGTTCCTGGGGTACGGCGGCACTTGGTGCGGAAGAATGCCAAAGGGTCTGTGCAG
AE2 Human  GGCCACGGCCGACCTAGACCTCATGAAGAGTCACCGGTTTGAGGACGTTCCTGGGGTGCGGCGGCACTTGGTGCGGAAGAATGCCAAAGGGTTCCACACAG
AE2 Mouse  GGCCACAGCCGACCTTGACCTCATGAAAAGTCACCGATTTGAGGATGTTCCTGGGGTACGGCGACACTTGGTGAGGAAGAATGCCAAAGGGTCTACACAG
AE2 Rat    GGCCACAGCTGACCTCGACCTCATGAAAAGTCACCGGTTTGAGGACGTTCCGGGGGGTACGGCGACATTTGGTGAGGAAGAATGCCAAAGGGTCTACACAG
AE2 Rabbit GGCCACAGCCGACCTAGACCTCATGAAGAGTCACCGGTTTGAGGACGTCCCCTGGGGTGCGGCGGCACTTGGTGCGGAAGAATGCCAAAGGGTCAGCTCAG
Consensus  GGCCACAGCCGACCTAGACCTCATGAAGAGTCACCGGTTTGAGGACGTTCCTGGGGTACGGCGGCACTTGGTGCGGAAGAATGCCAAAGGGTCTACACAG
           901                                                                                            1000
AE2 Horse  AGTGGCCGGGAAGGGCGAGAGCCTGGCCCCACGCCTCGGGCCCCGGCCCCGGGGCCCCCCACAAGCCCCATGAGGTGTTCGTGGAACTGAATGAGTTGCTGC
AE2 Human  AGTGGCCGAGAAGGGCGGGAGCCTGGCCCCACACCTCGGGCTCGACCCCGGGGCCCCCCACAAGCCCCATGAGGTGTTTGTGGAGCTGAATGAGTTGCTCC
AE2 Mouse  GCTGCCCGGGAAGGTCGAGAGCCTGGCCCCACACCTCGGGCACGGCCACGGGCCCCCGCATAAGCCCCATGAGGTGTTTGTGGAGCTGAATGAGCTGCTGT
AE2 Rat    GCTGCCCGGGAAGGCCGAGAGCCTGGCCCCACACCTCGGGCACGACCACGGGCCCCCCATAAGCCTCATGAGGTGTTCGTAGAGCTGAATGAATTGCAGT
AE2 Rabbit AGCAGCCGGGAAGGGCGAGAGCCTGGCCCCACGCCTCGCTCGCGACCCCGGGCCCCCCACAAGCCCCACGAGGTGTTTGTGGAGCTGAACGAGTTGCTCT
Consensus  AGTGGCCGGGAAGGGCGAGAGCCTGGCCCCACACCTCGGGC CGACCCCGGGCCCCCCACAAGCCCCATGAGGTGTTTGTGGAGCTGAATGAGTTGCTGT
           1001                                                                                           1100
AE2 Horse  TGGACAAAAAACCAGGAGCCCCAGTGGCGGGAGACGGCGCGCTGGATCAAGTTTGAGGAGGACGTGGAGGAGGAGACAGAGCGCTGGGGGAAGCCCCACGT
AE2 Human  TGGACAAAAAACCAGGAGCCCCAGTGGCGGGAGACAGCTCGCTGGATCAAATTTGAAGAGGACGTGGAGGAGGAGACCGAGCGCTGGGGGAAGCCCCACGT
AE2 Mouse  TGGACAAAAAACCAGGAGCCTCAGTGGCGGGAGACAGCCCGCTGGATAAAAATTCGAGGAGGATGTGGAAGAGGAGACTGAGCGCTGGGGGAAGCCTCATGT
AE2 Rat    TGGACAAAAAACCAGGAGCCTCAGTGGCGGGAGACAGCCCGGTGGATAAAAATTTGAGGAGGACGTGGAAGAGGAGACTGAGCGCTGGGGCAAGCCTCACGT
AE2 Rabbit TGGACAAGAACCAGGAGCCTCAGTGGCGGGAGACGGCTCGCTGGATCAAATTTGAGGAGGACGTGGAGGAGGAGACGGAGCGCTGGGGGAAGCCTCACGT
Consensus  TGGACAAAAAACCAGGAGCCTCAGTGGCGGGAGACAGC CGCTGGATCAAATTTGAGGAGGACGTGGAGGAGGAGAC GAGCGCTGGGGGAAGCCTCACGT
           1101                                                                                           1200
AE2 Horse  GGCCTCCCTGTCCTTCCGCAGCCTCCTGGAGCTCCGCCGGACCCTGGCCCACGGGGCCGTGCTCTTGGACCTGGACCAGCAGACCCTACCTGGGGTGGCT
AE2 Human  GGCCTCCCTCTCCTTCCGCAGTCTCCTGGAGCTCCGCCAGGACCCTGGCCCATGGGGCTGTGCTCTTGGATCTGGACCAGCAGACCCTGCCCGGAGTGGCC
AE2 Mouse  GGCCTCACTGTCCTTCCGTAGCCTCCTGGAGCTCCGCCAGGACTCTGGCCCATGGAGCTGTGCTCTTAGACCTCGATCAGCAGACCCTGCCTGGGGTGGCC
AE2 Rat    GGCATCACTGTCCTTCCGCAGCCTCCTGGAGCTCCGCCAGGACACTGGCCCATGGAGCTGTGCTCTTGGACCTCGATCAGCAGACCCTGCCTGGGGTGGCC
AE2 Rabbit CGCCTCTCTGTCGTTCCGCAGTCTCCTGGAGCTCCGCGCGGACCCTGGCCCATGGGGCTGTGCTCTTGGACCTGGACCAGCAGACCCTGCCTGGGGTGGCC
Consensus  GGCCTC CTGTCCTTCCGCAGCCTCCTGGAGCTCCGCCAGGACCCTGGCCCATGGGGCTGTGCTCTTGGACCTGGACCAGCAGACCCTGCCTGGGGTGGCC
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163

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            1201                                                                                        1300
AE2 Horse   CACCAGGTGGTGGAGCAGATGGTCATCTCTGACCAGATCAAGGCCGAGGACAGAGCCGATGTGCTGCGGGCCCTGCTGCTAAAACACAGCCACCCAAGTG
AE2 Human   CACCAGGTGGTGGAGCAGATGGTCATCTCTGACCAGATCAAGGCCGAGGATAGGGCCAACGTGCTGCGGGCTCTGCTGTTGAAACACAGCCACCCAAGTG
AE2 Mouse   CATCAGGTGGTCGAGCAGATGGTCATCTCTGACCAGATCAAGGCAGAGGATAGAGCCAATGTGCTACGGGCCCTCCTGCTAAAGCACAGCCACCCAAGTG
AE2 Rat     CATCAGGTGGTCGAGCAGATGGTTATCTCTGACCAGATCAAAGCAGAGGACAGAGCCAATGTGCTACGAGCCCTTCTGCTGAAACACAGCCACCCAAGTG
AE2 Rabbit  CACCAGGTGGTGGAGCAGATGGTCATCTCTGACCAGATCAAGGCCGAGGACAGAGCCAACGTGCTGCGGGCCCTGCTGCTGAAACACAGCCACCCGAGTG
Consensus   ACCAGGTGGTGGAGCAGATGGTCATCTCTGACCAGATCAAGGCCGAGGACAGAGCCAATGTGCTGCGGGCCCTGCTGCTGAAACACAGCCACCCAAGTG

            1301                                                                                        1400
AE2 Horse   ACGAGAAGGACTTCTCCTTCCCCCGCAATATCTCGGCTGGCTCCCTGGGCTCCCTGCTGGGGCATCACCATGGCCAGGGAGCCGAGAGTGACCCCCACGT
AE2 Human   ATGAGAAGGACTTCTCCTTCCCCCGCAACATCTCAGCTGGCTCCCTGGGCTCCCTGCTGGGGCATCACCATGGTCAGGGGGCTGAGAGTGACCCCCACGT
AE2 Mouse   ACGAGAAAGAGTTCTCCTTCCCCCGAAACATCTCAGCGGGCTCTCTAGGCTCTCTACTGGGGCATCACCATGCCCAGGGGACCGAGAGTGATCCTCATGT
AE2 Rat     ATGAGAAAGAATTCTCCTTCCCCCGGAACATCTCAGCGGGCTCTCTGGGCTCTCTCCTGGGGCATCACCACGCCCAGGGGACTGAGAGTGATCCTCACGT
AE2 Rabbit  ATGAGAAGGACTTTTCCTTCCCCCGCAACATCTCGGCCGGCTCCCTGGGCTCCCTGCTGGGGCATCACCACGCGCCAGGGGGCCGAGAGCGACCCTCATGT
Consensus   ATGAGAAGGACTTCTCCTTCCCCCGCAACATCTCAGC GGCTCCCTGGGCTCCCTGCTGGGGCATCACCATGGCCAGGGGGCCGAGAGTGACCCTCACGT

            1401                                                                                        1500
AE2 Horse   CACCGAGCCTCTCATTGGAGGTGTTCCCGAGACCCGGCTGGAAGTGGAGCGAGAGCGTGAGCTATCACCTCCGGCTCCACCAGCTGGCATCACTCGCTCC
AE2 Human   CACCGAGCCTCTCATGGGAGGTGTTCCTGAGACCCGGCTGGAGGTGGAGCGAGAGCGTGACGTGCCGCCCCAGCACCACCAGCTGGCATCACCCGCTCC
AE2 Mouse   CACTGAGCCTCTCATTGGTGGTGTTCCTGAGACCCGACTGGAGGTGGATAGAGAGCGTGAGCTACCACCCCCAGCACCACCTGCAGGTATTACCCGCTCC
AE2 Rat     CACTGAGCCTCTCATCGGTGGTGTTCCTGAGACCCGGCTGGAGGTGGATAGAGAGCGTGAGCTGCCGCCCCCAGCCCCACCTGCAGGTATTACCCGCTCC
AE2 Rabbit  CACTGAGCCCCTCATTGGAGGAATTCCCGAGACCCGGCTGGATGTGGAACGAGAGCGCGACGTGCCCCCCTCGGCCCCGCCGGCCGGCATCACACGTTCC
Consensus   CACTGAGCCTCTCATTGGAGGTGTTCCTGAGACCCGGCTGGAGGTGGA CGAGAGCGTGAGCTGCC CCCCCAGC CCACC GC GGCATCACCCGCTCC

            1501                                                                                        1600
AE2 Horse   AAGTCCAAGCACGAGCTGAAGCTGCTGGAGAAGATCCCCGAGAACGCCGAGGCCACGGTGGTCCTCGTGGGCTGCGTGGAGTTCCTCTCCCGCCCCACCA
AE2 Human   AAGTCCAAGCACGAGCTGAAACTGCTGGAGAAGATTCCTGAGAATGCCGAGGCCACGGTGGTCCTTGTGGGCTGCGTGGAGTTCCTCTCCCGCCCCACCA
AE2 Mouse   AAGTCCAAGCATGAGCTGAAGCTGCTGGAGAAGATCCCTGAGAATGCGGAGGCTACAGTGGTCCTCGTGGGCTGTGTGGAGTTCCTCTCCCGCCCTACCA
AE2 Rat     AAGTCCAAGCATGAGCTGAAGCTGCTGGAGAAGATCCCTGAGAATGCAGAGGCCACAGTGGTCCTCGTGGGCTGTGTGGAGTTCCTCTCCCGCCCCACCA
AE2 Rabbit  AAGTCCAAGCACGAGCTGAAGCTGCTGGAGAAGATCCCGGAGAACGCCGAGGCCACGGTGGTCCTTGTGGGCTGCGTGGAGTTCCTCTCCCGCCCCACCA
Consensus   AAGTCCAAGCACGAGCTGAAGCTGCTGGAGAAGATCCCTGAGAATGCCGAGGCCACGGTGGTCCTCGTGGGCTGCGTGGAGTTCCTCTCCCGCCCCACCA

            1601                                                                                        1700
AE2 Horse   TGGCCTTTGTGCGGCTACGGGAGGCGGTGGAGCTGGATGCAGTGCTGGAGGTGCCTGTGCCTGTGCGCTTCCTCTTCCTGCTCCTGGGCCCGAGCAGCGC
AE2 Human   TGGCCTTTGTGCGGCTCCGGGAGGCTGTGGAGTTGGACGCAGTGTTGGAGGTGCCGGTGCCTGTGCGTTTCCTCTTCCTGCTGCTGGGCCCGAGTAGTGC
AE2 Mouse   TGGCCTTCGTGCGGGTTGCGGGAGGCTGTGGAGCTGGATGCCGTGCTAGAGGTGCCTGTGCCTGTGCGCTTCCTCTTCTTGCTGCTGGGACCCAGCAGTGC
AE2 Rat     TGGCCTTTGTGCGCCTGCGGGAGGCTGTGGAACTGGATGCAGTACTGGAGGTGCCTGTGCCTGTGCGCTTCCTCTTCCTGCTGCTGGGGCCCAGCAGCGC
AE2 Rabbit  TGGCCTTCGTGCGGGCTGCGGGAGGCTGTGGAACTGGATGCTGTGCTGGAGGTGCCCGTGCCTGTGCGCTTCCTCTTCCTGCTGCTGGGGCCCGAGCAGCGC
Consensus   TGGCCTTTGTGCGGCTGCGGGAGGCTGTGGAGCTGGATGCAGTGCTGGAGGTGCCTGTGCCTGTGCGCTTCCTCTTCCTGCTGCTGGGCCCGAGCAGCGC

            1701                                                                                        1800
AE2 Horse   CAACATGGACTACCACGAGATCGGCCGCTCCATCTCCACCCTCATGTCGGACAAGCAATTCCACGAGGCAGCCTACCTGGCAGACGAGCGGGAGGACCTG
AE2 Human   CAACATGGACTACCACGAGATCGGCCGCTCCATCTCCACCCTCATGTCAGACAAGCAATTCCACGAGGCAGCCTACCTGGCTGACGAGCGGGAGGACCTG
AE2 Mouse   TAACATGGACTACCATGAGATCGGCCGCTCCATTTCCACCCTCATGTCTGACAAGCAATTTCATGAGGCAGCCTACCTGGCGGATGAACGAGACGACTTG
AE2 Rat     CAACATGGACTACCATGAGATTGGCCGATCCATCTCCACCCTCATGTCTGACAAGCAATTCCACGAGGCAGCCTACCTGGCAGATGAACGGGATGACTTG
AE2 Rabbit  CAACATGGACTACCACGAGATCGGCCGCTCCATCTCCACCCTCATGTCGGACAAGCAATTCCACGAGGCGGCCTACCTGGCAGACGAGCGCGAGGACCTG
Consensus   CAACATGGACTACCACGAGATCGGCCGCTCCATCTCCACCCTCATGTC GACAAGCAATTCCACGAGGCAGCCTACCTGGCAGACGAGCGGGAGGACCTG
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                   1801                                                                                    1900
AE2 Horse     CTGACCGCCATCAACGCCTTTCTGGACTGCAGCGTGGTGCTGCCGCCCTCGGAGGTGCAGGGCGAGGAGTTGCTGCGATCCGTCGCTCACTTCCAGCGCC
AE2 Human     CTGACGGCCATCAACGCCTTCCTGGACTGCAGCGTGGTGCTGCCGCCTTCAGAAGTGCAGGGCGAGGAGCTGCTGCGCTCTGTGGCCCACTTCCAGCGCC
AE2 Mouse     CTGACTGCTATCAATGCCTTCCTGGACTGCAGTGTTGTGCTACCGCCTTCTGAAGTGCAGGGCGAGGAGCTGCTGCGTTCTGTTGCCCATTTCCAACGCC
AE2 Rat       CTGACTGCTATCAATGCCTTCCTGGACTGCAGTGTTGTGCTACCGCCTTCTGAAGTGCAGGGCGAGGAGCTGCTGCGTTCTGTTGCCCATTTCCAGCGCC
AE2 Rabbit    CTGACCGCCATCAACGCCTTCCTGGACTGCAGTGTGGTGCTGCCGCCCTCCGAAGTGCAGGGCGAGGAGCTGCTGCGCTCCGTCGCCCATTTCCAGCGCC
Consensus     CTGAC GCCATCAACGCCTTCCTGGACTGCAGTGTGGTGCTGCCGCCTTC GAAGTGCAGGGCGAGGAGCTGCTGCG TCTGT GCCCATTTCCAGCGCC
                   1901                                                                                    2000
AE2 Horse     AGATGCTCAAGAAGCGGGAGGAGCAGGGCCGGCTGTTGCCCACGGGGGCTGGGCTGGAGCCCAAGTCGGCCCAAGATAAGGCGCTCCTGCAGATGGTAGA
AE2 Human     AGATGCTCAAGAAGCGAGAGGAGCAGGGCCGGCTGCTACCTACAGGGGCTGGGCTGGAGCCCAAATCTGCCCAAGATAAGGCGCTCCTGCAGATGGTAGA
AE2 Mouse     AGATGCTAAAGAAGCGAGAGGAGCAGGGCCGCCTGCTGCCCCCAGGGGCTGGGCTAGAGCCCAAGTCTGCCCAAGATAAGGCACTCCTGCAGATGGTAGA
AE2 Rat       AGATGCTAAAGAAGCGAGAGGAGCAGGGCCGCCTGCTGCCCCCCTGGTGCTGGGCTAGAGCCCAAGTCTGCCCAAGATAAGGCACTCCTGCAGATGGTAGA
AE2 Rabbit    AGATGCTCAAGAAGCGGGAGGAGCAGGGCCGGCTGCTGCCCCCGGGGCTGGGCCTGGAGCCCAAGTCTGCCCAAGATAAGGCGCTCCTGCAGATGGTAGA
Consensus     AGATGCTCAAGAAGCGAGAGGAGCAGGGCCGGCTGCTGCCCCC GGGGCTGGGCTGGAGCCCAAGTCTGCCCAAGATAAGGCGCTCCTGCAGATGGTAGA
                   2001                                                                                    2100
AE2 Horse     GGCGGCGGGTGCAGTTGAAGACGATCCCCTTCGGCGGACAGGCCGACCGTTCGGGGGGCTGATACGAGATGTGCGGCGCCGCTATCCCCACTATCTGAGT
AE2 Human     GGCGGCAGGGGCAGCTGAAGATGATCCCCTTCGGCGGACGGGGCGGCCCTTTGGGGGGCTGATCCGAGATGTGCGGCGCCGCTATCCCCACTACCTGAGT
AE2 Mouse     GGTGGCAGGTGCAGCTGAAGATGATCCCCTTCGGAGGACAGGCCGGCCCTTTGGGGGGCTGATCCGTGACGTGCGGCGGCGCTACCCCCACTACCTAAGT
AE2 Rat       GGTGGCAGGTGCAGCTGAAGATGATCCCCTTCGGAGGACAGGCCGGCCCTTTGGGGGGCTGATCCGTGACGTGCGGCGGCGCTACCCGCACTACCTGAGT
AE2 Rabbit    GGCAGCAGGTGCAGCTGAAGATGATCCCCTGCGGCGGACTGGCCGGCCCTTCGGGGGCCTGATCCGTGACGTGCGGCGCCGCTACCCACACTACCTGAGC
Consensus     GGCGGCAGGTGCAGCTGAAGATGATCCCCTTCGGCGGACAGGCCGGCCCTTTGGGGGGCTGATCCGTGACGTGCGGCGCCGCTACCCCCACTACCTGAGT
                   2101                                                                                    2200
AE2 Horse     GACTTCCGAGACGCACTCGACCCCCAGTGCCTGGCTGCCGTCATCTTCATCTACTTTGCGGCCCTGTCTCCTGCTATCACCTTTGGGGGGCTGCTGGGAG
AE2 Human     GACTTCCGAGATGCACTTGACCCTCAGTGCCTGGCCGCAGTCATCTTCATCTACTTTGCCGCCCTGTCTCCTGCCATCACCTTTGGGGGGCTGCTGGGAG
AE2 Mouse     GACTTCCGCGATGCACTTGACCCCCAGTGCCTGGCTGCTGTTATTTTCATCTACTTTGCCGCCCTGTCTCCTGCCATCACCTTTGGGGGGCTACTGGGGG
AE2 Rat       GACTTCCGAGATGCACTAGACCCCCAGTGCCTGGCTGCTGTCATTTTCATCTACTTTGCCGCCCTGTCGCCTGCCATCACTTTTGGGGGGCTACTGGGGG
AE2 Rabbit    GACTTCCGGGACGCGCTCGACCCCCAGTGTGTGGCTGCTGTCATCTTCATCTACTTTGCCGCCCTGTCTCCTGCCATCACCTTTGGGGGGCTGCTGGGAG
Consensus     GACTTCCGAGATGCACT GACCCCCAGTGCCTGGCTGCTGTCATCTTCATCTACTTTGCCGCCCTGTCTCCTGCCATCACCTTTGGGGGGCTGCTGGGAG
                   2201                                                                                    2300
AE2 Horse     AGAAGACGCAGGACCTGATCGGGGTGTCAGAGCTGATCATGTCCACAGCGCTCCAGGGCGTGGTCTTCTGCCTGCTGGGTGCCCAGCCGCTGCTGGTGAT
AE2 Human     AGAAGACACAGGACCTGATAGGGGTGTCGGAGCTGATTATGTCCACAGCGCTCCAGGGCGTGGTCTTCTGCCTGCTGGGTGCCCAGCCCCTGTTGGTGAT
AE2 Mouse     AGAAGACAAAGGACCTGATAGGAGTGTCAGAGCTGATCATGTCCACAGCGCTGCAGGGAGTGGTCTTCTGCCTGCTGGGGGGCTCAGCCCCTGCTGGTCAT
AE2 Rat       AGAAGACACAGGACCTGATAGGAGTGTCAGAGCTGATCATGTCCACAGCGCTCCAGGGAGTGATCTTCTGCCTGCTGGGCGCCCAGCCACTGCTGGTGAT
AE2 Rabbit    AGAAGACGCAGGACCTGATAGGGGTGTCGGAGCTGATCATGTCCACGGCGCTGCAGGGTGTGATCTTCTGCCTGCTGGGGGGCTCAGCCACTGCTGGTGAT
Consensus     AGAAGACACAGGACCTGATAGGGGTGTCAGAGCTGATCATGTCCACAGCGCTCCAGGG GTGGTCTTCTGCCTGCTGGG GCCCAGCC CTGCTGGTGAT
                   2301                                                                                    2400
AE2 Horse     CGGCTTTTCAGGGCCTCTGCTGGTCTTTGAGGAGGCCTTCTTCTCGTTCTGCAGCAGCAATGACCTAGAGTACCTGGTGGGCCGTGTGTGGATCGGCTTC
AE2 Human     CGGCTTCTCAGGGCCCCTGCTGGTCTTTGAGGAGGCCTTCTTCTCGTTCTGTAGCAGCAACCACCTGGAGTACCTGGTGGGCCGTGTGTGGATCGGCTTC
AE2 Mouse     CGGCTTTTCTGGGCCTCTGCTGGTCTTCGAGGAGGCCTTCTTCTCGTTCTGCAGTAGCAATGAGTTGGAGTACTTGGTGGGCCGAGTGTGGATTGGCTTC
AE2 Rat       CGGCTTCTCGGGGCCTCTGCTGGTCTTCGAGGAGGCCTTCTTCTCGTTCTGCAAGAGCAACCAGTTGGAGTACTTGGTGGGCCGAGTTTGGATTGGCTTC
AE2 Rabbit    CGGCTTCTCCGGGCCTTTGCTGGTCTTCGAGGAGGCCTTCTTCACGTTCTGTAGCAGCAACCAGCTGGAGTACCTGGTGGGCCGCGTGTGGATCGGCTTC
Consensus     CGGCTTCTC GGGCCTCTGCTGGTCTTCGAGGAGGCCTTCTTCTCGTTCTGCAGCAGCAACCAGCTGGAGTACCTGGTGGGCCG GTGTGGATCGGCTTC
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165

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            2401                                                                                    2500
AE2 Horse   TGGCTGGTGCTCCTGGCCCTGCTCATGGTGGCCTTGGAGGGGAGCTTCCTGGTGCGCTTCGTCTCCCGTTTCACTCAGGAGATCTTCGCCTTCCTCATCT
AE2 Human   TGGCTGGTGTTCCTGGCCCTGCTCATGGTGGCCCTGGAGGGGAGCTTCCTGGTCCGCTTCGTCTCCCGCTTCACCCAGGAGATCTTCGCCTTCTTGATCT
AE2 Mouse   TGGCTGGTGTTCCTGGCCCTGCTCATGGTGGCTCTGGAGGGGAGCTTCCTGGTCCGCTTTGTATCCCGATTCACCCAGGAGATCTTTGCCTTCCTCATAT
AE2 Rat     TGGCTGGTGCTCCTGGCCCTGCTCATGGTGGCTCTGGAGGGGAGCTTCCTGGTCCGCTTTGTCTCCCGATTCACCCAGGAGATCTTTGCCTTCCTCATAT
AE2 Rabbit  TGGCTGGTGCTGCTGGCGCTGCTCATGGTGGCCCTGGAGGGCAGCTTCCTGGTCCGCTTTGTCTCCCGCTTCACCCAGGAGATCTTCGCCTTTCTCATCT
Consensus   TGGCTGGTGCTCCTGGCCCTGCTCATGGTGGCCCTGGAGGGGAGCTTCCTGGTCCGCTTTGTCTCCCG TTCACCCAGGAGATCTTCGCCTTCCTCATCT
            2501                                                                                    2600
AE2 Horse   CCCTCATCTTCATCTACGAGACCTTCTACAAGCTGGTGAAGATCTTCCAGGAACACCCCCTCCACGGCTGTTCAGTCTCCAACAGCTCTGAGGCAGACAG
AE2 Human   CACTCATCTTCATCTATGAGACCTTCTACAAGCTGGTGAAGATCTTCCAGGAGCACCCCCTGCATGGCTGCTCAGCCTCCAACAGCTCAGAGGTGGACGG
AE2 Mouse   CACTCATCTTCATCTACGAGACCTTCTATAAGCTGATCAAGATCTTCCAGGAGCACCCACTCCATGGCTGCTCAGGCTCCAACGACTCAGAGGCAGGCAG
AE2 Rat     CACTCATCTTCATCTATGAGACCTTCTATAAGCTGATCAAGATCTTTCAGGAGCACCCCCTCCATGGCTGCTCAGTCTCCAACGACTCAGAGGCAGACAG
AE2 Rabbit  CCCTCATCTTCATCTACGAGACCTTCTACAAGCTGATCAAGATCTTCCAGGAACACCCGCTGCACGGCTGTTCCGTCTCCAACAGCTCAGAGACAGACAG
Consensus   CACTCATCTTCATCTACGAGACCTTCTACAAGCTGATCAAGATCTTCCAGGAGCACCCCCTCCATGGCTGCTCAGTCTCCAACAGCTCAGAGGCAGACAG
            2601                                                                                    2700
AE2 Horse   TGGCGACAATGCCACGTGGGCTGGAACAAGAGTTACGCTGGGCCTGGGAAACGGGAGCTCGGCTGGGCCGGCTGGGCAGGGGAGGCCCCGGGGCCAGCCC
AE2 Human   CGGTGAGAACATGACATGGGCCGGGGCAAGACCCACGCTGGGGCCGGGCAACAGGAGCTTGGCTGGGCAGTCTGGGCAGGGGAAGCCCCGGGGCCAGCCC
AE2 Mouse   CAGCAGCAGCAGCAATATGACATGGGCAACAACCATACTGGTACCAGACAACAGCAGCGCTTCTGGGCAGTCTGGGCAGGAGAAGCCCCGGGGCCAGCCC
AE2 Rat     CAGCAGTAACA---ATATGACTTGGGCAGCAACCACACTGGCACCAGACAACAGCAGTGC---------GTCTGGGCAGGAGAGGCCCCGGGGCCAGCCT
AE2 Rabbit  CAGCGAGAACGCCACCTGGGCCGGGGCAGGATCCACGCTGGGGCCGGCCAACAGGAGCTCAGCTGGACAGGCCGGGCAGGGGAGGCCCCGGGGCCAGCCC
Consensus   CAGCGA AACA CAC TGGGC GGGGCAAGA CCACGCTGGG CCGG CAACAGGAGCTC GCTGGGCAGTCTGGGCAGGGGAGGCCCCGGGGCCAGCCC
            2701                                                                                    2800
AE2 Horse   AACACAGCCCTGCTGTCGCTGGTGCTCATGGCCGGCACTTTCTTCATTGCCTTCTTCCTGCGCAAATTCAAGAACGGCCGGTTCTTCCCTGGCCGGGGTAC
AE2 Human   AACACGGCCCTGCTGTCGCTGGTGCTCATGGCCGGCACCTTCTTCATCGCCTTCTTCCTGCGCAAATTCAAGAACAGCCGGTTCTTTCCTGGCCGGGATCC
AE2 Mouse   AACACAGCTTTGCTATCGCTGGTGCTAATGGCTGGCACTTTCTTCATTGCCTTCTTCCTGCGCAAGTTCAAGAACAGCCGGTTCTTCCCTGGCCGGGATCC
AE2 Rat     AACACTGCCTTGCTATCACTGGTGCTGATGGCCGGCACTTTCTTCATCGCCTTCTTCCTGCGCAAATTCAAGAACAGCCGGTTCTTCCCTGGCCGGGATCC
AE2 Rabbit  AACACAGCCCTGCTGTCGCTGGTGCTCATGGCTGGCACCTTCTTCATTGCCTTCTTCCTGCGCAAATTCAAGAACAGCCGGTTCTTCCCTGGCCGGGATCC
Consensus   AACACAGCCCTGCTGTCGCTGGTGCTCATGGCCGGCACTTTCTTCATTGCCTTCTTCCTGCGCAAATTCAAGAACAGCCGGTTCTTCCCTGGCCGGATCC
            2801                                                                                    2900
AE2 Horse   GGCGGGTGATTGGGGACTTTGGGGTGCCCATCGCGATCCTCATCATGGTGCTTGTGGATTACAGTATTGAGGACACCTACACCCAGAAGCTGAGTGTGCC
AE2 Human   GGCGGGTGATTGGGGACTTTGGGGTGCCCATCGCCATCCTCATCATGGTGCTTGTGGATTACAGTATTGAGGACACCTATACCCAGAAGCTGAGCGTTCC
AE2 Mouse   GGCGGGTAATTGGGGACTTTGGGGTGCCCATCGCGATCCTCATCATGGTGCTTGTGGATTACAGTATTGAGGACACCTACACCCAGAAACTGAGTGTGCC
AE2 Rat     GGCGGGTAATTGGGGACTTTGGGGTGCCCATCGCGATCCTCATCATGGTGCTTGTGGATTACAGTATTGAGGATACCTACACCCAGAAGCTAAGTGTGCC
AE2 Rabbit  GGCGGGTGATTGGGGACTTTGGGGTGCCCATTGCAATCCTCATCATGGTGCTTGTGGATTACAGCATTGAGGACACCTATACCCAGAAACTGAGTGTCCC
Consensus   GGCGGGTGATTGGGGACTTTGGGGTGCCCATCGCGATCCTCATCATGGTGCTTGTGGATTACAGTATTGAGGACACCTACACCCAGAAGCTGAGTGTGCC
            2901                                                                                    3000
AE2 Horse   CAGCGGATTCTCGGTGACAGCCCCAGAAAAGCGAGGCTGGGTCATCAACCCCCTGGGGGAGAAGAGCTCCTTCCCCGTGTGGATGATGGTTGCCAGCCTG
AE2 Human   CAGTGGATTCTCGGTGACTGCCCCAGAAAAGAGGGGCTGGGTCATCAACCCCCTGGGAGAGAAGAGCCCCTTCCCTGTGTGGATGATGGTTGCCAGCCTG
AE2 Mouse   CAGCGGATTCTCAGTGACAGCCCCAGACAAGCGGGGCTGGGTCATCAACCCCCTTGGAGAAAAGACCCCCTTCCCTGTGTGGATGATGGTGGCCAGCCTG
AE2 Rat     CAGTGGGTTCTCAGTGACAGCCCCAGACAAGCGGGGCTGGGTCATCAACCCCCTTGGAGAGAAGACTCCTTTCCCTGTGTGGATGATGGTGGCCAGCCTG
AE2 Rabbit  AAGTGGATTCTCAGTGACAGCCCCTGACAAGCGGGGCTGGGTCATCAACCCCCTGGGCGAGAAGAGCCCCTTCCCAGTGTGGATGATGGTGGCCAGCCTG
Consensus   CAGTGGATTCTCAGTGACAGCCCCAGACAAGCGGGGCTGGGTCATCAACCCCCTGGGAGAGAAGAGCCCCTTCCCTGTGTGGATGATGGTGGCCAGCCTG
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              3001                                                                                   3100
AE2 Horse     CTGCCCGCCATCCTGGTCTTCATCCTCATCTTCATGGAGACGCAAATCACCACGCTGATCATCTCCAAGAAGGAGCGCATGCTGCAGAAGGGCTCCGGTT
AE2 Human     CTGCCCGCCATCCTGGTCTTCATTCTCATCTTCATGGAGACACAGATCACCACGCTCATCATCTCCAAGAAGGAGCGCATGCTGCAGAAGGGCTCCGGCT
AE2 Mouse     CTGCCTGCTGTTCTGGTGTTCATCCTCATCTTCATGGAGACACAGATCACCACGCTGATCATCTCCAAGAAAGAGAGGATGCTGCAGAAGGGCTCTGGCT
AE2 Rat       TTGCCTGCCGTTCTGGTGTTCATCCTCATCTTCATGGAGACACAGATCACCACGTTGATCATCTCCAAGAAAGAGCGGATGCTTCAGAAGGGCTCTGGCT
AE2 Rabbit    CTGCCTGCCATCCTAGTCTTCATCCTCATCTTCATGGAGACGCAGATCACCACGCTGATCATCTCCAAGAAGGAGCGAATGCTGCAGAAGGGCTCCGGCT
Consensus     CTGCCTGCCATCCTGGTCTTCATCCTCATCTTCATGGAGACACAGATCACCACGCTGATCATCTCCAAGAAGGAGCG ATGCTGCAGAAGGGCTCCGGCT
              3101                                                                                   3200
AE2 Horse     TCCACCTGGACCTGCTGCTCATTGTGGCCATGGGTGGCATCTGTGCCCTCTTTGGCCTGCCCTGGTTGGCTGCTGCCACTGTCCGCTCCGTCACTCATGC
AE2 Human     TCCACCTGGACCTGCTGCTCATCGTGGCCATGGGCGGCATCTGTGCCCTCTTTGGCCTGCCCTGGTTGGCTGCTGCCACTGTCCGCTCTGTCACTCACGC
AE2 Mouse     TCCATCTCGACCTGTTGCTCATTGTAGCCATGGGTGGCATCTGTGCCCTCTTTGGCCTGCCCTTGGTTGGCCGCTGCCACTGTCCGCTCTGTCACCCATGC
AE2 Rat       TCCACCTTGATCTGTTGCTCATTGTAGCCATGGGTGGCATCTGTGCCCTCTTTGGCCTGCCCTTGGTTGGCTGCTGCCACTGTCCGCTCTGTCACTCATGC
AE2 Rabbit    TCCACCTGGACTTGCTGCTCATCGTGGCTATGGGGGGCATCTGTGCCCTCTTTGGCCTGCCCTTGGTTGGCCGCTGCCACCGTGCGCTCTGTCACTCACGC
Consensus     TCCACCTGGACCTGCTGCTCATTGTGGCCATGGGTGGCATCTGTGCCCTCTTTGGCCTGCCCTTGGTTGGCTGCTGCCACTGTCCGCTCTGTCACTCATGC
              3201                                                                                   3300
AE2 Horse     CAACGCGCTCACCGTCATGAGCAAGGCTGTGGCGCCGGGGGACAAGCCCAAGATCCAGGAAGTCAAAGAGCAGCGGGTGACGGGGCTGCTGGTTGCCCTG
AE2 Human     CAACGCGCTCACTGTCATGAGCAAGGCTGTGGCACCTGGGGACAAGCCCAAGATTCAGGAAGTCAAGGAGCAGCGGGTGACGGGGCTGCTGGTTGCCCTG
AE2 Mouse     CAATGCACTCACTGTCATGAGCAAGGCTGTGGCACCTGGGGACAAACCCAAGATTCAGGAAGTCAAGGAACAGCGTGTGACAGGGCTGCTGGTGGCCCTG
AE2 Rat       CAATGCACTCACTGTCATGAGCAAGGCTGTGGCACCTGGTGACAAACCCAAGATTCAGGAAGTCAAGGAGCAGCGGGTGACGGGGCTGCTGGTGGCCCTG
AE2 Rabbit    CAATGCACTCACCGTCATGAGCAAGGCTGTGGCGCCCGGGGACAAGCCCAAGATTCAGGAGGTCAAGGAGCAGCGGGTGACGGGGCTGCTGGTGGCCCTG
Consensus     CAATGCACTCACTGTCATGAGCAAGGCTGTGGCACCTGGGGACAAGCCCAAGATTCAGGAAGTCAAGGAGCAGCGGGTGACGGGGCTGCTGGTGGCCCTG
              3301                                                                                   3400
AE2 Horse     CTTGTGGGCCTCTCCATAGTTATTGGAGATCTGCTCCGGCAGATACCCCTGGCCGTGCTCTTTGGAATCTTCCTGTACATGGGAGTTACCTCCCTTAACG
AE2 Human     CTTGTGGGCCTCTCCATAGTTATCGGGGATCTGCTCCGGCAGATCCCCCTGGCCGTGCTCTTTGGAATTTTCCTGTACATGGGAGTCACCTCCCTTAACG
AE2 Mouse     CTTGTGGGCCTCTCCATGGTCATTGGGGACCTCCTGCGGCAGATCCCCCTGGCTGTGCTCTTTGGCATTTTCTTGTACATGGGAGTCACCTCCCTCAATG
AE2 Rat       CTTGTGGGACTCTCCATGGTCATTGGGGACCTACTGCGGCAGATCCCCCTGGCTGTGCTCTTTGGCATTTTCTTATACATGGGAGTTACTTCCCTTAATG
AE2 Rabbit    CTTGTAGGCCTCTCCATCGTTATCGGGGACCTGCTCCGGCAGATCCCCCTGGCCGTGCTCTTTGGAATTTTCCTCTACATGGGCGTCACATCTCTTAATG
Consensus     CTTGTGGGCCTCTCCAT GTTATTGGGGACCTGCTCCGGCAGATCCCCCTGGCCGTGCTCTTTGGAATTTTCCTGTACATGGGAGTCACCTCCCTTAATG
              3401                                                                                   3500
AE2 Horse     GGATCCAGTTCTATGAGCGGCTGCACCTGCTGCTCATGCCACCCAAACACCACCCAGATGTCACCTATGTCAAGAAGGTTCGGACCCTCCGTATGCACCT
AE2 Human     GGATCCAGTTCTATGAGCGGCTGCATCTGCTGCTCATGCCGCCCAAACACCACCCAGATGTCACTTACGTCAAGAAGGTCCGGACCCTCCGTATGCACCT
AE2 Mouse     GGATCCAGTTCTACGAGCGGCTGCACCTGCTGCTCATGCCGCCCAAACACCACCCAGATGTCACCTATGTCAAAAAGGTTCGGACCATGCGGATGCACCT
AE2 Rat       GGATCCAATTCTACGAGCGGTTGCACCTGCTACTCATGCCGCCCAAACACCACCCAGATGTTACCTATGTCAAGAAGGTTCGGACCATGCGGATGCACCT
AE2 Rabbit    GGATTCAGTTCTATGAGCGGCTGCACCTGCTGCTGATGCCGCCCAAGCACCACCCCGACGTCACTTACGTCAAGAAGGTTCGGACCCTGCGGATGCACTT
Consensus     GGATCCAGTTCTATGAGCGGCTGCACCTGCTGCTCATGCCGCCCAAACACCACCCAGATGTCACCTATGTCAAGAAGGTTCGGACCCTGCGGATGCACCT
              3501                                                                                   3600
AE2 Horse     GTTCACAGCCCTGCAGCTGCTCTGCCTGGCCCTGCTCTGGGCCGTCATGTCCACAGCAGCCTCCCTGGCCTTCCCCTTCATCCTCATCCTCACAGTGCCG
AE2 Human     GTTCACGGCCCTGCAGCTGCTCTGCCTGGCCCTGCTCTGGGCCGTCATGTCCACAGCTGCCTCCCTGGCCTTCCCCTTCATCCTCATCCTCACAGTGCCG
AE2 Mouse     GTTCACTGCCCTTGCAGTTGCTCTGCCTGGCCCTGCTTTGGGCAGTCATGTCCACAGCCGCTTCCCTGGCCTTCCCCTTCATCCTCATCCTCACAGTGCCT
AE2 Rat       GTTCACTGCCCTTGCAGCTGCTCTGTCTGGCCCTGCTTTGGGCGGTCATGTCCACAGCTGCTTCCCTGGCCTTCCCCTTCATCCTCATCCTCACAGTGCCT
AE2 Rabbit    GTTCACGGCCCTGCAGCTGCTCTGCCTGGCCCTGCTCTGGGCTGTCATGTCCACAGCTGCTTCCCTGGCCTTCCCCTTCATCCTCATCCTCACGGTGCCG
Consensus     GTTCAC GCCCTGCAGCTGCTCTGCCTGGCCCTGCTCTGGGC GTCATGTCCACAGCTGCTTCCCTGGCCTTCCCCTTCATCCTCATCCTCACAGTGCCG
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            3601                                                                                    3700
AE2 Horse   CTCCGCATGGTCGTGCTCACCCGCATCTTCACCGAGCGAGAGATGAAATGCCTGGATGCGAATGAGGCGGAGCCGGTGTTTGATGAGCGGGAGGGTGTGG
AE2 Human   CTCCGCATGGTGGTGCTCACCCGTATCTTCACCGACCGAGAGATGAAATGTCTGGATGCTAACGAGGCAGAGCCGGTGTTTGATGAGCGGGAGGGTGTGG
AE2 Mouse   TTGCGCATGGTGGTACTTACCCGAATCTTCACTGAGCGAGAAATGAAATGTCTGGATGCTAATGAGGCAGAGCCAGTGTTTGACGAGTGTGAAGGTGTGG
AE2 Rat     TTGCGCATGGTGGTACTTACCCGGATCTTCACTGAGCGAGAAATGAAATGTCTGGATGCTAATGAGGCAGAGCCAGTGTTTGATGAGTGTGAAGGTGTGG
AE2 Rabbit  CTCCGCATGGTGGTGCTCACCCGCATCTTCACTGAGCGAGAGATGAAATGCCTGGACGCTAATGAGGCGGAGCCCGTGTTTGATGAGCGGGAGGGTGTGG
Consensus   CTCCGCATGGTGGTGCTCACCCG ATCTTCACTGAGCGAGAGATGAAATGTCTGGATGCTAATGAGGCAGAGCC GTGTTTGATGAGCGGGAGGGTGTGG
            3701
AE2 Horse   ACGAGTACAATGAGATGCCCATGCCTGTG
AE2 Human   ACGAGTACAATGAGATGCCCATGCCTGTG
AE2 Mouse   ATGAGTACAACGAGATGCCCATGCCTGTG
AE2 Rat     ACGAGTACAACGAGATGCCCATGCCTGTG
AE2 Rabbit  ATGAGTACAACGAGATGCCCATGCCTGTG
Consensus   ACGAGTACAACGAGATGCCCATGCCTGTG
```

Figure VI.4. *Alignment of the nucleotide coding sequence of the equine AE2.*

It was compared with the sequence of human, mouse, rat, and rabbit.

VI.6. Anion exchanger AE2 and butyrate transport in the equine colonic BLM

Uptake studies were performed to test the potential involvement of anion exchanger isoform 2 in the uptake of butyrate in the basolateral membranes of the colon. As shown previously (see chapter IV), the initial rate of $[^{14}C]$-butyrate was inhibited in the presence of 20 mM of chloride when present in the extravesicular medium. For that reason AE2 was suggested as a potential candidate transporter for butyrate. In addition, it was suggested by Yabuuchi *et al.* (1998) that the butyrate transporter in HEK 293 cell line may belong to AE family (AE2).

For this, four conditions for transport studies were performed:

- Control: BLM vesicles were loaded with bicarbonate buffered at pH 7.5 and incubated in medium containing 1 mM of butyrate buffered at pH 5.5.

- Second condition: BLMV were loaded with bicarbonate buffered at pH 7.5 and incubated in a media (pH 5.5) containing both 1 mM butyrate and 20 mM of chloride.

- Third condition: BLMV were loaded with both HCO_3^- and chloride pH 7.5 and incubated in a media containing both butyrate and 20 mM of HCO_3^- as described in the figure legend.

- In the fourth condition inhibition study was carried out, but this time using low concentration (50 µM) stilbene derivatives, SITS and DIDS.
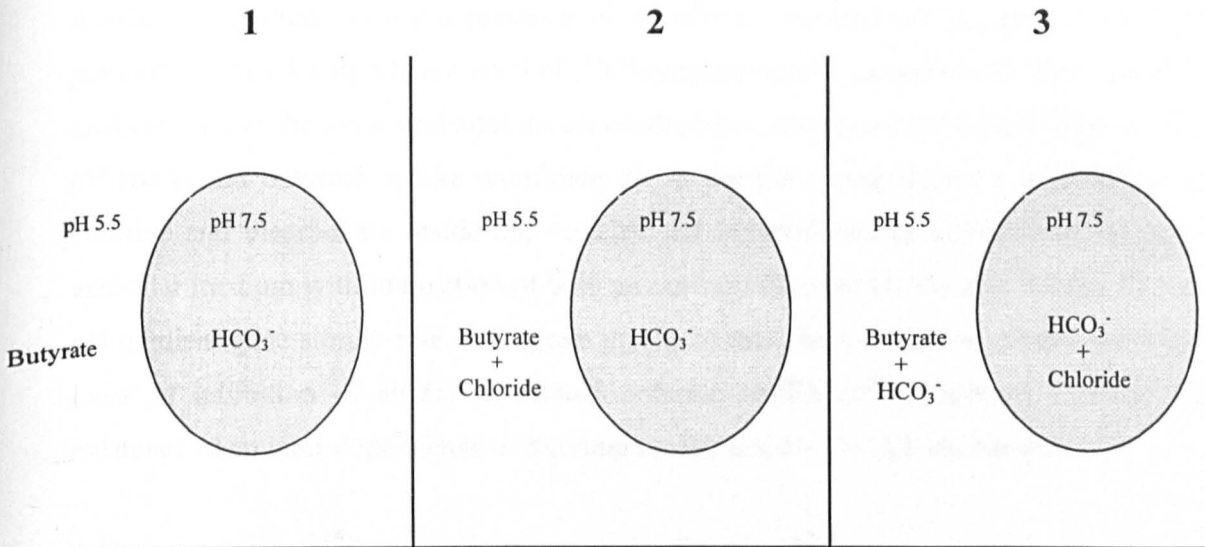
Figure VI.5 Schematic representation of the transport studies for the determination of the mechanism of butyrate uptake in BLMV isolated from horse colon.
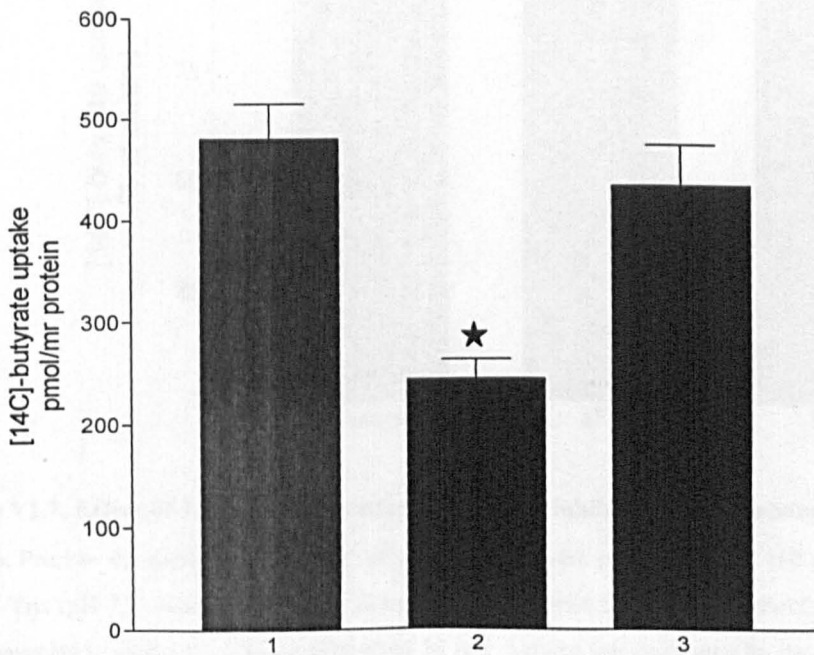
Figure VI.6. Effect of anions on butyrate uptake. The horse colonic BLMV were preloaded with 20 mM hepes/ Tris (pH 7.5) and either 150 mM KHCO₃ or 130 mM KHCO₃ and 20 mM KCl. Uptake was measured at 38^0C by incubating membrane vesicles (100 μg protein per assay) in medium containing 20 mM Mes/ Tris (pH 5.5), 1mM [^{14}C]-butyrate and either 150 mM K-gluconate, 130 mM K-gluconate and 20 mM KCl or 130 mM K-gluconate and 20 mM KHCO₃. The reaction was terminated by the addition of 1 ml of ice-cold stop solution after 3 seconds. Uptake was expressed as a percentage of control. Values are presented as means ± S.E.M for two separate experiments.

As shown in figure VI.6 the presence of an outward-directed HCO_3^- gradient and pH gradient produced a significant level of $[^{14}C]$-butyrate uptake (condition 1). Addition of 20 mM chloride to the extra-vesicular media resulted in a significant inhibition of the HCO_3^-/ pH-stimulated butyrate uptake (condition 2). In the third experiment, a combination of chloride and bicarbonate inside the vesicles and butyrate and bicarbonate in the extra-vesicular medium with imposition of both an outward directed HCO_3^- and inward directed pH gradient gave similar rate of butyrate uptake to that observed in the control condition. Lack of inhibition of HCO_3^--stimulated butyrate uptake (3^{rd} condition) excludes the existence of an inter-dependence of butyrate uptake and HCO_3^-/ Cl^- exchanger.
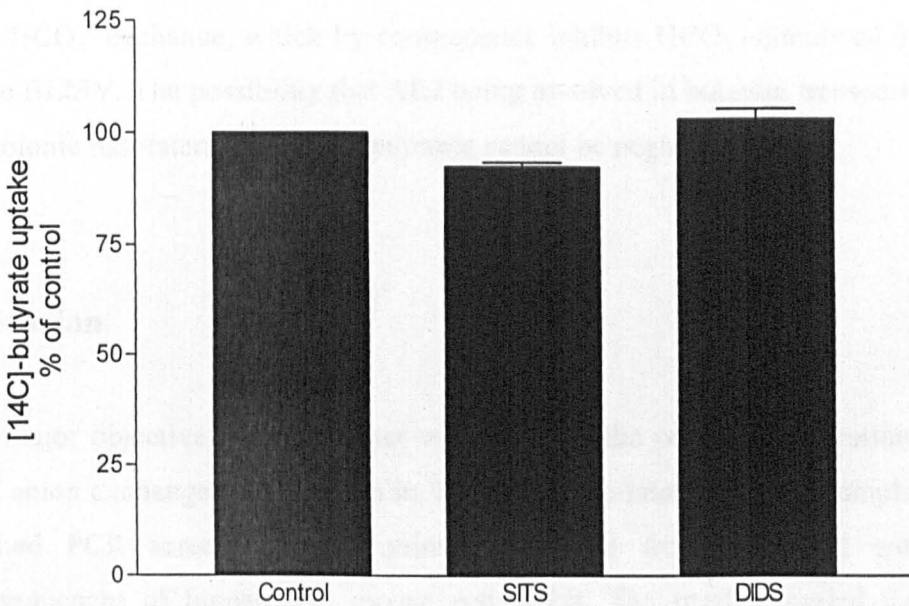
Figure VI.7. Effect of low concentration transport inhibitors on bicarbonate-stimulated butyrate uptake. Prior to the experiment, BLMV of equine colon were preloaded with 150 mM $KHCO_3$ and 20 mM Hepes/ Tris (pH 7.5) and incubated for 30 minutes at 4^0C with the anion inhibitors; SIDS, SITS were added to the vesicles to give a final concentration of 50 μM. Uptake was measured by incubating the vesicles (100 μg of protein per assay) in media containing 20 mM Mes/ Tris (pH 5.5), 1mM $[^{14}C]$-butyrate and 150 mM K-gluconate. After 3 seconds, the reaction was stopped by the addition of 1 ml of ice-cold stop solution. Values are presented as means ± S.E.M for two experiments set in triplicate.

In the fourth condition, the BLMV were incubated in a media containing 50 µM of SITS and DIDS prior to the experiment. At low SITS and DIDS concentrations, inhibition of the initial rate of butyrate uptake is not observed (figure VI.7) as was the case at the high SITS and DIDS concentrations (500 µM) where inhibition was between 60 – 70 % (see section IV.9), however, inhibition of butyrate transport at high concentration could be the subject of tissue specificity.In a previous study, DIDS was demonstrated to have high affinity to the Cl⁻/HCO₃⁻ exchanger (Janas *et al.* 1989). The authors have shown that DIDS bind irreversibly to the erythrocytes protein so that low concentration of 0.4 µM resulted in a 93 % inhibition. The uptake studies result suggestes that butyrate and Cl⁻ are taken up by two distinct exchangers. As a result, Cl⁻/HCO₃⁻ exchange dissipates the HCO₃⁻ gradient for butyrate/HCO₃⁻ exchange, which by consequence inhibits HCO₃⁻-stimulated butyrate uptake in the BLMV. The possibility that AE2 being involved in butyrate transport across the equine colonic basolateral plasma membranes cannot be neglected.

VI.7. Discussion

The major objective of this chapter was to assess the potential expression of the members of anion exchanger family (AE) in the horse large intestine. To accomplish this, we performed PCR screening using primers designed from published consensus nucleotide sequences of human, rat, mouse and rabbit. The result revealed that AE2 isoform but not AE1 or AE3 (brain subtype), was detected in the equine colon. It has been shown that AE isoforms mediate the Cl⁻/HCO₃⁻ exchange (Alper *et al.* 2002) which together with Na⁺/H⁺ exchange regulate cell volume (Shen *et al.* 2002., Tyagi *et al.* 2000., Rajendran and Binder, 2000).

AE1 is one of the best characterised erythrocyte-associated membrane proteins (Kopito, 1990), it was shown to be expressed in rat colon (Rajendran *et al.* 2000). Our study however failed to detect AE1 in the BLMV of horse colon. This agrees with the finding of Alrefai *et al* (2001) who could not detect AE1 at both RNA and protein level in the human colon.

Membrane studies of AE2 isoform have sown that the protein is present on the basolateral plasma membranes of polarised epithelial cells including human, mouse and rat colon (Alrefai *et al*. 2001., Ikuma *et al*. 2003., Alper *et al*. 1999). However Chow *et al* (1992) have shown by western blot analysis that AE2 was expressed on the brush-border membrane of ileal enterocytes. By using AE2 antibody for immunoblotting, the present study revealed that AE2 is localised at the protein level in basolateral membrane of equine colonocytes.

Another experiment was used to identify the potential expression of AE2 at the mRNA level. Therefore PCR-derived 550 bp cDNA fragment was used as a radiolabelled probe to establish the expression. As shown in figure VI.2, AE2 cDNA probe hybridised with the mRNA of an apparent size of 4.2 Kb.

Subsequently, we amplified, cloned and sequenced the entire AE2 transcript which was used later to generate the cRNA. The cRNA can be used for microinjection into xenopus oocytes, functional properties of AE2 cRNA-injected oocytes to potentially transport butyrate could be assessed.

The horse AE2 nucleotide and amino acid sequences were compared to AE2 sequence from other species (see figure VI.4). The data is summarised in table VI.1.

	AE2 Rat	AE2 Human	AE2 Mouse	AE2 Rabbit	AE2 Chicken
AE2 Horse (Nucleotide)	85.4	90.1	84.1	86.8	68.4
AE2 Horse (Amino Acids)	93.5	95.6	93.2	95.7	75.1

Table VI.1 *Comparison of the equine AE2 nucleotide and amino acid sequences with the AE2 sequence of human, mouse, rat, and rabbit. Values represent percentage.*

The complete nucleotide sequence of equine AE2 is characterised by its noticeable length. The horse nucleotide sequence exhibit high degree of homology with the human and to a lesser extent with the rat, mouse and rabbit with parentage of 85.4, 84.1 and 86.8 respectively. The chicken sequence (nucleotide and amino acid) is quite divergent compared to the horse sequence. The AE2 protein encoded by the cDNA is 1237 amino acids in length. Comparison of amino acid sequences for AE2 in various species exhibited high degree of identity ranging from 93.2 (mouse) to 95.7 (rabbit).

The horse AE2 nucleotide and amino acid sequences were also compared to the human AE isoforms, table VI.2.

	AE2 horse	AE1 Human	AE2 Human	bAE3 Human	cAE3 Human
Nucleotide	100	43.4	91.1	60.8	63.3
Amino Acids	100	43.2	95.6	57.2	56.4

Table VI.2. *Comparison of the equine AE2 nucleotide and amino acid sequences with the AE1, bAE3 and cAE3 sequence of human.. Values represent percentage identity.*

The objective of this chapter was to extend the available data regarding the mechanism of butyrate uptake in the equine colonic basolateral plasma membrane. From the abovementioned uptake studies, differences between HCO_3^--dependent Cl^- exchanger and butyrate uptake was highlighted.

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A

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