Title The Functional and Molecular Characterisation of the Pig Ileal NA^+/Bile Acid Co-Transport Protein

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THE FUNCTIONAL AND MOLECULAR CHARACTERISATION OF THE PIG ILEAL NA^+/BILE ACID CO-TRANSPORT PROTEIN

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Bile acids are essential for the efficient digestion and absorption of lipids, and are re-absorbed by a Na^+/bile acid co-transport protein within the ileum.

Investigation into the transport protein, using BBMV and *Xenopus laevis* oocytes, revealed that pig ileal Na^+/bile acid co-transport protein was not strictly dependent of the presence of an inwardly directed Na^+ gradient, as previously determined. Cations which had ionic radii between 0.8 – 1.33Å, most notably Na^+, K^+ and Ca^{2+}, stimulated secondary active transport of taurocholate. Taurocholate uptake in the presence of inwardly directed cation gradients of Na^+ and K^+, demonstrated Michealis Menten Kinetics, concentrative accumulation, competitive inhibition and was temperature sensitive. Preventing the translation of the gene encoding the Na^+/bile acid co-transport protein, abolished secondary active transport in the presence of both Na^+ and K^+.

Isolation and computer modelling of the gene which encoded the pig ileal Na^+/bile acid co-transport protein, revealed that this protein was composed of 8 transmembrane domains and lead to the identification of proposed cation and bile acid binding sites.

The ability of K^+ to stimulate the Na^+/bile acid co-transport protein could be of physiological importance *in vivo*, because of the depleted Na^+ concentration present within the ileum. Though, the rate of taurocholate transport in the presence of K^+ is reduced when compared to Na^+, the transport protein has very similar affinities for taurocholate in the presence of both cations and therefore could use K^+ for efficient re-absorption of taurocholate. Therefore, for this study it was proposed that the Na^+/bile acid co-transport protein had a preference for Na^+ rather than a strict dependence as previously concluded.

This study was undertaken in order to fulfil the requirements for the qualification of Doctor of Philosophy. The study involved the functional characterisation of the pig ileal Na^+/bile acid co-transport protein, and investigation into the gene which encodes for the protein.

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I declare this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Luton. It has not been submitted before for any degree or examination in any other University

Gillian Knight 30.09.00

INTRODUCTION

CHAPTER ONE:

1. The morphology and physiology of the gastrointestinal system

The function of the gastrointestinal system is the digestion and absorption of ingested food. The alimentary canal is a long muscular tube beginning at the lips and ending at the rectum, encompassing the mouth, pharynx, oesophagus, stomach, small intestine, large intestine, and glandular organs (salivary glands, liver, gall bladder and pancreas).

1.1 Physiology of the mouth

Digestion begins at the mouth, where the actions of salivary secretions start to digest food. In a normal adult, the salivary glands secrete about 500mls/ day of a hypotonic solution (Johnson, 1991). Saliva primarily consists of Na^+, K^+, Cl^- and HCO_3^- ions, with the salivary acinar cells actively secreting K^+, HCO_3^- and Li^+ from the blood into the saliva. The final salivary secretions consists of 8-40 mM K^+, 5-100 mM Na^+, 1.5-2 mM Ca^{2+}, 5.5-14 mM PO_4^-, 5-70 mM Cl^- (Hold *et al.*,1983). Therefore, the saliva entering the stomach consists of a high concentration of electrolytes, which will be utilised in digestion and absorption in the stomach and small intestine.

1.2 Physiology of the stomach

The stomach is a sack like organ where ingested food is stored partially digested and then slowly moved into the small intestine by peristalsis. The mucosa of the stomach is primarily composed of columnar mucus secreting epithelial cells and oxyntic and pyloric glands. The oxyntic glands contain chief cells and parietal cells that secrete the enzyme precursor pepsinogen and HCl respectively. In humans, the parietal cells

also secrete an intrinsic factor needed for vitamin B12 absorption in the terminal ileum (Davenport, 1975). The pyloric glands contain gastrin producing G cells and mucus cells which secrete a small amount of pepsinogen (Johnson, 1991).

1.2.1 Gastric secretions

1.2.1.1 Gastrin

Gastrin secretion by G cells occurs in the stomach and the duodenum. The G cells are stimulated by neural distension, the presence of proteins and gastrin releasing peptide (GRP). Within the gastrointestinal system, there are two forms of gastrin, with the larger 17 amino acid form (G34) being broken down to G17 and heptadecapeptide by the action of trypsin (Johnson, 1991). Gastrin has a range of effects as shown in table 1.1 but its most important effect is the stimulation of acid secretion (Campbell *et al.*, 1994). However, it can only function at a pH above 3 so after the release of HCl, gastrin secretion is inhibited by the release of somatostatin.

1.2.1.2 Hydrochloric acid secretion (HCl)

Parietal cells of the oxyntic mucosa are stimulated to secrete HCl by gastrin, histamine and acetylcholine (a mediator between vagal nerves and parietal cells). HCl is secreted by H^+ being actively pumped (down its electrical gradient) into the stomach lumen by an H^+, K^+ ATPase. Gastric inhibitory protein (GIP), which is released from the duodenum and jejunum, prevents HCl secretion (Campbell *et al.*, 1994).

1.2.1.3 Pepsin

Pepsinogen (precursor of pepsin) has two different forms, pepsinogen I which is

secreted by the chief and mucus cells of the oxyntic glands and pepsinogen II secreted by mucus cells present in the pyloric glands, oxyntic glands and duodenum. The strongest stimulation of pepsinogen secretion is caused by acetyl choline (ACH), which stimulates parietal cells to release H^+, which in turn activates chief cells to release pepsinogen. The H^+ then activates the enzyme, by degrading pepsinogen (42.5 kDa) into pepsin (35 kDa). Gastrin and secretin are also able to directly stimulate the chief cells (Davenport, 1975).

Table 1.1, The effects of gastrointestinal hormones

Action of hormone	Gastrin	CCK	Secretin
Acid secretion	✔	✓	x
Gastric emptying	x	x	x
Pancreatic HCO_3^- secretion	✓	✔	✔
Pancreatic enzyme secretion	✓	✔	✓
Bile HCO_3^- secretion	✓	✓	✓
Gall bladder contraction	✓	✓	✔
Gastric motility	✓	✓	x
Intestinal motility	✓	✓	x
Insulin release	✓	✓	✓
Mucosal growth	✔	✓	x
Pancreatic growth	✓	✔	✔

Key: ✓ = stimulates ✔ = important function x = inhibits

Adapted from Johnson, 1991

1.2.1.4 Electrolyte secretion

Within the stomach, an electrolyte solution is secreted. At low secretory rates, the

solution is mainly composed of NaCl with a low concentration of H^+ and K^+. As the secretion rate increases so does the concentration of H^+ with a corresponding decrease in Na^+ secretion. At peak secretory rates, the solution is mainly composed of HCl and low concentrations of Na^+ and K^+ (Johnson, 1991). Though the concentrations of the major electrolytes within the stomach are variable throughout different stages of digestion, the gastric juices remain isotonic with respect to the plasma (Davenport, 1975).

Therefore, in addition to the electrolytes entering the stomach from the saliva there is further secretion of the cations Na^+, K^+, H^+ and the anion Cl^- from the stomach. The presence of these ions entering the small intestine will be of vital importance for the reabsorption processes in the small intestine.

1.3 Physiology of the pancreas

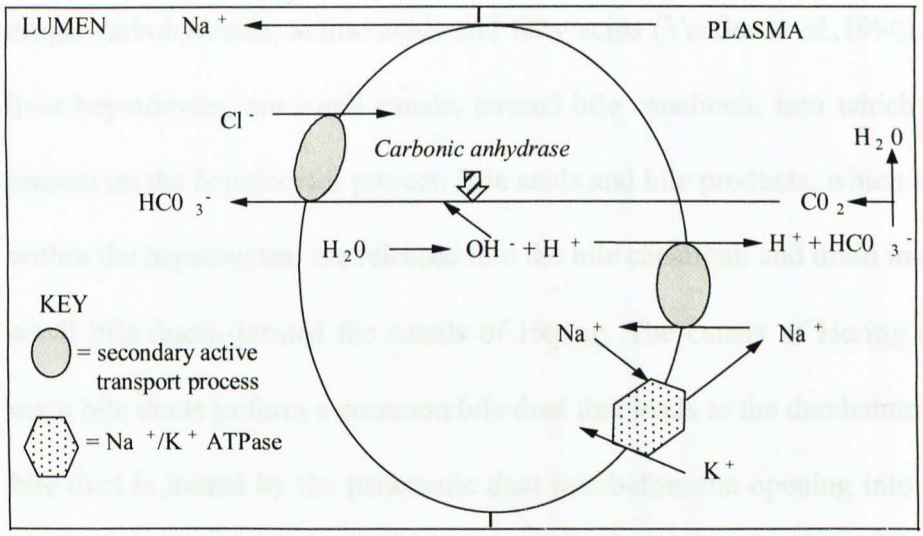
The functions of the pancreas are the exocrine secretion of enzymes and a bicarbonate solution. The secretions come from acinar cells which are stimulated by the presence of gastrin and CCK, though gastrin is only half as potent a stimulator as CCK (Jonhson,1991).

1.3.1 Electrolyte secretions

The pancreas, along with the stomach, also secretes an isotonic electrolyte solution which enters the small intestine. At low secretion rates, Na^+ and Cl^- are the predominant ions, whilst at high rates, Cl^- is replaced by HCO_3^-. K^+ is present in all secretions at the same concentration as the plasma (Davenport, 1975; Johnson, 1991). Figure 1.1 summarises the transport processes occurring within the ductule and acinar

cells.

Figure 1.1, Schematic diagram of electrolyte secretion in the pancreas



Adapted from Johnson, 1991

1.3.2 Pancreatic enzymes

There are a wide range of enzymes secreted by the acinar cells of the pancreas, all of which are involved in the digestion of food within the intestine. Proteolytic enzymes, which include trypsinogen, chymotrypsinogen, procarboxypeptidase, proelastase and pro-aminopeptidase are secreted in an inactive form and are activated by trypsin. The inactive form of trypsin, trypsinogen, is activated by duodenal enterokinase, by the cleavage of a hexapeptide from the N-terminal end (Johnson, 1991).

1.4 Physiology of the liver

The liver is the largest mass of glandular tissue in the body with a diverse range of functions, from exocrine and endocrine secretions to metabolism and toxin degradation. It is divided into four lobes, each containing hepatocytes and a stroma of connective tissue that has blood vessels, nerves, ducts and lymphatics (Ross and

Romrell, 1989). The hepatocytes have numerous functions including protein synthesis, lipoprotein synthesis, bile production as well as metabolism of lipid soluble drugs, carbohydrates, amino acids and fatty acids (Vander *et al.*,1990). Between the liver hepatocytes, are small canals, termed bile canaliculi, into which the microvilli present on the hepatocytes project. Bile acids and bile products, which are synthesised within the hepatocytes, are released into the bile canaliculi and drain into a network of small bile ducts, termed the canals of Hering. The canals of Hering connect to the main bile ducts to form a common bile duct that leads to the duodenum. The common bile duct is joined by the pancreatic duct just before the opening into the duodenum (Johnson, 1991).

1.5 Physiology of the gallbladder

The mucosa is lined with a thin layer of absorptive columnar epithelial cells that are separated by intercellular spaces and are involved in the reabsorption of electrolytes and water. The epithelial cells have numerous small microvilli on their surfaces, for the transport of water and solutes from the bile (Ross and Romrell, 1989). The absorption of Na^+ is an active process, which is coupled to the flow of an anion. Water reabsorption is a passive process but is dependent on the active absorption of NaCl and $NaHCO_3$ to provide the osmotic gradient. The absorption of water results in a concentrated bile solution within the lumen of the gall bladder (Johnson, 1991). The absorption of Na^+, Cl^-, and HCO_3^- leaves behind other electrolytes such as K^+ and Ca^{2+}. The concentration of cations, bile acids, cholesterol, phospholipids and mixed bile acid-lecithin-cholesterol micelles within the gallbladder, results in 40-60 ml of a

concentrated isotonic bile solution with a high composition of K^+ and Ca^{2+} (Davenport, 1975). The arrival of chyme from the stomach, containing lipids, into the duodenum stimulates the release of CCK (cholecystokinin) from the duodenal mucosa. CCK in turn stimulates the gallbladder to release the concentrated bile into the duodenum (Ross and Romrell, 1989).

1.6 Physiology of the large intestine

The mucosa of the large bowel contains unbranched crypts of Lieberkühn, endocrine cells, absorptive cells and goblet cells (Ross and Romerall, 1989). The epithelial cells of the large intestine are mainly involved in the absorption of water from the lumen of the bowel, though absorption of nutrients can occur within the proximal region. The presence of high concentrations of bile acids can inhibit water absorption and cause diarrhoea. Excessive concentrations of bile acids in the large bowel is usually due to disruption of enterohepatic circulation, such as ileal resection, thereby allowing the detergent-like bile acids to have an adverse effect on the epithelium of the large bowel (Eusufzai, 1995; Singh *et al.*,1996).

CHAPTER TWO:

2. The morphology and physiology of the small intestine

2.1 Anatomy of the small intestine

The small intestine is involved in the completion of digestion and absorption of ingested food. Starting at the pylorus and ending at the ileo-caecal junction, the small intestine is divided into the duodenum, jejunum and ileum. Maximum digestion and absorption occurs in the duodenum and jejunum, with pancreatic and liver secretions emptying through a duct into the duodenum (Ross and Romerall, 1989;Vander *et al.*, 1990). Therefore, the proximal region of the small intestine has the highest concentration of cations such as Na^+, so most of the intestinal enzymes and transport proteins are found within this region.

2.2 Morphology of the small intestine

To increase the rate of absorption, the small intestine is very long and has a large surface area due to the presence of villi (0.5-1 mm in length), microvilli and circular or spiral folding of the mucosa membrane (*plicae circulares*). Intestinal villi vary in shape, duodenal villi are broad and leaf shaped with maximal surface area, jejunal have a tongue-like shape and ileal villi resemble short finger-like projections (Trier and Madara, 1981). In the centre of each villus is the lacteal, which is involved fat absorption, and a capillary network that transports amino acids and carbohydrates to the hepatic portal vein.

2.3 Epithelium of the small intestine

Due to the numerous functions of the small intestine, the epithelium covering the villi

is a highly differentiated structure with a range of cells that have specific morphology and functions (Trier and Madara, 1981).

2.3.1 Crypts of Leiberkühn

The crypts of Leiberkühn contain a number of mature cells, such as caveolated and endocrine cells, and undifferentiated cells that mature as they migrate towards the tip of the villi (Ross and Romrell, 1989).

2.3.1.1 Paneth cells

Paneth cells occur in the lower half of the crypts of Leiberkühn and mature as they migrate from the base of the crypts (Cairnie, 1970; Bry *et al.*, 1994). These cells are present in humans, primates and some rodents but not rabbits or pigs. The density of paneth cells are highest in the ileum followed by the duodenum (Singh, 1971). Paneth cells release growth factors, digestive enzymes and antimicrobial peptides, which are able to digest the cell walls of certain groups of bacteria (Bry *et al.*, 1994). Furthermore, paneth cells are able to phagocytise certain bacteria (Ross and Romrell, 1989).

2.3.2 Brunner glands

Brunner glands penetrate the duodenal mucosa, and in the presence of chyme, secrete an HCO_3^- rich solution, along with a thin alkaline mucus, which helps neutralise the acidic chyme and protects the duodenal tissue from autodigestion (Trier and Madara, 1981).

2.3.3 Goblet cells

Small intestinal goblet cells are mucus secreting cells that are present thoughout the

small intestine and which increase in concentration down the small intestine. These cells have a turnover time of less than 40 hours by which time the cells will have migrated to the tip of the villus (Cairnie, 1970; Trier and Madara, 1981; Ross and Romrell, 1989).

2.3.4 Peyer's patches

The surface of the ileal epithelium contains the lymphoid Peyer's patches (Trier and Madara, 1981). These cells are precursors to plasma cells that secrete immunoglobulins particularly IgA and are stimulated to proliferate and differentiate by contact with intestinal antigens that penetrate the epithelium (Walker and Isselbacher, 1977). Specialised epithelial M cells have been proposed to facilitate the contact between intraluminal antigens and the Peyer's patches, by transporting antigens which are unable to penetrate the epithelium (Bockman and Cooper,1973; Walker and Isselbacher, 1977).

2.3.5 Enterocytes

Enterocytes are the major component of intestinal villi and are involved in the absorption of digested food and also the secretion of certain enzymes used in digestion. Enterocytes contain supranuclear golgi complexes, abundant mitochondria and basally located nuclei (Ross and Romrell, 1989). The apical membrane is composed of densely packaged microvilli, which are 0.5-1.5 μm in length and 0.1 μm in width (Trier and Madara,1981).These microvilli, termed the brush border membrane, have been estimated to increase the intestinal surface area by 14-40 fold. The basolateral membrane is thinner than the apical membrane and allows solutes to

pass from the enterocyte into the blood or lymph supply of the villus (Ross and Romrell, 1989).

The epithelium of the villus has a rapid proliferation rate with enterocytes being found in various stages of development due to cells migrating from the crypts of the villus to the tip. Mitotic division only occurs in the crypts of the villi, with the estimated time of the cycle taking 24 hours in the duodenum and jejunum, compared to 9 hours in the ileum (Holmes and Lobley, 1989)

2.3.5.1 Brush border membrane

The brush border membrane contains numerous intramembrane particles, which vary in size and composition. The membrane is rich in cholesterol and glycoproteins which increases its hydrophobicity (Kim and Perdomo, 1974; Holmes and Lobley, 1989). Important digestive processes have been localised to the brush border membrane, with a number of enzymes including various disaccharidases and peptidases being distributed along the microvillus. Furthermore, nonenzymatic proteins are present in the brush border membrane, such as receptors which can selectively bind vitamin B12 and Ca^{2+}, as well as transport proteins responsible for the co-transport of D-glucose, amino acids and bile acids (Wright, 1993; Aoshima *et al.*, 1988; Kramer *et al.*, 1983). The various proteins of the brush border membrane differ in the extent to which they are anchored in the hydrophobic core of the membrane. In the case of the larger molecular weight proteins, such as sucrase-isomaltase, the glycosylated portion of the enzyme protrudes into the intestinal lumen with only a small portion of the enzyme anchored in the hydrophobic membrane (Trier and Madara, 1981; Holmes

and Lobley, 1989). However, the small molecular weight membrane proteins, such as alkaline phosphatase, ATPase, trehalase and co-transport proteins are more deeply embedded (Trier and Madara, 1981; Holmes and Lobley, 1989).

2.4 Secretions of the small intestine

The maximal rate of digestion and absorption occurs in the proximal regions of the small intestine. The major secretions of the small intestine are mainly involved with the stimulation of pancreatic and bile acid secretion. Secretin release from S cells in the duodenum, is stimulated by the presence of H^+ and long chain fatty acids. Secretin's main action is the stimulation of gallbladder and pancreas secretions, as shown in table 1.1. Gastrin releasing peptide (GRP) is involved in the stimulation of gastrin production from G cells located in the stomach and the duodenum (Johnson, 1991). However, there is also evidence that GRP has an effect on ion transport in pig jejunum, with the stimulation of active electrogenic Cl^- secretion (Chandan *et al.*, 1988). Cholecystokinin (CCK) secretion occurs in the first 90 cm of the small intestine and is released in response to fatty acids and L- isomers of amino acids. This enzyme is a major stimulant of pancreas secretion (Johnson,1991).

CHAPTER THREE:

3. Digestion within the small intestine

3.1. Carbohydrate digestion

Starch is ingested in two principal forms, amylose and amylopectin which are polysaccharides consisting of α (1-4) linked glucose and α (1-4) and α (1-6) linked glucose units respectively (Gray, 1981).

3.1.1 Enzymes involved in carbohydrate digestion

3.1.1.1 Salivary and pancreatic enzymes

Within the mouth, the enzyme α amylase (ptyalin) cleaves internal α (1-4) glycosidic bonds present in starch to produce the oligosaccharides maltose and maltotriose and also α limit dextrins (Abdullah *et al.*, 1977).

Pancreatic α amylase is released into the duodenum and cleaves the internal α (1-4) links of polysaccharides to produce the oligosaccharides maltotriose and maltose. However, pancreatic α amylase is unable to cleave α (1-6) links in polysaccharides which also leads to the formation of α limit dextrins (Vander *et al.*, 1990).

3.1.1.2 Intestinal enzymes

Within the small intestine, disaccharides, such as lactose or sucrose, are hydrolysed to monosaccharides by enzymes present on the brush border membrane (Trier and Madara, 1981). These enzymes can hydrolyse the carbohydrates after they are released into the lumen or whilst still embedded in the membrane (Trier and Madara, 1981; Holmes and Lobely,1989).

The enzymes, sucrase-isomaltase, maltase-glucoamylase, trehalase and lactase are all

embedded in the brush border membrane by a peptide anchor which is cleaved to allow release (Holmes and Lobely, 1989).

Table 3.1 Summary of the actions of major enzymes in carbohydrate digestion

Enzyme	Substrate	Action on substrate	Final product
Lactase	Lactose	Cleaves α(1-4) linkages	glucose and galactose
Sucrase	Sucrose	Cleaves α (1-4) & α(1-6) linkages	glucose and fructose
Glucoamlyase	α dextrins	Cleaves α (1-4) linkages	malto-oligosaccharide, then hydrolysed to glucose by sucrase

Once the digested carbohydrates have reached the brush border membrane they are absorbed across the intestinal membrane into the enterocytes.

3.2 Protein digestion

3.2.1.1 Stomach and pancreatic enzymes

Protein digestion begins in the stomach by the action of pepsin, an endopeptidease with a specificity for peptide bonds involving aromatic amino acids. The action of pepsin terminates upon entry to the small intestine (Adibi & Young, 1981).

Within the small intestine pancreatic endopeptidases hydrolyse the interior peptide bonds of proteins. Trypsin attacks peptide bonds consisting of basic amino acids at the C-terminal end of the protein, whilst elastase degrades peptide bonds of neutral aliphatic amino acids (Davenport, 1975; Johnson, 1991).The majority of peptidases are secreted as pro-enzymes which become activated in the small intestine by tryspin. Protein hydrolysis by the action of pepsin and pancreatic peptidase results in the formation of large peptides, which are further broken down by the brush border

membrane peptidase.

3.2.1.2 Intestinal enzymes

The exopeptidase enzymes, located in the brush border membrane and cytoplasm of enterocytes, hydrolyse external N-terminal peptide bonds of polypeptides releasing free amino acids (Davenport, 1975; Siamak and Young, 1981; Johnson, 1991).

The peptidases present on the brush border membrane are mainly involved in the hydrolysis of long chain tri- and tetra-peptides (Johnson, 1991) and have an active site facing the lumen of the gut with a small hydrophobic anchoring peptide embedded in the brush border membrane (Siamak and Young, 1981).

The cytoplasmic peptidases of enterocytes are largely involved in the hydrolysis of di-peptides, especially those containing proline (Siamak and Young, 1981).

3.3. Lipid digestion

3.3.1. Lipid absorption within the stomach

Within the stomach, the digestion of short chain fatty acids occurs. These lipids are less hydrophobic than long chain fatty acids, so are less likely to form aggregates which impede digestion and absorption (Stryer, 1988).

Lipid digestion within the stomach occurs by means of lipases, which are either present in the ingested food, such as carboxylic ester hydrolyse (CEH) present in human milk, or lingual lipase which is secreted by the mouth. Lingual lipase acts on triglyceride esters producing diglycerides and fatty acids (Hamosh *et al.*,1978).

3.3.2 Lipid digestion within the small intestine

Lipid digestion within the small intestine involves a range of lipids derived from

either an exogenous or an endogenous source.

3.3.2.1 Triglycerides

Triglycerides are important lipids and are mainly derived from an exogenous source. These lipids are composed of uncharged esters of glycerol with fatty acids attached to the glycerol moiety. The majority of triglycerides contain long chain fatty acids, resulting in very hydrophobic lipids that can only be digested after they have formed mixed micelles with bile acids (Voet and Voet, 1995).

Triglycerides are digested by pancreatic lipases which hydrolyse the 1 and 3 ester bonds resulting in the formation of free fatty acids and 2-monoglycerides (Ahrens and Borgstrom, 1955; Tso and Balint, 1986). Pancreatic lipase is secreted in an active form, but this can be inhibited by bile acids. To prevent this inhibition, co-lipase, is secreted from the pancreas which aids in the binding of lipase to the lipid (Davenport, 1975; Thomson and Dietschy, 1981).

3.3.2.2 Phospholipids

There are two forms of phospholipids, glycerophospholipids (exogenous source) and sphingolipids (endogenous source) (Voet and Voet, 1995). Glycerophospholipids are derivatives of glycerol and sphingolipids are derived from sphingosine, an amino alcohol, which contains a long unsaturated hydrocarbon chain. Phospholipids are very important in the solubilisation of certain lipid products and so are abundant in bile. The phospholipid lecithin is a polar molecule present in the normal diet and abundant in bile, where it is used for the solubilisation of biliary cholesterol (Davenport, 1975). Phospholipids are digested by pancreatic lipase which attack the bond between the

glycerol or sphingosine and fatty acids, to release the fatty acids (Thomson and Diestchy, 1981).

3.3.2.3 Cholesterol

Cholesterol is the most abundant steroid in animals and is a major component of plasma, bile acids and cell membranes (Voet and Voet, 1995). Cholesterol comes from both an exogenous and endogenous source, with endogenous cholesterol being present in the bile (Lamzini and Northfiled, 1988; Harnett *et al.*,1989; Dawson, 1999). Cholesterol contains hydroxyl groups and so is able to form esters with fatty acids; these esters need to be hydrolysed before uptake into the intestinal brush border membrane (Thomson and Dietschy, 1981). Human pancreatic esterase hydrolyses esters of cholesterol and also vitamins A, D and E. However, cholesterol esterase is only active against cholesterol incorporated into mixed bile acid micelles (Thomson and Dietschy, 1981). Cholesterol esterase is also present on the brush border membrane of intestinal enterocytes and is able to resynthesis cholesterol for the formation of chylomicrons (Brindley, 1978; Johnson, 1991).

Endogenous cholesterol is more likely to be absorbed in the jejunum whilst dietary cholesterol absorption takes place throughout the small intestine (Lutton, 1976).

3.3.2.4 Fatty acids

Fatty acids are the major components of lipid structure and are utilised in the synthesis of phospholipids, glycolipids and triglycerides.

Short chain fatty acids (SCFA's) are present in the diet in limited concentrations, however lipase from the pancreas and the intestinal mucosa preferentially hydrolyses

the triglycerides containing short chain fatty acids. These SCFA's which are present in the lumen of the small intestine are readily absorbed from the jejunum, ileum and colon (Greenberger *et al.*, 1966). Long chain fatty acids are more common and have the effect of increasing the hydrophobicity and melting point of lipids such as triglycerides and phospholipids.

3.4 The requirement of bile acids in the digestion of lipids

In the hydrophilic environment of the small intestine, hydrophobic lipids have poor solubility. Even when hydrolysed to simple constituents, lipids tend to aggregate together to form larger complexes. Though the mechanical action of the small intestine breaks up the larger lipid aggregates, digestion still proceeds slowly, which can lead to the malabsorption of lipids (Davenport, 1975). This problem is overcome by the interaction of bile acid micelles and lipids within the small intestine. Emulsification of most dietary fat occurs in the duodenum, and requires a neutral environment and a detergent in the form of bile acids. The acidic chyme entering the duodenum from the stomach is neutralised by bicarbonate secretions that are released from the pancreas into the small intestine. Twenty minutes after the beginning of a meal the gall bladder contracts and releases bile containing concentrated bile acids into the duodenum. The bile acids emulsify lipid droplets by the formation of mixed micelles, thereby allowing nearly all the lipids to be digested in the small intestine (Hofmann *et al.*, 1991).

3.4.1 Formation of mixed micelles in the lumen of the small intestine

Bile acids have an amphipathic nature due to being composed of a hydrophobic

steroid nucleus and methyl groups and one or more hydrophilic hydroxyl side chains. Due to this structure, bile acids are able to form micelles, which are composed of a lipid-like hydrophobic core and a surface of polar headgroups (Thomson and Dietschy, 1981).

Being detergents, all bile acids have a critical micellar concentration (CMC), which is a narrow concentration range below which no micelles are detected and above which virtually all additional surfactants form micelles (Thomson and Dietschy, 1981). Conjugated bile acids have a lower CMC than unconjugated bile acids, with glycine conjugates giving the lowest CMC. In most mammals, all bile acids have CMC less than 10 mM, therefore allowing micellar formation to occur in the gall bladder and small intestine (Dietschy, 1968; Wilson, 1981).

As the concentration of bile acids increases to CMC, spontaneous association of monomers occurs, and then the formation of dimers and tetramers. Monomer micelles initially consist of bile acids, 2-monoglycerides and free fatty acids. The bile acid/monoglyceride micelle incorporates other lipids such as fatty acids, phospholipids and diglycerides along with free and esterified cholesterol into its hydrophobic centre (Vander *et al.*, 1990). The hydrophilic surface of the micelle contains partially ionised fatty acids, less hydrophobic monoglycerides and bile acids (Dietschy, 1968; Davenport, 1975). Bile acid micelles also facilitate the absorption of fat-soluble vitamins, such as vitamin A, D, E and K, by the incorporation of these non-polar vitamins into the mixed micelle (Thomson and Dietschy, 1981).

Each micelle is about 4-6 nm in diameter and contains about 20 fat molecules

(Davenport, 1975). The size of a mixed micelle is not static and alters in relation to the properties of lipids present in the micelles (Fondacaro and Rodgers, 1978). Solubilisation in mixed micelles is competitive, with the more hydrophilic lipids being solubilised quicker than the strongly hydrophobic lipids (Wilson 1981). The movement of lipid molecules from the micelles occurs at a fast rate, with the mean residence time being around 10 milliseconds. This means that as free fatty acids and 2-monoglycerides enter the aqueous lumen and diffuse through the brush border membrane, more lipids rapidly enter the aqueous phase. This is termed dynamic equilibrium (Davenport, 1975; Thomson and Dietschy, 1981; Wilson,1981).

There is some evidence that bile acids are able to form micelles with heavy metals present in the bile, liver and intestines. Small bile acid /heavy metal aggregates are formed, with increased stability and solubility in the presence of particular heavy metals. The stability of the micelles increased in the presence of $Cd^{2+} > Fe^{2+} > Pb^{2+} > Zn^{2+} > Cu^{2+}$, with solubility increasing from $Fe^{2+} > Pb^{2+} > Cd^{2+} > Zn^{2+}$ (Feroci *et al.*, 1996).

3.5 Unstirred water layer

The unstirred water layer (UWL) is present in the lumen of the small intestine, and surrounds the intestinal villi. The UWL can be thought of a series of water lamellae extending outward from the brush border membrane, each progressively more stirred, until they blend with the bulk water phase. The thickness of the UWL varies between 100-500μm in various epithelial surfaces *in vitro* (Hingson and Diamond, 1972). The UWL is thinner at the tips of the villi which leads to an increased rate of absorption

(Thomson and Dietschy, 1981; Pohl *et al.*, 1998).

Movement across the UWL only occurs by diffusion and for hydrophobic lipids, amino acids, sugars and bile acids the UWL can be the rate limiting step in the process of absorption. Therefore, the UWL is of major importance in determining the passive movement of a molecule (Wilson, 1981). The presence of the UWL in perfused intestine results in artificially low permeability coefficients for passive transport processes and higher Km values for active transport processes. Therefore, *in vivo* transport studies have to take into account the UWL in relation to Km values (Wilson and Dietschy, 1974 ; Wilson and Treanor, 1975; Wilson, 1981).

CHAPTER FOUR:

4. Absorption of substrates and electrolytes throughout the small intestine

The small intestine is involved in the absorption and transport of exogenous and endogenous substrates, with amino acids, fatty acids and glucose mainly being absorbed in the duodenum and jejunum, whilst the ileum is primarily involved in the reabsorption of bile acids and vitamin B12 (Neale, 1990).

4.1 Electrolyte absorption within the small intestine

Small intestine epithelia have a very high passive permeability to small ions and water, so the majority of cation absorption occurs within the small intestine (Schultz, 1981).

4.1.1 K^+ absorption within the small intestine

In the lumen of the duodenum and jejunum, the concentration of K^+ is relatively low (342mg/L), due to stomach and pancreatic secretions not containing a high K^+ concentration. However, the concentration of K^+ within proximal enterocytes is usually at a higher concentration than in the lumen, due to K^+ being pumped into the enterocyte from the basolateral ATPase pump. Therefore, in the duodenum and jejunum, K^+ diffuses out of the enterocytes into the intestinal lumen (Johnson, 1991). Within the lumen of ileum the concentration of K^+ increases to 3420mg/L resulting in a cation gradient inwardly directed from the lumen to the enterocyte (Johnson, 1991).

4.1.2 Ca^{2+} absorption within the small intestine

The transport of Ca^{2+} into the brush border membrane is initiated by the presence of dihydroxy-vitamin D_3. Within the brush border membrane of the small intestine, two

vitamin D-dependent Ca^{2+} binding proteins have been identified, a 12.5 kDa protein in the duodenum and a 27 kDa protein present in the jejunum and ileum. The duodenal protein displays a greater affinity for Ca^{2+} and is more dependent on the presence of vitamin D (Miller *et al.*, 1979).

4.1.3 Na^+ absorption within the small intestine

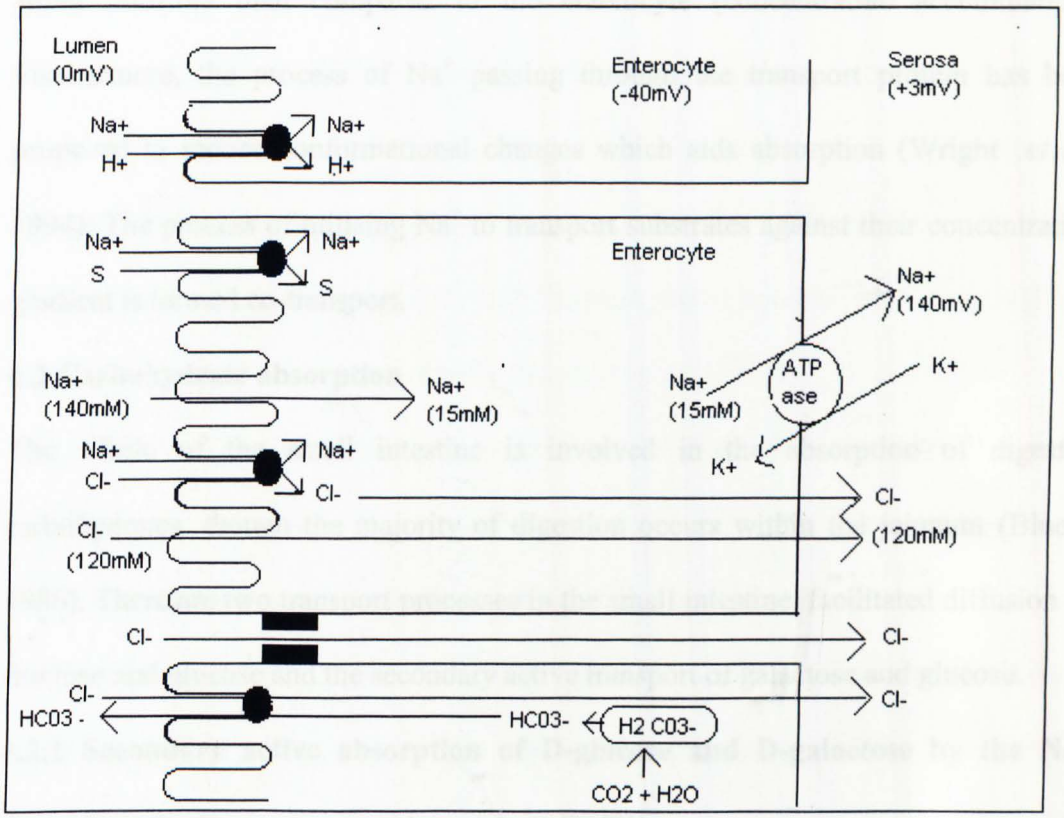
Na^+ is an essential ion for all mammals and is utilised in a wide range of metabolic processes including active transport and coupled transport. In the duodenum, the Na^+ concentration is around 3220 mg/L which decreases to 2875 mg/L in the jejunum and 920 mg/L in the ileum (Johnson, 1991).

4.1.3.1 Active (electrogenic) transport

The mechanism of active transport of Na^+ occurs by simple electrical and chemical diffusion as shown in figure 4.1(Johnson, 1991). There is some evidence that the addition of ethanol inhibits the absorption of Na^+, by dissipation of the Na^+ electrochemical gradient (Gunther, 1978; Hunter *et al.*, 1983).

The exit of Na^+ from the basolateral membrane of the enterocyte occurs against a steep chemical and electro-potential gradient which requires energy provided by ATP to drive the basolateral Na^+/K^+ ATPase (Schultz, 1981;Weinberg, 1986; Clausen, 1998).

Figure 4.1, Cation transport through intestinal enterocytes



Key: S = substrate coupled to Na^+

Adapted from Schultz, 1981 Salt and water absorption by mammalian small intestine

4.1.3.2. Secondary active (coupled) absorption

Within the small intestine, the absorption of substrates such as glucose, amino acids and bile acids are generally coupled to the absorption of Na^+. In most cases, the coupled absorption of a substrate with Na^+ occurs by means of a specific transport protein located in the brush border membrane of the enterocytes.

The process of Na^+ entering the cell by passive diffusion supplies sufficient

electrochemical energy for a substrate to be transported through the brush border membrane, even when the substrate has a lower concentration within the lumen of the small intestine than compared to the enterocyte (concentrative accumulation). Furthermore, the process of Na^+ passing through the transport protein has been proposed to induce conformational changes which aids absorption (Wright *et al.*, 1994). The process of utilising Na^+ to transport substrates against their concentration gradient is termed co-transport.

4.2 Carbohydrate absorption

The whole of the small intestine is involved in the absorption of digested carbohydrates, though the majority of digestion occurs within the jejunum (Bluett, 1986). There are two transport processes in the small intestine, facilitated diffusion of fructose and glucose and the secondary active transport of galactose and glucose.

4.2.1 Secondary active absorption of D-glucose and D-galactose by the Na^+ dependent D-glucose transport protein (SGLT1)

The active transport of D-glucose and D-galactose was one of the first intestinal transport processes to be characterised. Crane *et al.*, (1962) proposed that D-glucose was actively transported across the brush border membrane of enterocytes against its concentration gradient. This transport mechanism was shown to be a secondary active process dependent on the presence of a Na^+ gradient, inwardly directed from the lumen of the intestine into the enterocytes (Lucke *et al.*, 1978a; Kimmich, 1981; Dorando and Crane, 1984). However, further work has shown that, though the transport protein is dependent on the presence of Na^+, it also utilises H^+ for secondary

active transport. At an acidic pH of 5.5, the presence of H^+ induced a higher rate of D-glucose uptake when compared to Na^+ dependent D-glucose transport at that pH. Furthermore, the Na^+ dependent D-glucose transport protein (SGLT1) had a higher affinity for H^+ than for Na^+, but the H^+ loaded protein had a lower affinity for the D-glucose than the Na^+ loaded protein. Secondary active D-glucose transport has been shown to be an electrogenic process (Murer and Hopfer, 1974; Ikeda *et al.*, 1989; Mullins *et al.*, 1992; Wright, 1994) with the transport of two Na^+ or H^+ ions required for the transport of one sugar molecule (Kauntiz *et al.*, 1992).

The SGTL1 is selective with respect to the structure of the substrate, only transporting hexose sugars (such as D-glucose and D-galactose) that have a hydroxyl group at position 2, whilst the configuration at positions 1, 3 and 4 on the hexose ring are less important (Crane, 1962; Ikeda *et al.*, 1989). However, the Na^+ dependent D-glucose transport system can be competitively inhibited by the presence of phlorizin (a plant glycoside) which shares a similar structure to D-glucose (Harig *et al.*, 1989; Umbach *et al.*, 1990; Ikeda *et al.*, 1989; Wright, 1993; 1994). In secondary active transport processes, competition arises between the substrates for the transport protein, with the structure of the substrate being very important in the determination of rate and affinity of that particular substrate for the transport process.

SGLT1 has also been shown to display Michaelis Menten kinetics (Dorando and Crane, 1984; Kaunitz *et al.*, 1992; Wolfram *et al.*, 1986; Heidger *et al.*, 1987a; Mullins *et al.*,1992). The active transport of a substrate, in this case D-glucose, has a maximal rate (Vmax) of transport because the transport protein is only able to absorb

a finite amount of substrate at a given time. The affinity of the substrate (Km) for the transport process is also important because the affinity of the substrate affects the rate of transport.

SGLT1 is a member of a family of 12-14 transmembrane domain proteins that are divided into facilitative (uniporters) and co-transporters (Turk *et al.*, 1991; Wright *et al.*, 1994). SGLT1 is composed of 662-665 amino acids, with a molecular weight of 57 - 63 kDa which increases to 71-73 kDa after glycosylation (Hediger *et al.*, 1987a&b; Wright,1994). The SGLT1 protein has 14 transmembrane alpha-helices each composed of 21 residues (Turk *et al.*, 1997) with a large hydrophilic link between transmembranes helices 11 -12 and small links between transmembranes 3-4, 5-6 and 7-8 (Wright, 1992; 1993).

The expression of intestinal mRNA using *Xenopus laevis* oocytes has shown that the SGLT1 mRNA is present in mRNA throughout the small intestine, but has the highest abundance in the jejunum and then the ileum (Hediger *et al.*, 1987 b; Dyer *et al.*, 1997a). These findings correspond to previous observations that the rate of secondary active D-glucose transport is highest in the jejunum, then the ileum and finally the duodenum (Bluett, 1986; Harig *et al.*, 1989; Mullins *et al.*, 1992). The expression of SGLT1 can also be altered by a change in diet and in disease (Hirsch *et al.*, 1996). In diabetic rats there was an increase of SGLT1 activity due to a specific increase in protein expression (Dyer *et al.*, 1997b), whilst in ruminant sheep the expression of SGLT1 varied between a number of animals on different diets, with grass consuming ruminants having no expression of SGLT1 due to little or no production of D-glucose

(Rowell *et al.*, 1997). The ontogenetic development of SGLT1 has been shown to exhibit prenatal expression (Buddington and Diamond, 1989), with expression and transport rates varying in different mammals (Wolfram *et al.*, 1986; Shirazi-Beechey *et al.*, 1989).

The SGLT1 gene has been mapped to human chromosome 22q 11-2 by Southern blotting and fluorescence *in situ* hybridisation (FISH) (Hediger *et al.*, 1989; Turk *et al.*,1991). Furthermore, a single base change of guanine to an adenine at position 92 has been shown to result in the loss of Na^+ dependent glucose transport in *Xenopus laevis* oocytes when mutated human mRNA was expressed (Turk *et al.*, 1991).

4.2.1.2 Facilitated carbohydrate transport (GLUT's)

Within the small intestine are a family of structurally related facilitated transport proteins, involved in the facilitated diffusion of carbohydrates. Five facilitated D-glucose transporters have been localised to erythrocytes (GLUT 1), hepatocytes (GLUT 2), brain tissue (GLUT 3), muscle fibres (GLUT 4) and enterocytes (GLUT 5). However all these transporters are present in varying degrees in human small intestine (Davidson *et al.*, 1992).

4.3 Peptide absorption

4.3.1 Small peptide absorption

The absorption of short peptides in the jejunum and ileum occurs by an active process which displays concentrative accumulation, Michaelis Menten kinetics, inhibition by metabolic inhibitors and dependence on a cation gradient (Siamak and Young, 1981; Johnson, 1991). The structure of the peptide being absorbed is very important,

peptides that have an L-isomer structure are absorbed at a higher rate in the jejunum than the D-isomer form (Siamak and Young, 1981).

4.3.2 Amino acid absorption

Free amino acids (basic, neutral and acidic) are absorbed by secondary active transport across the brush border membrane. There are a number of transport processes involved in the transport of either neutral, basic, acidic amino acids and imino acids, with the standard structural configuration for these amino acids being a positively charged amino or imino group and the presence of a carboxyl group (Munck, 1981; Stryer, 1988).

In most cases, the amino acid transport processes in the small intestine are dependent on the presence of an inwardly directed Na^+ gradient (Wright *et al.*, 1996).

However, the intestinal absorption of neutral amino acids, such as leucine, occurs by a secondary active transport process which can utilise both Na^+ and K^+ for the active transport of amino acids (Sacchi and Perego, 1994). The active transport of glutamate is an electrogenic process with respect to Na^+ and uses K^+ or H^+ for counter transport (Wright *et al.*, 1996: Castagne *et al.*, 1997, 1998). For the absorption of one molecule of glutamate, two Na^+ are transported into the enterocyte, with one K^+ or H^+ being transported out (Wright *et al.*, 1996).

4.4 Lipid transport through the brush border membrane and enterocyte

The majority of lipids are absorbed in the upper jejunum of the small intestine, but the ileum is also important in lipid absorption (Booth *et al.*, 1961). Most studies indicate that digested lipids pass through the border membrane by simple diffusion (Ahrens

and Borgstrom, 1955;Tso and Balint, 1986). However, some data indicate that there could be carrier-mediated transport of lipids, with the transport of unbound oleic acid being shown to demonstrate saturation kinetics (Prieto *et al.*, 1996).

4.4.1 Lipid transport through the cytoplasm of intestinal enterocytes.

Lipid transport in the liver and intestine has been shown to occur by means of fatty acid binding proteins, such as Y,Y' and Z in the liver (Takikawa *et al.*,1992). These binding proteins have molecular weights of 15 kDa (127-134 amino acids) and are members of the family of fatty acid binding proteins identified in the heart and adipocyte (Green *et al.*, 1992 ; Richieri, 1992).

Sequence analysis of the ileal fatty acid binding protein has revealed that it has the same sequence (Stengelin *et al.*, 1996) as the proposed ileal bile acid binding protein present in the cytoplasm of terminal ileal enterocytes (Vodenlich *et al.*, 1991; Gong *et al.*, 1994; Lin *et al.*, 1991; Stengelin *et al.*, 1996).

After entry into the enterocytes, fatty acids and 2 monoglycerides are re-synthesised to triglycerides (Voet and Voet, 1995), which produces a concentration gradient for more free fatty acids and 2 monoglycerides to diffuse through the brush border membrane into the enterocyte (Stryer, 1988).

4.4.2. Formation of chylomicrons

During passage through the enterocytes the absorbed lipids combine together to form chylomicrons which are re-synthesised by means of agranual endoplasmic reticulum (ER). Triglycerides join with phospholipids, cholesterol and fat soluble vitamins in the formation of chylomicrons, which are about 750-5000A in diameter and exist as

an emulsion within enterocytes. The newly synthesised chylomicrons pinch off from the ER and proceed through the Golgi apparatus and eventually fuse with the basolateral plasma membrane of the epithelial cell. Chylomicrons are absorbed into the intestinal lymphatic vessels and transported through the thoracic duct into the blood (Thomson and Dietschy, 1981).

In normal faeces less than 6 gm of fat are excreted a day, with an excess of 6 gm being defined as steatorrhea. In steatorrhea, the amount of faecal fat is roughly proportional to the amount of dietary fat, but more is formed from free fatty acids, with very few short chain and medium chain fatty acids being reabsorbed (Davenport, 1975).

4.5 Vitamin absorption

As previously described, fat soluble vitamins (A, D, E and K) are passively absorbed through the brush border membrane. However, water soluble vitamins are too hydrophilic to diffuse through the hydrophobic membrane, so a number of transport processes are used for the absorption of hydrophilic vitamins.

In humans, lower primates, and guinea pigs, vitamin C is actively absorbed by a Na^+ dependent co-transport process present in the ileum (Rose, 1981). Secondary active transport processes for biotin, thiamine, inositol and nicotinic acid are present in the jejunum. Folic acid, pyridoxine and p-aminobenzoic acid (PABA), are absorbed by passive and facilitated diffusion (Rose, 1981).

In the stomach, vitamin B12 forms a complex with an R protein, which is degraded by pancreatic enzymes in the small intestine. The released B12 vitamins bind to an

intrinsic factor (IF) (Ramasamy *et al.*, 1989; Neal,1990) and the complex is then absorbed in the ileum (Shaw *et al.*, 1989; Sriram *et al.*, 1989).

CHAPTER FIVE:

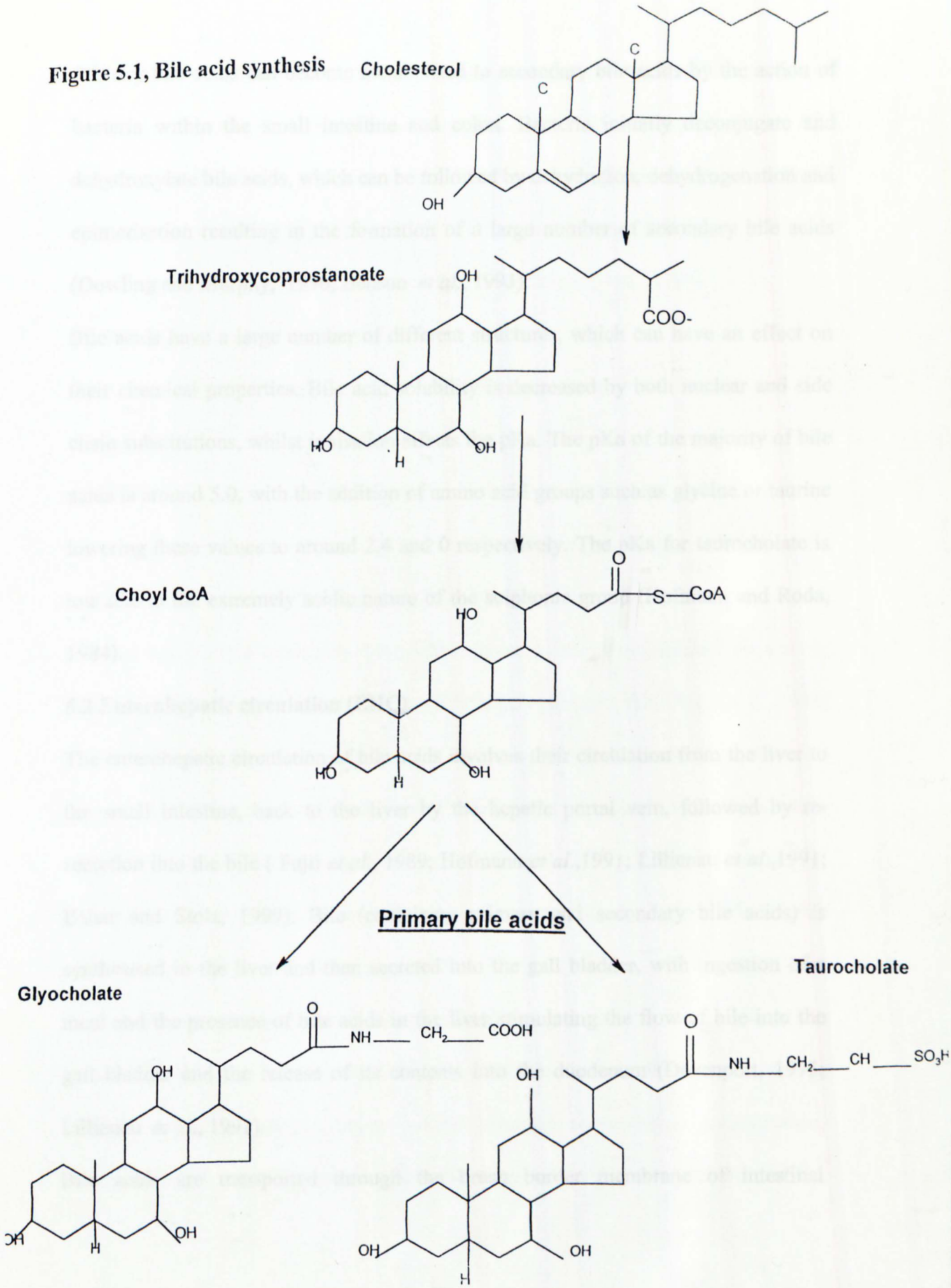
5. Functional characteristics of bile acid transport in the gastrointestinal system

To ensure sufficient concentrations of bile acids for lipid digestion, all mammals synthesis their own bile acids in the liver, and subsequently reabsorb them in the small intestine.

5.1 Synthesis of bile acids

Bile acids are synthesised in the liver from cholesterol (figure 5.1), by the action of 7α hydroxylatase (cyp7) which hydroxylates the cholesterol nucleus, followed by oxidation of the cholesterol side chain and addition of a carboxyl side chain (Dowling and Murphy, 1996). The cyp7 enzyme can be inhibited by bile acids so thereby allowing regulation of bile acid synthesis. Furthermore, the abundance of this enzyme has been shown to be substrate dependent, with up regulation of the cyp7 mRNA being observed after an increased concentration of cholesterol was fed to mice (Torchia *et al.*, 1996). Two main bile acids are produced from cholesterol; cholate and chenodeoxycholate. Cholate can react with the amino groups of glycine or taurine to produce the conjugated trihydroxy bile acids glycocholate and taurocholate respectively (Hofmann, 1991; Morsiani *et al.*, 1998). An alternative pathway of bile acid synthesis also occurs in the liver. It begins with oxidation of the cholesterol side chain and ends with hydroxylation of the nucleus producing monohydroxy bile acids, such as lithocholate (Dowling and Murphy, 1995).

Figure 5.1, Bile acid synthesis



Primary bile acids can become metabolised to secondary bile acids by the action of bacteria within the small intestine and colon. Bacteria initially deconjugate and dehydroxylate bile acids, which can be followed by dehydration, dehydrogenation and epimerisation resulting in the formation of a large number of secondary bile acids (Dowling and Murphy, 1996; Benson *et al.*, 1993).

Bile acids have a large number of different structures, which can have an effect on their chemical properties. Bile acid solubility is decreased by both nuclear and side chain substitutions, whilst ionisation affects the pKa. The pKa of the majority of bile acids is around 5.0, with the addition of amino acid groups such as glycine or taurine lowering these values to around 2.4 and 0 respectively. The pKa for taurocholate is low due to the extremely acidic nature of the sulphonic group (Hofmann and Roda, 1984).

5.2 Enterohepatic circulation (EHC)

The enterohepatic circulation of bile acids involves their circulation from the liver to the small intestine, back to the liver by the hepatic portal vein, followed by re-secretion into the bile (Fujii *et al.*, 1989; Hofmann *et al.*,1991; Lillienau *et al.*,1991; Bahar and Stolz, 1999). Bile (containing primary and secondary bile acids) is synthesised in the liver and then secreted into the gall bladder, with ingestion of a meal and the presence of bile acids in the liver stimulating the flow of bile into the gall bladder and the release of its contents into the duodenum (Davenport, 1975; Lillienau *et al.*, 1991).

Bile acids are transported through the brush border membrane of intestinal

enterocytes by passive and active transport processes, where they pass through the cell and leave the basolateral membrane via a Na^+ independent transport system. The active transport process on the basolateral membrane has been proposed to be an anion exchanger (Weinberg *et al.*, 1986), with a 40 fold higher affinity for bile acids than the brush border membrane transport process (Simon *et al.*, 1990).

5.3 Bile acid transport within the liver

5.3.1 Bile acid transport through the sinusoidal membrane

Transport of bile acids from the blood through the sinusoidal membrane into hepatocytes occurs by a Na^+ dependent transport protein, with the inwardly directed Na^+ gradient being maintained by a Na^+/K^+ ATPase present on the sinusoidal membrane (Frimmer and Ziegler, 1988). The Na^+ dependent transport process displays competitive inhibition, Michaelis Menten kinetics, is inhibited by anionic transport inhibitors and is an electroneutral transport process (Von Dippe *et al.*,1986; Frimmer and Ziegler,1988; Hagenbuch *et al.*,1991). The Na^+ dependent co-transport protein is a 7-8 transmembrane domain protein, which consists of 1086 nucleotides, with a 1.7 kb mRNA reading frame and a molecular weight of 49 kDa with 5 glycosylation sites (Kramer *et al.*,1982; Ananthanarayanan *et al.*,1988, 1991; Levy and Von Dippe,1989; Hagenbuch *et al.*,1990, 1991).

There is some evidence that a Na^+ independent transport process is also present on the sinusoidal membrane (Frimmer and Ziegler, 1988). In the presence of an outwardly directed hydroxyl gradient, cholate uptake displayed concentrative accumulation, which indicated that a hydroxyl/cholate exchange mechanism was present on the

sinusoidal membrane (Blitzer *et al.*, 1986). A multispecific organic anion transporter is also present on the sinusoidal membrane, which mediates the Na^+ independent transport of conjugated and deconjugated bile acids. This transport process is temperature sensitive and displays Michaelis Menten kinetics (Kullak-Ublick *et al.*, 1996).

In addition to bile acids, organic anions, nonesterified fatty acids and non bile acidic cholephils are also taken up by the liver. In most cases these substrates are absorbed by Na^+ dependent transport systems on the sinusoidal membrane. The molecular weight for the bilirubin carrier is 60-55 kDa, whilst the nonesterified fatty acid transporter has a molecular weight of 40 kDa (Frimmer and Ziegler, 1988).

5.3.2 Bile acid transport through the cytoplasm of hepatocytes

Glutathione S-transferases (45-50 kDa) are a family of dimeric proteins (Y_a and Y_b), that have the ability to bind bile acids, with Y_a subunit binding bilirubin and lithocholate with a very high affinity (Stolz *et al.*, 1989; Tuchweber *et al.*, 1996). Bile acids are also bound by a 33 kDa Y' bile acid binder protein, which is a member of the family of organic acid binding proteins. This family includes a 34 kDa protein that binds organic anions and a phenolic steroid sulphotransferase (32.5 kDa) that adds sulphate groups to estradiol, oesteron and bile acids at the 3-OH position (Stolz *et al.*, 1989; Tuchweber *et al.*, 1996). Within the liver, fatty acid binding proteins that bind bile acids are also present, as mentioned in the previous chapter (Stolz *et al.*, 1989; Tuchweber *et al.*, 1996).

5.3.3 Bile acid transport through the canalicular membrane

Transport of bile acids out of the hepatocyte and into the bile duct occurs by 2 different proteins and is the rate limiting step in bile acid secretion (Sippel *et al.*, 1994). An ATP dependent bile acid transporter (100 kDa) has been localised to the canalicular membrane, and demonstrates Michaelis Menten kinetics, competitive inhibition, and ontogenic development (Ruetz *et al.*,1987; Adachi *et al.*, 1991; Novak *et al.*,1991; Stieger *et al.*,1992; Sippel *et al.*,1990; Gerloff *et al.*,1998). The bile acid canalicular transport protein is proposed to have 12 transmembrane domains and belongs to the family of ABC (ATP binding cassette) transporters (Gerloff *et al.*, 1998).

Ecto-ATPase is a two transmembrane domain protein, that uses a phosphorylated serine and tyrosine at amino acid positions 503 and 488 respectively, to transport bile acids (Sippel *et al.*,1991; 1994). The protein has structural identity to the glycoprotein cell adhesion molecule (CAM105) and it has been proposed that the canalicular ecto-ATPase and the CAM105 are in fact the same protein (Lin *et al.*, 1991).

There is some evidence that a secondary active transport process exists in the luminal plasma membrane of rat biliary epithelial cells. Using the rat ileal Na^+/bile acid co-transport protein as a probe, a mRNA of 5 Kb was detected, which was identical to the ileal Na^+/bile acid co-transport protein (Elsing *et al.*, 1999).

5.4 Passive transport of bile acids within the small intestine

5.4.1 Regional distribution

The passive diffusion of bile acids, down their concentration gradients, from the lumen of the gut into the intestinal enterocyte occurs throughout the small intestine (Marcus *et al.*, 1991, Stiehl *et al.*, 1995). There are two types of passive transport processes in the small intestine, ionic and non-ionic diffusion (Dietschy, 1968; Wilson, 1981).

5.4.1.1 Ionic Diffusion

Ionic diffusion involves the transport of conjugated bile acids (Hoffmann and Roda, 1984), though membranes are relatively impermeable to charged molecules so conjugated bile acids have a slow rate of diffusion (Dietschy, 1968). Ionic diffusion only accounts for a limited amount of bile acid reabsorption for a given length of tissue (Wilson, 1981). However, over the full length of the small intestine the overall contribution of passive transport may be more significant (Dakka *et al.*, 1995), with a higher rate of passive diffusion in the terminal ileum due to the higher cholesterol-to-phospholipid ratio of the ileal brush border membrane (Aldini *et al.*, 1995, 1996).

5.4.1.2 Non ionic diffusion

Non-ionic diffusion involves the transport of unionised bile acids, which penetrate the brush border membrane faster than ionised forms. However, nearly all bile acids in the proximal small intestine are conjugated. Therefore, non ionic diffusion does not occur to a significant amount in the jejunum, but within the ileum and colon there is an increased concentration of deconjugated bile acids leading to highly efficient non

ionic diffusion (Dietschy, 1968; Aldini *et al.*, 1992; Aiso *et al.*, 1996). Deconjugated bile acids have been shown to cause a secretory effect on the ileum and jejunum, which can reduce active transport of nutrients in the small intestine (Berant *et al.*, 1988; Fasano *et al.*, 1994) and can lead to dose dependent mucosal damage (Armstrong *et al.*,1994).

5.4.1.3 Effect of the structure of bile acids on passive diffusion

The permeability coefficients of protonated and ionised monomers are related to the number of hydrogen bonds that particular bile acids form in water, with a decreased permeability for each additional group added to the bile acid molecule. Protonated monomers have a higher rate of diffusion (Schiff *et al.*, 1972; Krag and Phillips, 1974) and deconjugated bile acids are more permeable than conjugated, though glycine conjugates possess a higher permeability than taurine (Wilson and Treanor, 1975).

5.5 Active transport of bile acids within the small intestine

The low uptake of conjugated bile acids by passive transport mechanisms necessitates the existence of another transport system that reabsorbs the abundant conjugated bile acids, in order to maintain efficient enterohepatic circulation.

5.5.1 Regional distribution of active bile acid transport

Initial experiments, using everted intestinal sacs, showed that conjugated bile acids were only transported against their concentration gradients in the ileum (Lack and Weiner, 1961; Holt *et al.*, 1964; Wilson and Treanor, 1979; Rouse and Lack, 1979). Furthermore, ileal transport of trihydroxy and dihydroxy bile acids (after correction

with the passive permeability values) exhibited a hyperbolic function representative of a carrier mediated transport system, whilst the jejunum displayed a linear relationship typical of passive transport (Schiff *et al.*, 1972). These early experiments seemed to indicate that an active transport process was present in the ileum of mammals, which was involved in the reabsorption of bile acids at a higher rate than that observed for the passive diffusion of conjugated bile acids.

The isolation of brush border membrane vesicles (BBMV) from the small intestine enabled further investigation of the mechanisms of active transport of bile acids, offering the opportunity to study properties of the transport process under controlled conditions without interference from intracellular metabolism (Louvard *et al.*, 1973; Schmitz *et al.*, 1973; Shirazi-Beechey *et al.*,1990). In most cases the uptake of a radiolabelled bile acid into BBMV was measured by use of a rapid filtration technique.

Using BBMV, active transport of bile acids was found to be temperature dependent, with a reduction in temperature to 4°C reducing the rate of active bile acid transport to that observed with passive diffusion. Furthermore, bile acid reabsorption in the ileum displayed concentrative accumulation and competitive inhibition between different bile acids (Wilson and Treanor, 1975; Lack *et al.*, 1977, Lucke *et al.*, 1978; Beesley and Faust, 1979; Schwenk *et al.*,1982, 1983; Sorscher *et al.*, 1992; Stiehl, 1995). More recent work has shown the intestinal bile acid transport process to be bi-directional. The Na^+/bile acid co-transport protein was able to actively transport bile acids out of oocytes expressing the transport protein (Weinman *et al.*, 1998).

Therefore, experiments using BBMV and isolated gut tissue indicated that a secondary active bile acid transport mechanism was present in the small intestine, but restricted to the terminal ileum.

However, expression of the Na^+/ bile acid co-transport protein in *Xenopus laevis* oocytes by injection of mRNA isolated from the duodenum, jejunum and ileum of adult pigs, all led to the expression of Na^+-dependent bile acid uptake. This uptake was inhibited by the presence of taurodeoxycholate, and possessed similar transport affinities to the native brush border transporter. Therefore, it appeared that mRNA encoding the active bile acid transport protein was present throughout the small intestine, whilst the transport protein itself was found to be restricted to the ileum of adult pigs indicating post-transcriptional regional regulation (Mullins *et al.*, 1992). These findings are supported by the observation that secondary active bile acid absorption extends to the jejunum in the sheep (Wolffram *et al.*,1988).

5.5.2 Cation dependency of active bile acid transport

In bile acid transport, Na^+ plays an important physiological role, with a 6-7 fold increased rate of ileal taurocholate uptake in the presence of Na^+ compared to uptake in the absence of a cation gradient (Holt, 1964; Gallagher *et al.*, 1975; Lucke *et al.*, 1978; Bessely and Faust, 1979; Schwenk *et al.*,1983; Kramer *et al.*, 1982; 1994; Mullins *et al.*, 1992; Dawson *et al.*, 1994).

To determine whether the active transport of bile acids was coupled to the flow of Na^+, the cellular electrochemical potential was manipulated by the addition of monactin. This led to an increase in cation permeability of the membrane, which

prevented the concentrative accumulation of bile acids indicating the disruption of the active transport mechanism. This observation provided strong evidence for a co-transport system between Na^+ and bile acids, with Na^+ being essential for the active re-absorption of bile acids (Holt, 1964; Lucke *et al.*, 1978).

The addition of valinomycin can also alter the membrane potential by causing an increased membrane permeability to K^+. In ileal BBMV treated with valinomycin, the presence of an inwardly directed Na^+ gradient and an outwardly directed K^+ gradient, enhanced taurocholate uptake 1.4 fold. This finding not only indicated that an increase in electrical potential across the brush border membrane can enhance Na^+ dependent bile acid uptake (Lucke *et al.*, 1978), but also indicated a role for K^+ in bile acid absorption.

Investigations of the cation dependency of the Na^+/ bile acid co-transport protein have been influenced by the early cation dependence experiments, that determined the bile acid transport protein could only use Na^+ for secondary active transport (Lack and Weiner, 1961; Holt *et al.*, 1964; Wilson and Treanor, 1979; Rouse and Lack, 1979). The majority of studies investigating the cation dependency of the Na^+ /bile acid co-transport protein were limited in terms of the experimental conditions tested, only replacing Na^+ with one other cation, usually K^+, and noting the resulting decrease in the transport rate. However, the few experiments that have investigated a wider range of cations have shown increased bile acid uptake in the presence of K^+ when compared to other cations such as Li^+, Rb^+, and Cs^+ (Lucke *et al.*,1979, Wilson 1981). Furthermore, investigation into the regional distribution of the Na^+/bile acid

co-transport protein showed that within the ileum the rate of taurocholate uptake in the presence of K^+ was higher when compared to uptake rate in the jejunum and duodenum (Kramer *et al.*, 1992). However, this observation was not discussed in the paper and Kramer and his colleagues determined that the Na^+/bile acid co-transport protein could only use Na^+ for secondary active transport. Therefore, the cation dependency of the intestinal bile acid transport needs further investigation.

5.5.3 Na^+/K^+ ATPase present on the basolateral membrane

The electrochemical Na^+ gradient that enables the active transport of bile acids is maintained by active extrusion of the cation across the basolateral cell membrane with energy derived from ATP hydrolysis by an Na^+/K^+ ATPase (Wilson and Treanor, 1975, 1979; Reymann *et al.*, 1989; Clausen, 1998). Abolishment of the ATPase, by the addition of ouabain, had the effect of reducing the rate of taurocholate transport to passive diffusion levels due to the depletion of the inwardly directed Na^+ gradient. This indicated that the active bile acid transport system in the ileum was operating by indirect energetic coupling to the cell metabolism via the establishment of the transmembrane Na^+ gradient (Schwenk *et al.*, 1983).

5.5.4 Stoichiometry of bile acid transport

The discovery of a secondary active bile acid transport process which was reliant on an electrochemical gradient of Na^+ lead to the investigation of whether the system was electrogenic, like Na^+ dependent D-glucose transport, or electroneutral.

In Na^+ dependent D-glucose transport, the anion coupled to Na^+ can have an effect on the rate of D-glucose transport, with the replacement of chlorine with more permeable

anions leading to an increased rate of D-glucose absorption (Kimmich, 1981; Wright, 1994; Mullins *et al.*, 1992). Taurocholate uptake in rat ileal BBMV was increased by the presence of more permeable anions, which indicated that Na^+ dependent bile acid transport was an electrogenic process, with the negative charge of the bile acid being overcompensated by two Na^+ ions (Lucke *et al.*, 1978). Furthermore, voltage clamping of oocytes expressing the transport protein showed a 3 fold reduction in extracellular Na^+ producing a negative 52 mV shift of flux voltage which was consistent with a 2:1 Na^+: bile acid stoichiometry (Weinman *et al.*, 1998).

In contrast, the replacement of chlorine in guinea pig ileal BBMV, with other anions did not effect the Na^+ dependent transport of taurocholate (Rouse and Lack, 1979). These findings were supported in pig and human ileal BBMV, which also showed no variation in Na^+ dependent bile acid transport with the substitution of different anions, indicating an electroneutral transport process consistent with a 1:1 transport of Na^+ and bile acid (Barnard and Ghishan, 1986,1987; Mullins *et al.*, 1992).

Therefore, at this present time, the stoichiometry of the secondary active bile acid transport process is unclear.

5.5.5 Kinetic analysis of bile acid absorption

The Na^+ dependent bile acid co-transport protein displays Michaelis Menten kinetics, with the rate of transport (Vmax) being dependent on the number of hydroxyl groups present on the steroid nucleus of the bile acid. The Vmax for trihydroxy bile acids were 1543-1906 pmoles/min per cm, compared to 114-512 pmoles/min per cm for dihydroxy bile acids and 45-57 pmoles/min per cm for monohydroxy bile acids. The

affinity of the bile acids for the transport protein (Km) were found to be dependent on conjugation, with conjugated bile acids having Km's of 0.12-0.23 mM compared to 0.37-0.49 mM for deconjugated bile acids (Schiff *et al.*, 1972). Furthermore, decreasing the length of the side chain resulted in an increase in Vmax and Km (Marcus *et al.*, 1991).

Expression of pig ileal mRNA in *Xenopus laevis* oocytes displayed saturation kinetics, with a Km of 48 μM compared to 40 μM for the native protein in pig ileal BBMV. Expression of size fractionated pig ileal mRNA also revealed that two transcripts encoded the Na^+/bile acid co-transport protein. The smaller mRNA transcript at 3.8-4.5 kb had a Vmax of 5.1 pmol/oocyte/hr, whilst the larger transcript (11-13 kb) had a Vmax of 7.6 pmol/oocyte/hr. In both cases the Km values were the same at 37 – 40 μM, which corresponded to the Km observed with unfractionated mRNA (Mullins *et al.*, 1992, 1996). Therefore, this finding indicated that more than one mRNA transcript codes for the transport protein.

Kinetic analysis of a cDNA clone proposed to encode the Na^+/bile acid co-transport protein (when transfected into COS cells) displayed a similar Km to previous findings of 33μM and a very high Vmax of 396 pmol / min / mg protein (Wong *et al.*, 1994).

5.5.6 Carrier-mediated jejunal transport

It has been suggested for many years now that jejunal absorption of bile acids is too high to be accounted for by diffusion alone (Angelin *et al.*, 1976; Steiger *et al.*, 1984; Lewis and Root, 1990). Using perfused guinea pig jejunum, the uptake rates of the dihydroxy and trihydroxy bile acids were investigated. Only dihydroxy conjugates

demonstrated saturation kinetics and competitive inhibition which indicated two transport mechanisms were occurring in guinea pig jejunum, carrier mediated transport of dihydroxy conjugates (which was independent of Na^+) and passive transport of trihydroxy conjugates (Amelsberg *et al.*, 1995). An outward anion gradient of bicarbonate ions has also been shown to stimulate absorption of taurocholate and taurochenodeoxycholate in rat jejunal BBMV. This anion exchanger displays saturation kinetics, substrate specificity and competitive inhibition (Amelsberg *et al.*, 1996).

5.5.7 Temporal development of active transport

The development of intestinal secondary active bile acid transport in all species matures soon after weaning. In rat ileal BBMV, only the adult rats (21 days old) showed an intravesicular accumulation of taurocholate, Na^+-stimulation of taurocholate was first observed after 17 days and the first concentrative accumulation observed after 18 days, which reached its maximum in adult animals (Moyer *et al.*, 1986). Furthermore, kinetically viable data was only observed in 3 weeks (Km = 0.59mM, Vmax = 844 pmol/mg protein per 120 seconds) and 6 weeks old rats (Km = 0.66mM,Vmax = 884 pmol/mg protein per 120 seconds), with the addition of methylprendisolone accelerating postnatal development (Barnard and Ghishan, 1985;1986).

However, in neonatal and newly born pigs (no older than 12 hours), Na^+ dependent bile acid transport was demonstrated throughout the small intestine, which was lost after 12 hours. Active transport was only regained after weaning (30 day old pigs) and

then appeared to be restricted to the ileum (Buddington and Puchal Personal Communication; Buddington and Diamond, 1989).

Ontogenic development corresponded to an increase in mRNA encoding the Na^+/bile acid co-transport protein from 14 to 28 day old rats. No mRNA coding the secondary active transporter was detected in 7 day old rats (Schneider *et al.*, 1995). These findings suggest that developmental regulation of the active transport protein occurs at the transcriptional level.

It has been proposed that an observed change in the villi during weaning could have some effect on secondary active bile acid transport. The development from weaning to adult, in rabbits, resulted in an increase in villus height, width and number of villi per unit serosal length and a decrease in membrane fluidity (Thomson *et al.*,1987). Active taurocholate uptake was first observed in 21 day old rabbits (start of weaning) and reached its maximum after 35 days, with a reduction of membrane fluidity observed over this time period (Schwarz *et al.*,1989, 1990). However, in rats no significant changes in membrane fluidity were observed, leading to the conclusion that changes in microvillus membrane fluidity were unlikely to modulate the postnatal development of active ileal bile acid transport (Heubi *et al.*, 1985; Heubi and Fellows, 1990).

5.5.8 Substrate regulation of active transport

The abundance of transport proteins present in the brush border membrane can be regulated by alterations in the concentration of substrate. In rats, the ileal Na^+/bile acid co-transport protein and mRNA (5Kb) encoding the Na^+/bile acid co-transport

protein was increased by the addition of taurocholate and cholate (Stravitz *et al.*, 1996; 1997; Nowicki *et al.*, 1997), with similar findings being observed with the liver Na^+/bile acid co-transport protein (Higgins *et al.*, 1993). Glucocorticoids also increased Na^+ dependent taurocholate uptake, from 148 pmol/mg prot at 30s in the control group to 264 pmol/mg prot at 30s in the steroid injected rats.

However, other studies have shown that the abundance of the transport protein was down regulated in the presence of increased substrate. The down regulation of the protein has been proposed to decrease intestinal absorption of bile acids during cholestasis (a disease of the liver), thereby protecting hepatocytes against further bile acid accumulation and cell damage (Lillienau, 1993; Torchia *et al.*,1996; Sauer *et al.*, 1996). The addition of taurocholate and cholesterol have been shown to decrease the levels of mRNA encoding the Na^+/bile acid co-transport proteins (Torchia *et al.*, 1996).

However, it has also been shown that a change in the local bile acid concentration can either up or down regulate the number of bile acid carriers in the distal ileum (Van Tilburg *et al.*, 1990). More recent work, has shown that there is no increase or decrease in the number of bile acid transport proteins, in the ileum or the liver, after a decrease in the concentration of taurocholate (Sauer *et al.*, 2000). Therefore, the effects of substrate concentration on the abundance of the transport protein is still unclear.

5.5.9 Steroid recognition binding sites on the transport protein

The Na^+/bile acid co-transport protein has a specific structure, which will only allow

certain configurations of bile acids to be transported. The Na^+/bile acid co-transport protein has a cationic binding site and an anionic binding site, which bind the negatively charged bile acid and the positively charged cation respectively (Lack *et al.*, 1977; Wilson, 1981). The cationic site consists of positively charged lysine amino groups, which bind the negatively charged bile acid. The anionic site is composed of negatively charged cysteine residues that bind the positively charged cation (Hardison *et al.*, 1991; Kramer *et al.*, 1993a, 1994).

Using rabbit BBMV the chemical groups proposed to be involved in bile acid binding to the Na^+/bile acid co-transport protein, were modified using a range of chemicals. Only the modification of thiol groups and amino groups on the transport protein, especially cysteine and lysine, prevented secondary active uptake of taurocholate. Therefore, from these findings it was deduced that the positively charged lysine amino group on the transport protein was involved in the binding of the negatively bile acid and the negatively charged cysteine residue binds the positively charged cation (Hardison *et al.*, 1991; Kramer *et al.*, 1992).

To enable the bile acid to bind, it requires the presence of a negative charge on the bile acid side chain and at least one hydroxyl group in positions 3,7, or 12 on the steroid nucleus (Hardison *et al.*, 1991; Kramer *et al.*, 1993a, 1994; Swaan *et al.*, 1997). The presence of 2 hydroxy groups at positions 3,7 and 12 are optimal for absorption, whilst the addition of a third hydroxy group decreased affinity (Baringhaus *et al.*, 1999; Kramer *et al.*, 1999). The addition of a small peptide to the 3 position of the steroid nucleus was shown not to effect the Na^+ dependent uptake of

these modified bile acids, as long as the peptide chain was below 10 amino acids. Therefore, the ileal bile acid transport system has been proposed as a possible mechanism for the transport of small peptide drugs (Kramer *et al.*, 1993, 1994). By coupling together two bile acid analogues via a spacer, then a potent inhibitor of the Na^+/bile acid co-transport protein can also be produced (Kramer *et al.*, 1995a).

CHAPTER SIX:

6. Molecular characteristics of the Na^+/bile acid co-transport protein in the small intestine

6.1 Photoaffinity labelling of the Na^+/bile acid co-transport protein

The first attempts at finding the size of the Na^+/bile acid co-transport protein involved photoaffinity labelling methods, using photolabile bile acid derivatives. A synthetic bile acid 7,7-azo-taurocholate (TC) was used to determine whether the bile acid was transported by means of the same transport system as natural bile acids. The rate of 7,7-azo-TC uptake was stimulated two fold in the presence of an inwardly directed Na^+ gradient, with competitive inhibition of taurocholate uptake in the presence of 7,7-azo-TC. These findings indicated that the natural and synthetic bile acids were sharing a common transport mechanism, proposed to be the ileal Na^+/bile acid co-transport protein (Burckhardt *et al.*, 1983a & b).

Photoaffinity labelling of rat ileal BBMV with 7,7-azo-TC resulted in the incorporation of radioactivity into several polypeptides, with the molecular weights of 125, 99, 83 and 43 kDa. The 99 kDa polypeptide was the most prominently labelled, whilst photoaffinity labelling of rat jejunal BBMV resulted in the labelling of polypeptides with the apparent molecular weights of 125, 94, 83, and 43 kDa. Analysis by SDS-PAGE indicated an increased incorporation of radioactivity into the 99 kDa polypeptide when compared to the 94 kDa polypeptide, so it was proposed that the 99 kDa polypeptide identified in rat ileal BBMV was the polypeptide involved in Na^+ dependent bile acid transport (Kramer *et al.*,1983; Montagnani *et*

al.,1996). Antibodies raised against the 99 kDa protein, which inhibited Na^+ dependent taurocholate uptake, detected a 99 kDa protein in rat ileal and kidney brush border membranes, but not the jejunum and liver (Kato *et al.*,Personal communication; Burckhardt *et al.*, 1987; Gong *et al.*, 1991). The 93 kDa protein detected in the jejunum has been proposed to be the carrier mediated Na^+ independent transporter of bile acids (Kramer *et al.*,1993a).

The incorporation of 7,7-azo-TC into the basolateral membrane vesicles, the cytosolic fraction and mitochondria of rat BBMV, resulted in the labelling of polypeptides with different molecular weights. For the basolateral membrane, the greatest incorporation of radioactivity was seen in polypeptides with the molecular weights of 54 and 59 kDa (Lin *et al.*, 1988). Again, in the BBMV a 99 kDa polypeptide was labelled, whilst a 20 kDa polypeptide was labelled in the mitochondria fraction. The cytosolic fraction had the greatest incorporation of radioactivity, with the labelling of polypeptides with molecular weights of 14, 35, 43, 59 and 68 kDa, with the 68 and 43 kDa polypeptides being identified as albumin and actin respectively. These findings confirmed that the molecular weight of the transport protein present in brush border membrane was 99 kDa, and that the transport of bile acids across the brush border and basolateral membranes was mediated by different proteins. Furthermore, the study revealed the presence of previously unrecognised polypeptides, a 20 kDa microsomal and 14 and 35 kDa cytosolic proteins which appeared to be involved in the transcellular movement of bile acids (Lin *et al.*,1990). The 99 kDa brush border membrane protein had the highest initial labelling rate, followed in descending order

by the 43 kDa actin, 35 and 14 kDa cytosolic proteins, 54 kDa and 59 kDa basolateral membrane proteins (BLM), and finally the 20 kDa microsomal protein (Stolz *et al*,.1989). Based on these findings in rats and rabbits, Lin *et al* (1993) proposed a general model for the transcellular movements of conjugated bile acids in ileal enterocytes. According to this model, bile acids were taken across the brush border membrane by a 99 kDa Na^+ dependent active transport system. Once across the brush border membrane, bile acids were transported via actin (43 kDa protein) or the 14 and 35 kDa cytosolic proteins either to a microsomal 20 kDa protein or directly to the basolateral membrane. Bile acids could be transferred to the 59 kDa basolateral membrane - associated protein before they exited from the cell via the 54 kDa integral BLM protein (Lin *et al*., 1993; Kramer *et al*., 1993b & c, 1994). The use of intracellular transport proteins to move bile acids through the cytoplasm has recently been supported but the types of proteins involved in the intracellular movement have yet to be established (Agellon and Torchia, 2000).

To investigate the possibility of the formation of complexes containing more than one Na^+/bile acid co-transport protein, the protein was subjected to target inactivation analysis by high-energy electrons. The molecular weight of the whole arrangement was 451 ± 35 kDa, which indicated that at least four 93 kDa Na^+/bile acid co-transport protein could make up the functional bile acid transporter. Photoaffinity labelling of radiation-inactivated vesicles revealed a biphasic inactivation of labelling for the 93 kDa and 14 kDa protein, determining the target size of 229 ± 23 kDa for the 93 kDa protein and 132 ± 23 kDa for the 14 kDa protein. The functional

molecular size for the Na^+/bile acid co-transport system, indicated that it was a homotetramer composed of four 93 ± 14 kDa subunits, (Kramer *et al*, 1995b). Using a synthetic dimeric bile acid analogue, the transport of bile acids in the homotetramer was inhibited (Kramer, 1996).

6.1.1 Ileal Cytosolic bile acid binding protein

The 14 kDa polypeptide, first observed by Lin *et al.*, (1988), was initially identified as gastrotrophin (Vodenlich *et al.*, 1991; Kramer *et al.*, 1993), though later studies renamed it the ileal bile acid binding protein (Gong *et al.*, 1994). The bile acid binding protein has been proposed to be a member of the family of fatty-acid binding proteins because it consists of 128 amino acid residues with 4 exons and 3 introns, which have the same organisation as other members of the fatty-acid binding family (Green *et al.*, 1992; Oelkers & Dawson,1995; Stengelin *et al.*, 1996). However, other studies have proposed that the 14 kDa cytosolic bile acid binding protein is related to, but distinctly different from, the liver and intestinal fatty acid binding proteins (Lin *et al.*, 1991). The cystolic transport protein has been mapped to chromosome 5 and exhibits post-weaning development (Gong *et al.*, 1996; Hwang *et al.*, 2000).

6.2 Sequence analysis of the Na^+/bile acid co-transport protein

Sequencing of the ileal Na^+/bile acid co-transport protein has revealed a protein with a different molecular weight to that identified by photoaffinity labelling and western blotting. Sequencing of a hamster cDNA clone proposed to encode the Na^+/bile acid co-transport protein (ISBT), revealed a protein of 348 amino acids with a molecular mass of 37 kDa, and three potential N-linked glycosylation sites. Comparison of the

distribution of IBAT mRNA revealed a single mRNA of approximately 4kb was readily detected in hamster ileum, kidney and weakly in jejunum but none in the duodenum or liver (Wong *et al.*, 1994), although comparison of protein and nucleic acid data bases revealed that the hamster IBAT was 35% identical and 63% similar to rat LBAT (liver bile acid transporter). The 4 kb mRNA coincides with the expression of a 3.8 – 4.5 kb mRNA identified in oocyte expression studies (Mullins *et al.*, 1992). The identification and isolation of the hamster ileal Na^+/bile acid co-transport protein (ISBT) allowed the subsequent isolation of the rat and human ISBT cDNA by PCR. Sequence analysis of the clone isolated from rat cDNA revealed a protein of 348 amino acids with a molecular mass of 38 kDa, which when fully glycosylated increased to 48 kDa, by virtue of two potential N-linked glycosylation sites. Comparison of amino acid sequences of the rat ileal Na^+/bile acid co-transporter with the rat basolateral bile acid transporter and hamster ISBT revealed 37 and 88% identity (64 and 95% similarity) respectively (Hagenbuch *et al.*,1991; Wong *et al.*,1994). The isolated human cDNA also encoded a protein of 348 amino acids but with 1 potential N linked glycosylation sites, which shared 84% identity with the hamster ISBT (Dawson and Olelker, 1995). After transfection into COS cells, the hamster (ISBT) and human (HISBT) had a taurocholate uptake rate of 77 pmol min^{-1} mg $protein^{-1}$ and 24 pmol min^{-1} mg $protein^{-1}$ respectively (Dawson and Wong, 1994) and were able to transport both conjugated and deconjugated bile acids with a higher affinity (Km) for dihydroxy bile acids than trihydroxy (Craddock *et al.*, 1998). Northern blot analysis, using the hamster cDNA clone, detected 5kb bands in the

ileum, kidney, caecum and proximal colon, with a faint signal in the liver and jejunum. Western blotting detected a range of polypeptides (48, 75, 92 and 130 kDa) in ileal brush border membrane proteins, with the 48 and 92 kDa proteins being the most easily detected. Purification by SDS-PAGE and re-analysis by Western blotting did not result in the formation of the 92 kDa from the 48 kDa by dimerisation or the breakdown of the 92 to the 48 kDa (Schneider *et al.*, 1995). However, more recent work has determined that the 90-99 kDa protein is the dimer of the 46 kDa monomeric protein (Sauer *et al.*, 2000).

The human ileal Na^+ bile acid co-transporter gene has been localised to autosome 13q33 (Dawson and Wong, 1995; Wong *et al.*, 1996). However, the gene which encodes the hamster Na^+/bile acid co-transport protein has been localised to chromosome 8 (Lammert *et al.*, 1998). Sequencing of the bile acid transport protein has led to the identification of mutations that abolishes transport activity. In a patient with Crohn's disease, a single C to T transition in the region encoding amino acid 290 resulted in a proline to serine substitution which prevented bile acid uptake (Wong *et al.*,1995). An inherited mutation has been detected in the ileal Na^+/bile acid co-transporter that disrupts normal enterohepatic circulation and that may be responsible for some cases of bile acid malabsorption observed in familial hypertriglyceridemia (Love *et al.*,1996). In addition, primary bile acid malabsorption (PBAM) has been attributed to a defective ileal Na^+/bile acid co-transport protein. A patient with PBAM demonstrated a mutation on one allele (where AAgt was replaced with Cctt) and two missense mutations, which abolished taurocholate transport in COS cells (Dawson

and Oelkers, 1996; Oelkers, 1997, 1998).

6.3 Functional expression of the Na^+/bile acid co-transport protein

The functional expression of size fractionated pig ileal mRNA by *Xenopus laevis* oocytes resulted in the expression of Na^+ dependent taurocholate uptake by mRNA of two different sized transcripts, which indicated another possible link between the two different molecular weight proteins identified by photoaffinity labelling and sequencing. The smaller 3.8-4.5 kb mRNA was consistent with the 4 and 5 kb mRNA bands identified on Northern blots (Schneider *et al.*, 1995) and is likely to represent the 37 kDa protein. However, a 1.5-3.0 kb sized transcript of mRNA has been shown to encode the 33-35 kDa Na^+ dependent bile acid transporter in hepatocytes (Hagenbuch *et al.*, 1990, 1991). The larger 11-13 kb mRNA identified on Northern blots of intestinal mRNA (Hagenbuch *et al.*, 1991) and expressed in oocytes (Mullins *et al.*, 1992) remains uncharacterised. The larger transcript may correspond to the 90-99 kDa protein identified by photoaffinity labelling, or it may code for the smaller 35-40 kDa protein along with a long untranslated region (Mullins *et al.*, 1996).

Recent studies into the distribution of ISBT mRNA, has shown that highest concentration of mRNA encoding the hamster ileal Na^+/bile acid co-transport protein is detected 1-4cm before the ileocecal valve, with the concentration decreasing proximally and distally (Stelzner *et al.*,2000).

6.4 Predicted transmembrane arrangement of the Na^+/bile acid co-transport protein

The Na^+/bile acid co-transport protein was initially predicted to be composed of seven

transmembrane domains (Wong *et al.*,1994; Dawson and Wong, 1995; Oelkers *et al.*,1997), with transmembrane domains 2, 3 and 4 showing the greatest sequence homology between different transport proteins (Dawson and Oelkers, 1996). However, *In vitro* translation of segments of the human ileal Na^+/bile acid co-transporter and hydropathy analysis showed the presence of eight membrane insertion domains (Hallen *et al.*, 1996). Further modelling of the Na^+/bile acid co-transport proteins present in the liver, intestine and kidney have indicated that the transport proteins have 9 transmembrane domains with an exoplasmic N terminus and a cytoplasmic C terminus (Hallen *et al.*, 1999), which coincides with the observation that the protein has an odd number of transmembrane domains (Bayle *et al.*, 1997). Therefore, from the translation data it has been determined Na^+/bile acid co-transport protein has between seven to nine transmembrane domains (Oelkers *et al.*, 1997). The cytoplasmic tail of the ileal Na^+/ bile acid co-transport protein has been shown to be important in the insertion of the transport protein into the brush border membrane. Removal of the cytoplasmic region of the protein abolished Na^+ dependent taurocholate uptake and also prevented the protein from becoming embedded into the membrane. However, removal of the cytoplasmic region from the liver Na^+/ bile acid co-transport protein (which is present in the basolateral membrane of hepatocytes) did not result in a significant decrease in taurocholate uptake and the protein was embedded normally. Therefore, it was concluded that the C terminus of the ileal Na^+/ bile acid co-transporter was important for the protein to be embedded into the brush border membrane (apical membrane) in particular (Sun *et al.*, 1998).

6.5 Objectives of this study

6.5.1 Background to intestinal bile acid transport

6.5.1.1 Cation dependency of bile acid transport

Previous investigations into the cation dependency of the Na^+/ bile acid co-transport protein have determined it to be strictly dependent on the presence of Na^+ to enable secondary active transport of bile acids (Lack and Weiner, 1961; Holt *et al.*, 1964; Wilson and Treanor, 1979; Rouse and Lack, 1979). However, more recent work has shown that other transport process previously determined to only use Na^+, can in fact use other cations. D-glucose transport protein (SGLT1) can utilise H^+ for secondary active transport, with the SGLT1 having a higher affinity for H^+ than for Na^+(Wright *et al.*, 1994) . Investigation into amino acid transport has also revealed that these transport proteins can utilise a wide range of cations for secondary active transport. A group of Na^+ and K^+ dependent transporters have been identified which are involved in the transport of glutamate and neutral amino acids (Hediger *et al.*, 1995). A metal ion transporter has also been identified in the intestine of rats, which are able to utilise the cations Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Pb^{2+} (Gunshin *et al.*, 1997). Therefore, the observations that other transport proteins, which were initially determined to be strictly dependent on Na^+, could utilise other cations for secondary active transport indicated that secondary active intestinal transport of bile acids could probably occur using other cations.

6.5.1.2 Molecular characteristics of the Na^+/bile acid co-transport protein

Recent work into the molecular characteristics of the transport protein has produced a

wide range of conflicting data. Photoaffinity labelling of the transport protein revealed a protein with the molecular weight of 90-99 kDa (Kramer *et al.*,1983; Kramer *et al*, 1995B; Montagnani *et al.*,1996) whilst sequence analysis of the transport protein revealed a protein of 38 kDa (Wong *et al.*, 1994; Schneider *et al.*, 1995). Furthermore, computer modelling of the ileal Na^+/bile acid co-transport protein produced conflicting data, with the possibility that the transport protein could have between 7-9 transmembrane domains (Dawson and Oelkers, 1996;Oelkers *et al.*, 1997; Hallen *et al.*, 1999). Therefore, further investigation into the functional and molecular characteristic of the gene that encodes the Na^+/bile acid co-transport is required.

6.5.2 Objectives of the study

1) To investigate the cation dependency of the pig ileal Na^+/bile acid co-transport protein. This was achieved by:

- Investigation into regional distribution of the ileal Na^+/bile acid co-transport protein using BBMV and *Xenopus laevis* oocytes.
- Determination of the effects of abolishing either the cation or taurocholate gradients on taurocholate uptake in the presence of a range of cations, using BBMV.
- Inhibition of taurocholate transport by another bile acid in the presence of a range of cations, using BBMV and *Xenopus laevis* oocytes.
- Determination of Km and Vmax for taurocholate transport by the Na^+/bile acid co-transport protein in the presence of a range of cations, using BBMV and

Xenopus laevis oocytes.

- Investigation into the temperature sensitivity of the transport protein using BBMV.
- Determination of the stoichiometry of the transport process using BBMV.
- Investigation into whether the size or charge of the cation is important in taurocholate transport, using BBMV.
- Hybrid arrest of the pig ileal Na^+/bile acid co-transport protein by the hamster Na^+/bile acid co-transport protein, to determine whether the same transport protein was utilising the same cations, by using *Xenopus laevis* oocytes.

2) Determination of the sequence of the pig ileal Na^+/bile acid co-transport protein and its structural arrangement within the membrane. This was achieved by:

- Designing primers complementary to the Na^+/bile acid co-transport protein to isolate the region of DNA that encoded the transport protein.
- Amplification of the region of DNA encoding the Na^+/bile acid co-transport protein by polymearase chain reaction (PCR).
- Determination of whether the isolated PCR product encoded the Na^+/bile acid co-transport protein, by hybrid arrest of the pig ileal Na^+/bile acid co-transport protein using the isolated PCR product.
- Cloning of the region of DNA encoding the Na^+/bile acid co-transport protein and sequencing of the gene
- Computer modelling of the nucleotide sequence to determine the structural arrangement of the transport protein in the membrane.

CHAPTER SEVEN:

7.1 MATERIALS

7.1.1 Removal and storage of intestinal tissue

Duodenal, jejunal and ileal tissue was removed from adult pigs within 3 minutes of death, to prevent degradation of the tissue. The first metre after the pyloric sphincter was taken as the duodenum. The metre immediately prior to the ileo-caecal valve was used as the ileum, and a metre of small intestine mid-way between the duodenum and ileum was taken as jejunum. The sections were removed and cut into segments 10 cm in length. They were flushed with ice-cold 0.9% (w/v) NaCl (pH 7), cut open longitudinally and washed again with the saline. The segments were wrapped in aluminium foil and dropped in liquid nitrogen. They were kept at – 70°C until use.

It has been shown that results obtained from material stored in this way were similar in terms of Na^+-dependent D-glucose and taurocholate transport to those obtained with fresh material (Mullins *et al.*,1992)

7.1.2 Radioisotopes

^{14}C D-glucose (297mCi/mmol) and ^{14}C methyl-α-D-glucopyranoside (293mCi/mmol) were purchased from Amersham International, Buckinghamshire, England and were diluted as required.

^{3}H-[G]-taurocholate (250mCi/mol) was purchased from New England Nuclear Corporation, and diluted as required.

7.1.3 Chemicals

Unless otherwise stated, the chemicals used in the experiments were purchased from Sigma or BDH. For molecular biology work, all the chemicals were purchased from Sigma's molecular biology range.

7.2 METHODS

7.2.1 The isolation and characterisation of brush border membrane vesicles (BBMV)

7.2.1.1 Isolation of Brush Border Membrane Vesicles (BBMV)

To allow the characterisation of the transport processes in the pig small intestine, the brush border membrane was separated from the rest of the enterocyte.

The method was as described by Shirazi-Beechey *et al.*(1990). A section of pig small intestinal tissue (about 10 g) was removed from duodenum, jejunum or ileum. The frozen section was weighed and defrosted slowly on ice in 100 ml of a hypotonic buffer (100 mM mannitol, 2 mM HEPES/Tris, pH 7.1). The tissue was homogenised by an Ultra Turrax homogeniser (T25 basis, Labortechnik), which separated the epithelial cells from the underlying muscle. Homogenisation of the tissue was carried out in 5-10 second intervals until all the lumps of the tissue had been removed, The homogenate was then filtered through a Buchner funnel (2 mm pore size) to remove any large remaining fragments of tissue and muscle. An aliquot of the homogenate was removed and stored in liquid nitrogen, to be used later for assay of protein and marker enzyme activities.

To the remaining tissue, 10 mM $MgCl_2$ was added. The tissue was mixed thoroughly on ice (using a magnetic stirrer) for 30 minutes, and then centrifuged (Europa 24, MSE) at 2500 xg for 20 minutes at 4°C. The resulting pellet contained the contaminating organelles, such as mitochondria, Golgi bodies and endoplasmic reticulum. The supernatant (containing the brush border membrane and basolateral membrane) was further centrifuged at 30,000 xg for 30 minutes at 4°C. The brush border membrane pellet was resuspended in 25 ml of a buffer

containing 100 mM mannitol, 0.1 mM $MgSO_4$, 2 mM HEPES/Tris, pH 7.4. The suspension was centrifuged for a further 30 minutes at 30,000 xg and the final brush border membrane pellet was resuspended in 2.0 ml of a isotonic buffer (300 mM mannitol, 0.1 mM $MgSO_4$, 20 mM HEPES/Tris, pH 7.4). Passing the suspension of brush border membranes (BBM) through 23.4 and 25 gauge B-D microlance needles aided the formation of brush border membrane vesicles (BBMV). Aliquots of BBMV were snap frozen in liquid nitrogen, and were then stored in liquid nitrogen until use.

7.2.1.2 Experimental conditions for BBMV pre-loaded with either taurocholate or a cation

For the experiments involving loaded vesicles, the final BBMV fraction was resuspended in either 100 mM NaCl, 100 mM KCl, or 100 µM taurocholate, corrected to 340 mOsM by the addition of the appropriate concentration of mannitol and the pH fixed by addition of 20 mM Tris, pH 7.4.

For bi-directional experiments, the BBMV were pre-loaded with 100 µM ^3H taurocholate (37 Kbq) 300 mM mannitol, 0.1 mM $MgSO_4$, 20 mM HEPES/Tris, pH 7.4.

7.2.1.3 Electron microscopy of brush border membrane vesicles

To determine if the brush border membranes had formed vesicles after snap freezing in liquid nitrogen, the membrane preparations were examined by electron microscopy.

The BBMV were resuspended in either an isotonic mannitol buffer (300 mM mannitol, 0.1 mM $MgSO_4$, 20 mM HEPES/Tris, pH 7.4) or distilled water. The BBMV were loaded onto colodain counted grids (stabilised with carbon) and were

then stained with 2% sodium phosphotungstate (pH 6.8). The BBMV were examined in an electron microscope (Jeol 100S) and a photograph was taken at 40,000 x magnification.

7.2.1.4 Protein Assay

The protein concentration of both the BBMV and the homogenate was assayed, in order to determine enzyme activity and rates of D-glucose and taurocholate transport.

The protein assay method was adapted from Lowry (1951). Bovine serum albumin (2 mg/ml) was used as the standard and diluted with water to give a range from 0-600 µg/ml. Triplicate samples of homogenate and BBMV (5 and 10 µl) were diluted to 1.0 ml with distilled water.

To each tube, 5.0 ml of alkaline copper reagent was added. Alkaline copper reagent was freshly prepared with 50 parts of solution A (2% $NaCO_3$ (w/v), 0.1 mM NaOH) to 1 part of solution B (0.5% $CuSO_4$ (w/v), 1% (w/v) sodium tartrate). The tubes were mixed thoroughly and allowed to stand at room temperature for 10 minutes. 500 µl of Folin Ciocalteu reagent (Sigma) was added to each tube and the tubes left at room temperature for 30 minutes. Absorbance was measured in a spectrophotometer (Unicam 8625 UV/vis) at 750 nm versus a reagent blank.

7.2.1.5 Enzyme Assays

To ensure that the isolated BBMV were sufficiently enriched with mainly the brush border membrane, marker enzyme assays were carried out on the BBMV and the homogenate. The enzyme activities of the different marker enzymes were compared in the BBMV and the homogenate.

7.2.1.5.1 Alkaline Phosphatase

The activity of alkaline phosphatase was measured using the protocol described by Shirazi *et al.*, (1981). For both the homogenate and BBMV, a protein concentration range of 15-30 µg was needed. 50 µl aliquots of the diluted homogenate and BBMV were alkalinised by the addition of 450 µl of a carbonate buffer (100 mM Na_2CO_3 / Na_2HCO_3, 0.1 mM $MgSO_4$, pH10). The reaction was then started by the addition of 500 µl of 10 mM 4-nitrophenol phosphate (Sigma) made up in the same carbonate buffer. The tubes were incubated at 25°C for 10 minutes and the reaction stopped by the addition of 500 µl of 500 mM NaOH, and the absorbance was read in a spectrophotometer (Unicam 8625) at 410 nm against a reagent blank. The rate of reaction was measured by the formation of free 4-nitrophenol. By using the molar extinction coefficient for 4-nitrophenol of 17,000 l/mol/cm the enzyme activity could be expressed in µmol product formed/min/mg protein.

7.2.1.5.2 Sucrase/ isomaltase Assay

The activity of sucrase was assayed by a method described by Dahlqvist, (1964). Approximately 5 µg of protein (2-5 µl of homogenate and BBMV) were diluted in a volume of 50 µl of distilled water. The reaction was started by the addition of 500 µl of incubation buffer (56 mM sucrose, 100 mM maleate, 0.5 mM NaOH, pH 6) and the samples incubated at 37°C in a water bath for 30 minutes. A glucose standard (100 µl of 0.5 mM glucose) was also incubated for 30 minutes at 37°C. Placing the samples in boiling water for 2 minutes quenched the reaction. After cooling to room temperature, 1.5 ml of freshly prepared glucose oxidase /

peroxidase buffer (500 mM K_2HP0_4/KH_2P0_4, 0.005% (w/v) glucose oxidase, 0.005% (w/v) peroxidase, 0.1% (w/v) sodium azide, 0.035% (w/v) aminophenazae) was added. To each sample, 500 µl of 0.1% (w/v) phenol was added and the samples incubated in a water bath at 37°C for 10 minutes. Absorbance was read at 515 nm against the reagent blank. The enzyme activity was expressed in µmol glucose/min/mg protein.

7.2.1.5.3 Succinate Dehydrogenase Assay

Succinate dehydrogenase was measured by the method described by Pennington (1961). Approximately 100 µg of protein (50-100 µl homogenate or BBMV) was used. The reaction was started by the addition of 750 µl of 100 mM potassium phosphate buffer (100 mM K_2HPO_4/KH_2PO_4, pH 7.4) which contained 100 mM sodium succinate, 50 mM sucrose and 0.2% (w/v) *p*-iodo nitro tetrazolum violet (Sigma). The samples were incubated at 25°C for 15 minutes and the reaction was stopped by the addition of 750 µl of 10% (w/v) trichloroacetic acid. To each sample, 3.0 ml of ethyl acetate was added, the samples were mixed thoroughly and allowed to stand at room temperature until two distinct layers formed. The ethyl acetate layer (top layer) was decanted and absorbance read at 490 nm against the reagent blank. By using the molar extinction coefficient for iodo nitro tetrazolum violet of 20,100 l/mol/cm, the enzyme activity could be expressed in µmol product formed/min/mg protein.

7.2.1.5.4 α–Mannosidase Assay

The activity of α–mannosidase was assayed according to the method of Tulsiani *et al.*, (1977). To approximately 100 µg of protein (50-100 µl homogenate and

BBMV), 500 µl of 100 mM citrate buffer (50 mM sodium citrate/50 mM citric acid, pH 4.5) was added. The reaction was started by the addition of 500 µl of 10 mM 4-nitrophenyl α-D-mannopyranoside (Sigma), prepared in the 100 mM citrate buffer. The samples were incubated at 25°C in a water bath for 30 minutes and the reaction stopped by the addition of 2.0 ml of 200 mM borate buffer (adjusted to pH 9.8 with 2M NaOH). Absorbance was measured at 405 nm against the reagent blank. A molar extinction coefficient for 4-nitrophenol of 18,500 l/mol/cm was used, allowing activity to be expressed in µmol product formed/min/mg protein.

7.2.1.5.5 α-Glucosidase Assay

The activity of α-glucosidase was determined by the method of Peters (1976). 50 µl of a buffer containing 35 mM Tris/HCl, pH 8 and 50 mM glutathione (reduced form) was added to approximately 100 µg of protein (50-100 µl homogenate and BBMV) and the samples were pre-incubated for 15 minutes on ice. The reaction was started by the addition of 250 µl of 0.21 mM 4-nitro-α-D-glucopyranoside prepared in 100 mM sodium phosphate buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, pH 8.0) and 0.1% (w/v)Triton-X-100. The samples were incubated in a water bath at 37°C for 60 minutes and the reaction stopped by the addition of 2.0 ml of 1 M NaOH. The liberation of 4-nitrophenol was measured at 400 nm against a reagent blank. A molar extinction coefficient for 4-nitrophenol of 18,500 l/mol/cm was used, allowing enzyme activity to be expressed in µmol product formed/min/mg protein.

7.2.2 Transport of D-glucose and taurocholate into BBMV using the rapid stop filtration technique

The characterisation of D-glucose and taurocholate transport in pig small intestine, was carried out by the use of the appropriate radiolabelled substrates. The transport of these radiolabelled substrates into the BBMV was investigated under a range of conditions.

7.2.2.1 Assay of Na^+ dependent uptake of D-glucose into BBMV

Uptake of D-glucose was measured using a filtration stop technique adapted from Shirazi-Beechey *et al.*, (1988). Triplicate samples of 15-20 µg of protein (10-20 µl of BBMV) were incubated in a water bath at 37°C. The assay was started by the addition of 100 µl of glucose incubation medium in the presence of Na^+, K^+ and choline (100 mM NaCl, KCl or choline chloride, 100 mM mannitol, 100 µM D-glucose, 20 mM HEPES/Tris, pH 7.4), which contained 100 µM ^{14}C glucose (37 Kbq). A time period of 10 seconds was used to measure initial rates of transport because the rates were linear over this time period. Glucose uptake was stopped by the addition of 1.0 ml of ice cold stop solution (150 mM choline chloride, 20 mM HEPES/Tris, pH 7.4). The BBMV were separated from the incubation medium by placing 900 µl of the assay medium onto an acetate/nitrate filter (0.22 µm pore size (Millipore)) and filtered under vacuum. The filter was then washed 10 times with 1.0 ml aliquots of ice cold stop solution and placed in 5.0 ml of scintillation fluid (Packard Ultima Gold MV). Samples were counted in a 1215 beta rack scintillation counter (LKB Wallac) for 10 minutes each.

Controls were prepared by the addition of 1.0 ml of the ice stop solution to the BBMV prior to the addition of the ^{14}C glucose uptake medium. To assay the uptake of glucose that was not due to active transport, NaCl was replaced by 100 mM KCl or 100 mM choline chloride in the glucose uptake medium. The replacement of Na^+ with K^+ and choline halted any Na^+ dependent uptake that required an inwardly directed Na^+ gradient. All uptake assays were routinely performed in triplicate. The variation was never greater than 10% of the mean value.

7.2.2.2 Assay of secondary active uptake of taurocholate into BBMV

The uptake of taurocholate was measured using the filtration stop technique developed by Mullins *et al.*, (1992). The sodium incubation medium routinely contained 100 µM ^3H taurocholate (37 kbq), 100 mM NaCl, 100 mM mannitol and 20 mM HEPES/Tris, pH 7.4, with the substitution of NaCl by KCl or choline chloride. Triplicate samples of 15-20 µg of protein (10-20 µl of BBMV) were incubated in a water bath at 37°C. The assay was started by the addition of 100 µl of incubation buffer, with a time period of 10 seconds used to measure initial rates of transport. The uptake of taurocholate was stopped by the addition of ice cold stop solution (100 mM mannitol, 100 mM choline chloride, 20 mM HEPES/Tris, pH 7.4). Uptake at zero time was measured by adding the stop solution to the BBMV before adding the incubation medium.

The BBMV were separated from the incubation medium by placing 900 µl of the incubation medium onto an acetate/nitrate filter (0.22 µm pore size (Millipore)) and filtered under vacuum. The filter was then washed 10 times with 1.0 ml

aliquots of ice cold stop solution and placed in 5.0 ml of scintillation fluid (Packard Ultima Gold MV). Samples were counted in a 1215 beta rack scintillation counter for ten minutes each.

7.2.2.3 Further characterisation of secondary active taurocholate transport.

All the experiments described in this section were carried out using BBMV isolated from the pig ileum. Unless otherwise stated, the transport of taurocholate into the BBMV was allowed to proceed for 10 seconds. After that time period, the experiments were stopped by the addition of 1ml of ice cold stop solution. The experiments then proceeded as described in section 7.2.2.2.

For experiments using preloaded BBMV, refer to the methods section, the isolation of BBMV (7.2.1.2) which explains how the BBMV were preloaded.

7.2.2.3.1 Variation of the BBMV intravesicular volume by alterations in osmolarity

To determine if ^{3}H taurocholate was being transported into the BBMV or binding to the surface of the BBMV, the intravesicular volumes of the BBMV were altered. The BBMV were incubated at 37°C for 2 hours in 6 different incubation media varying from 240 to 490 mosM. The incubation media all contained 100 mM NaCl or KCl, 100 μM ^{3}H taurocholate (37 Kbq) and 20 mM HEPES/Tris pH 7.4, with 0, 50, 100, 150, 200 and 250 mM mannitol corresponding to osmolarity concentrations of 240, 290, 340, 390, 440 and 490 mosM respectively.

7.2.2.3.2 Elimination of secondary active transport by alteration in temperature

The ileal BBMV were incubated at either 15°C, 25°C, or 37°C for 1 hour, to determine that temperature at which secondary active bile acid transport ceased.

After the incubation period, 100 µl of incubation media (100 µM ^{3}H taurocholate (37 Kbq), 100 mM mannitol, 20 mM HEPES/Tris pH 7.4 and 100 mM NaCl, KCl or choline chloride) was added and uptake was measured after 10 seconds.

7.2.2.3.3 Time course for taurocholate uptake into BBMV

To determine if the ileal BBMV were able to accumulate taurocholate above equilibrium, taurocholate uptake was measured over 9 hours.

The BBMV were incubated at 37°C in 100 µl of uptake buffer (100 mM NaCl, KCl or choline chloride, 100 mM mannitol, 20 mM HEPES/Tris pH 7.4, 100 µM ^{3}H taurocholate (37 Kbq)). The reaction was stopped after 0, 5, 10, 20, 30 or 60 seconds, and after 30, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420, 480 and 540 minutes.

7.2.2.3.4 Inhibition of taurocholate transport by the addition of taurodeoxycholate

For the inhibition studies, 1 mM taurodeoxycholate was added to the standard taurocholate uptake medium (100 µM ^{3}H taurocholate (37 Kbq), 100 mM mannitol, 20 mM HEPES/Tris, pH 7.4). The ileal BBMV were incubated at 37°C and taurocholate uptake into the BBMV was assayed in the presence of either 100 mM NaCl, KCl or choline chloride.

7.2.2.3.5 Michaelis Menten kinetics of taurocholate transport

For investigations of substrate dependence, the concentration of taurocholate was varied over the range 10-2000 µM. Uptake into ileal BBMV was assayed in the presence of 10, 20, 50, 100, 200, 500, 1000 and 2000 µM taurocholate, the concentration being fixed by altering the amount of unlabelled taurocholate added.

The Michaelis Menten kinetics of taurocholate uptake were also assayed in the presence of different cation concentrations. Taurocholate uptake was assayed in the presence of 0, 20, 40, 60, 80, 100 and 120 mM of either NaCl or KCl. Varying the concentrations of mannitol maintained the standard isotonic osmolarity of 340 mOsM.

7.2.2.3.6 Determination of whether the transport process was electrogenic or electroneutral

For electronegativity experiments, valinomycin (a K^+ ionophore) was added to the uptake buffer (100 µM ^3H taurocholate (37 Kbq), 100 mM mannitol, 100 mM of NaCl or KCl, 20 mM HEPES/Tris pH 7.4) at the concentration of 1 µg of valinomycin/ 1 µg of protein.

For stoichiometry experiments, 100mM of NaSCN, $NaNO_3$, sodium isethionate, sodium glutamate or 100mM of KSCN, KNO_3, potassium isethionate, potassium glutamate was added to the uptake buffer (100 µM ^3H taurocholate (37 Kbq), 100 mM mannitol, 20 mM HEPES/Tris pH 7.4).

7.2.2.3.7 The effect of different ionic radii of bile acid transport

The BBMV were incubated at 37°C in 100 µl of uptake buffer (mannitol, 20 mM HEPES/Tris pH 7.4, 100 µM ^3H taurocholate (37 Kbq)) plus 100 mM of the respective chloride salt. The reaction then proceeded as previously stated.

7.2.2.4 Bi-directional transport of taurocholate by the Na^+/bile acid co-transport protein

Transport studies to determine if the ileal Na^+/bile acid co-transport protein was able to actively transport bile acids in an outward direction from the BBMV, were carried out. The BBMV were preloaded with 100 mM NaCl, KCl, choline

chloride or mannitol and 100 μM ^{3}H taurocholate (37 Kbq), by either resuspension of the BBMV with the cations (as previously described) or by allowing passive diffusion or secondary uptake of taurocholate into the BBMV. A choline chloride based uptake buffer was added to the pre loaded BBMV, to facilitate an outwardly directed cation gradient. For the choline chloride loaded vesicles, the uptake buffer contained 200 mM mannitol. The reaction was stopped after 10 seconds.

7.2.3 mRNA isolation and characterisation

7.2.3.1 RNase free environment

To avoid ribonuclease (RNase) contamination an RNase free environment was established and maintained according to guidelines issued by Promega UK. Disposable gloves were worn during all procedures involving RNA samples, and changed regularly. De-ionised water, which had been pre-treated with diethyl pyrocarbonate (DEPC), an RNase inhibitor, was used for the preparation of all solutions. A 0.1% DEPC concentration was routinely used except when the buffer contained Tris, which deactivated DEPC, when an increased concentration of 1% was required.

Sterile disposable plastic ware was routinely used and any glassware and non-disposable plasticware was thoroughly washed with 100% (w/v) ethanol and RNase zap^{TM} (Ambion) which eliminated ribonucleases from the equipment.

7.2.3.2 Extraction and purification of RNA

Within eukaroytes, RNA is primarily produced in the nucleus from DNA. The RNA, which is composed of tRNA, rRNA and pre-mRNA, is processed within the nucleus and then released into the cytoplasm. Within the cytoplasm the pre-

mRNA is spliced to remove any introns, which leaves a mRNA consisting of exons which is the reading frame for protein synthesis (Darnell *et al.*, 1990). Therefore, in order to express the ileal Na^+/bile acid co-transport protein, mRNA needed to be isolated from the intestinal cells.

To isolate mRNA, total cytoplasmic RNA initially needed to be isolated. The method of RNA extraction was based on that of Chomczynski and Saachi (1987). Total RNA from 1.5-2 g segments of adult pig duodenum, jejunum and ileum was isolated by a single step guanidium thiocyanate/phenol/chloroform extraction procedure. The frozen tissue was weighed and returned to liquid nitrogen in order to prevent it from defrosting. The tissue was removed from the liquid nitrogen, ground into a fine powder and was added to 25 ml of ice cold denaturing solution (4.7 M guanidium thiocyanate, 0.74 M sodium citrate, 0.1 M mercaptoethanol) and allowed to defrost on ice. The tissue was then homogenised by use of the Ultra Turrax homogeniser and the following reagents were added; 2.0 ml of 4 M sodium acetate, 20 ml of water saturated phenol, 4.0 ml of 98 % (w/v) chloroform: 25 % (w/v) iso-amyl alcohol. The contents were thoroughly mixed by inversion of the tube and then left on ice for 15 minutes. The homogenate was centrifuged (Europa 24, MSE) at 10,000 xg for 20 minutes at 4°C. Separation of RNA and DNA occurred, with the denser genomic DNA remaining in the lower phenol-chloroform layer. The aqueous top layer contained the RNA. A white layer at the interface contained cellular protein. The aqueous RNA layer was collected, transferred to a sterile tube and an equal volume of isopropanol was added. The RNA and isopropanol were mixed thoroughly and placed at -70°C for 2 hours. Precipitation of the RNA occurred within this time.

On removal, the RNA solution was centrifuged at 10,000 xg for 30 minutes at 4°C. The resulting supernatant was discarded and the RNA pellet was resuspended in 500 µl of isopropanol and transferred to 1.5 ml eppendorf vial. The resuspended RNA was centrifuged in a microcentrifuge (Denver Instruments) at 7,200 xg for 6 minutes and the isopropanol supernatant removed. The RNA pellet was resuspended in 750 µl of isopropanol and centrifuged again at 7,200 xg for 6 minutes. The supernatant was discarded and the RNA pellet was resuspended in 500 µl of DEPC-treated water and 750 µl of 8M LiCl. The suspension of RNA was centrifuged at 7,200 xg for 10 minutes. After removal of the supernatant, the RNA pellet was again resuspended in 750 µl of 8 M lithium chloride and centrifuged for a further 10 minutes at 7,200 xg. The supernatant was then discarded, the RNA pellet resuspended in 500 µl of isopropanol and centrifuged at 7,200 xg for 6 minutes. The supernatant was removed for a final time and the RNA pellet was resuspended in 250-500 µl of DEPC treated water, and was stored at -70°C. Purity and yield of the extracted RNA was determined by using a spectrophotometer (Unicam 8625 UV/vis) scanning between 230-320 nm. The absorbance value at 260 nm was used to calculate the yield of RNA and the ratio of absorbance values at 260 nm/280 nm were used to give an indication of RNA purity.

7.2.3.3 Purification of mRNA

Poly(A)$^+$ RNA (mRNA) was isolated using oligo (dT) - cellulose chromatography based on the technique described by Sambrook *et al.*, (1992). To 100 µg of oligo (dT) cellulose (Sigma), 1.0 ml of elution buffer (1 mM EDTA, 10 mM Tris/HCl

pH 7.5) was added, mixed thoroughly by inversion, and centrifuged in a microcentrifuge (Denver Instruments) at 300 xg for 1 minute. The supernatant was removed and 1.0 ml of 1x binding buffer (500 mM NaCl, 1 mM EDTA, 0.5% (w/v) SDS, 10 mM Tris/HCl, pH 7.5) was added. The oligo (dT) cellulose was centrifuged at 300 xg for one minute, the supernatant removed, and the oligo (dT) cellulose pellet was stored on ice. To 250-500 µl of RNA (RNA concentration of approximately 2-3 mg), 250-500 µl of 2x binding buffer (1 M NaCl, 2 mM EDTA, 1% (w/v) SDS , 20 mM Tris/HCl, pH 7.5) was added and the RNA was incubated at 65°C for 5 minutes, to break up any dimers of RNA. The RNA was added to the oligo (dT) cellulose pellet, mixed thoroughly, and centrifuged at 300 xg for 1 minute. The resulting pellet of oligo (dT) cellulose was resuspended with the RNA supernatant and centrifuged at 300 xg for 1 minute. This was repeated a further 2 times. The supernatant was then removed and incubated for 5 minutes at 65°C to break up any duplex RNA. The RNA was added back to the oligo (dT) cellulose pellet, was mixed thoroughly and centrifuged at 300 xg for 1 minute (this was repeated a further 3 times). After the final spin, the supernatant was removed, leaving a pellet, containing mRNA bound to the oligo (dT) cellulose. To the mRNA/oligo (dT), 1.0 ml of 1x binding buffer was added and the sample centrifuged at 300 xg for 1 minute. The supernatant was removed and the mRNA/oligo (dT) pellet resuspended in 1.0 ml of washing buffer (100 mM NaCl, 0.1 mM EDTA, 10 mM Tris/HCl pH 7.5) to remove any unbound tRNA and rRNA (this was repeated twice). After the final spin, the supernatant (which contained the washing buffer) was removed and the mRNA-oligo (dT) pellet was resuspended in 500 µl of elution buffer (1 mM EDTA, 10 mM Tris/HCl pH 7.5),

which removed the mRNA from the oligo (dT) cellulose. The mRNA-oligo (dT) pellet was centrifuged for 1 minute at 300 xg and the supernatant (containing unbound mRNA) was removed and labelled as fraction 1 of the mRNA. The remaining pellet was resuspended in another 500 µl of elution buffer and centrifuged again at 300 xg for 1 minute to produce fraction 2 of the mRNA, this was repeated twice more to produce fractions 3 and 4. To the mRNA fractions, 75 µl of 2 M sodium acetate and 1.0 ml of isopropanol were added. The mRNA and the above reagents were mixed thoroughly and then refrigerated at -70°C for 2 hours.

After this period of time, the mRNA fractions were centrifuged at 7,200 xg for 10 minutes resulting in a pooled mRNA pellet. The supernatant was then removed and the mRNA pellet was resuspended with 500 µl of isopropanol and centrifuged at 7,200 xg for 10 minutes. The supernatant was removed and the mRNA pellet was resuspended in 40 µl of DEPC treated water and stored at -70°C. Purification and yield of the mRNA extracted was determined by using a spectrophotometer scanning between 230-320 nm.

The absorbance value at 260 nm was used to calculate the yield of mRNA and the ratio of absorbance value at 260 nm/280 nm to give an indication of mRNA purity.

7.2.3.4 Electrophoretic analysis of RNA

The method was adapted from Amersham's website (nucleic acid labelling and detection). A 1.2% (w/v) agarose gel (50 ml) was prepared containing 10 ml of 10 x 3-[N-morpholino]propane sulfonic acid (MOPS) buffer (20 mM MOPS, 8 mM Na acetate, 1 mM EDTA, pH 5) and 2.25 ml of 2% (w/v) formaldehyde. A gel

loading buffer was prepared containing bromophenol blue (0.05% w/v), xylene cyanol (0.05% w/v), glycerol (50% v/v), 10mM EDTA (ethylene dismineletraacetic acid), made up to 10 ml in 1x MOPS (2mM MOPS, 0.8mM Na acetate, 0.1mM EDTA, pH 5) buffer. To each RNA sample, 2 µl of gel loading buffer was added. Each RNA sample contained either 10 µg of total RNA or 10-20 µg of mRNA, 3.3 µl of formaldehyde, 9 µl of formide and 1.8 µl of 10x MOPS. The samples were run at 55 volts against a 0.1-0.9 Kb RNA ladder (Promega) in 100 ml of running buffer containing 1x MOPS, until the bromophenol blue had migrated to within 1 cm from the end of the gel. The gels were stained with ethidium bromide (2 µg/µl) overnight in 100 ml of DEPC treated water.

7.2.4 Expression of mRNA in *Xenopus laevis* oocytes

Previous studies have shown that *Xenopus laevis* oocytes can be used as a protein expression system. The injection of mRNA into the cytoplasmic pole of the oocyte can lead to the translation of the mRNA to its corresponding proteins.

7.2.4.1 Microinjection of *Xenopus laevis* oocytes

Xenopus laevis oocytes were microinjected according to the procedure described by Colman (1986).

A mature female *Xenopus laevis* was buried in ice for an hour. The toad was then killed by a sharp blow to the head and the anterior part of the head was immediately removed. The toad's abdomen was cut open and the ovaries were removed. Individual oocytes were manually dissected and the follicular layer removed. The excised oocytes were maintained in a modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM HEPES, 0.3 mM $Ca(NO_3)_2$,

0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, pH 7.6 1 M NaOH). After an overnight incubation, healthy stage V-VI oocytes were selected and microinjected according to the procedure described by Colman (1986).

To ensure an RNase free environment, the microinjection equipment (World Precision Instruments) was cleaned using RNAse Zap ™. A glass capillary was heated and pulled into a fine needle and the capillary filled up with mineral oil. The capillary was then inserted onto the microinjection needle and an air tight seal was formed by the application of silicon grease.

The capillary was then washed out with RNase zap ™ followed by 0.1% DEPC water to ensure a RNase free environment. Using the glass capillary the mRNA was drawn into the capillary and the capillary could then be used to inject mRNA into the oocytes.

Oocytes were injected with 40-50 ng (in 46 nl) of either total mRNA into the cytoplasmic pole of the oocytes. Control oocytes were injected with 46 nl of DEPC treated water. Oocytes were cultured for 4 days at 20°C with a daily change of modified Barth's solution.

7.2.4.2 Hybrid arrest of the translation of pig ileal mRNA by hamster ileal cDNA

The hybrid arrest studies were carried out using the method described by St Germain (1990). In the hybrid arrest studies, 2 µg of mRNA was added to 4 µg of hamster ileal bile acid co-transporter cDNA (kindly donated by Paul Dawson), in a total reaction volume of 8 µl containing100 mM NaCl, 10 mM Tris/HCl, pH 7.5. The hybridisation mixture was incubated at 70°C for 5 minutes to break up any duplex mRNA and then 55°C for 20 minutes to allow the DNA to anneal to the

mRNA. The mixture was then rapidly cooled on ice. For the negative control, mRNA was incubated in 100 mM NaCl, 10 mM Tris/HCl, pH 7.5, without any DNA. Oocytes were injected with 46 ng of either mRNA or mRNA/cDNA and were cultured for 4 days at 20°C with a daily change of modified Barth's solution. For positive control experiments, Na^+-dependent hexose uptake was determined using 100 µM methyl-α-D-^{14}C glucopyranoside (Amersham, 37 KBq), in the presence of Na^+, K^+ and choline.

7.2.4.3 Assay of taurocholate transport into oocytes

In order to dispel any Na^+ or K^+ gradients the oocytes were incubated in a choline chloride based Barths solution (92.93 mM choline chloride, 15 mM HEPES/Tris, pH 7.6) for 1 hour at 30°C.

To measure Na^+-dependent taurocholate uptake, oocytes were incubated for 1 hour at 30°C in 500 µl of uptake medium consisting of the conventional NaCl based Barths solution (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/NaOH (pH 7.6), 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) along with 1.25 µl of ^{3}H taurocholate (37 Kbq) and 100 µM unlabelled taurocholate. For uptake in the presence of a K^+ gradient, the experiment was carried out in the same way, but with a solution containing 89mM KCl, 2.4mM $KHCO_3$, 15mM HEPES/KOH (pH 7.6), 0.3mM $Ca(NO_3)_2$, 0.41mM $CaCl_2$, 0.82mM $MgSO_4$. For uptake in the presence of choline, an uptake solution containing 92.93 mM choline chloride, 15mM HEPES/Tris (pH 7.6) was used.

Uptake was stopped by the addition of 5 ml of ice-cold stop solution composed of the choline chloride Barths solution, with the addition of 1 mM unlabelled taurocholate, added in order to reduce non-specific binding of radiolabelled

taurocholate. Oocytes were washed a further four times in 5 ml of cold stop solution. Individual oocytes were dissolved in 20 µl of formic acid, 4 ml of scintillation fluid added, and the radioactivity associated with each oocyte counted in a liquid scintillation counter (Wallac LKB).

For the taurocholate kinetic experiments, taurocholate concentrations ranging from 10 – 500 µM were used. The mRNA-injected and water-injected oocytes were assayed in the presence of NaCl, KCl and choline chloride with a taurocholate concentration of either 10, 20, 50, 100, 200 or 500 µM of unlabelled taurocholate, with each solution containing 37 KBq of ^{3}H taurocholate.

To determine the Michaelis Menten kinetics of taurocholate uptake in the presence of different cation concentrations, taurocholate uptake was assayed in the presence of 0, 20, 40, 60, 80 and 100 mM of either NaCl or KCl and 15 mM HEPES/Tris p.H 7.6. Varying the concentrations of choline chloride maintained the standard molarity of 107.93 mM.

Competitive inhibition studies were carried out in the presence of Na^{+}, K^{+} and choline uptake buffers plus 100 µM taurocholate or 100 µM taurocholate and 1 mM taurodeoxycholate.

For each of the above experiments, mRNA-induced taurocholate uptake in the presence of each cation was determined by the rate of taurocholate uptake in water-injected controls being subtracted from those injected with mRNA. Furthermore, to determine bile acid transport in the presence of Na^{+} and K^{+}, uptake rates for taurocholate transport in the presence of choline were subtracted from taurocholate uptake in the presence of Na^{+} and K^{+}.

7.2.5 Isolation and characterisation of genomic DNA from pig ileum

7.2.5.1 Isolation of pig ileal genomic DNA

The isolation of pig ileal DNA was adapted from the method of Herrington and McGee,(1992). The DNA was isolated for use in Polymerase Chain Reaction (PCR) experiments.

Pig ileum (0.5 g) was ground into a fine paste under liquid nitrogen, in order to isolate the genomic DNA by the disruption of the plasma and nuclear membranes of the intestinal cells. The pig ileum was not homogenised using an Ultra Turrax because this process tends to shear the DNA (Herrington and McGee, 1992). The DNA was added to 2.5 ml of extraction buffer (300 mM Sodium acetate, 50 mM EDTA, pH.7.5).

To the DNA/extraction buffer, 17.5 ml of hot phenol extraction buffer (7.5 ml of extraction buffer, 0.5% sodium sarcosine, 10 ml of buffer saturated phenol, pH 8) was added after the phenol was heated to 65°C. The DNA and phenol were mixed until an emulsion formed and was then allowed to settle at room temperature for 5 minutes. The DNA was then centrifuged (Europa 24, MSE) at 3500 xg for 5 minutes. After centrifugation, 10 ml of chloroform:isoamyl alcohol was added and the sample was mixed for 5 minutes at room temperature.

The DNA was then centrifuged (Europa 24, MSE) at 3500 xg for 6 minutes in order to separate the aqueous and protein phases. The aqueous and protein phases were then collected using a wide-bore pipette and added to 10 ml of chloroform:isoamyl alcohol. After mixing at room temperature for 5 minutes the DNA/chloroform was centrifuged (Europa 24, MSE) at 3500 xg for 5 minutes. After centrifugation the aqueous phase was transferred to a fresh tube, 10 ml of

chloroform:isoamyl alcohol was added and the sample was spun at 3500 xg for 5 minutes. The aqueous phase was again removed, added to 10 ml of chloroform:isoamyl alcohol, and centrifuged at 3500 xg. After centrifugation the aqueous phase was removed and added to an equal volume of isopropanol. The DNA and isopropanol were mixed until the DNA precipitated, the sample was then centrifuged at 3500 xg for 5 minutes in order to pellet the DNA.

After the supernatant was discarded, the DNA pellet was re-suspended in 1 ml of isopropanol and transferred to an eppendorf. The DNA was then centrifuged (Denver Instruments Microcentrifuge) at 3500 xg for 1 minute. The supernatant was then discarded and the DNA pellet was resuspended in 500 µl of DEPC treated water.

The absorbance value at 260 nm was used to calculate the yield of genomic DNA and the ratio of absorbance value at 260 nm/280 nm to give an indication of DNA purity.

7.2.5.2 Electrophoretic Analysis of Genomic DNA

In order to determine if the isolated genomic DNA was intact, electrophoresis of DNA was carried according to the method of Sambrook *et al.*, 1989. If the DNA needed to be isolated from the agarose gel to be used in other experiments, such as PCR and sequencing, then a Tris/EDTA and glacial acetic acid buffer (TAE buffer) was used. If the DNA run in the gel was not required for further use, then a Tris/EDTA and boric acid buffer (TBE) was used. The reason for using a TBE buffer for standard gel electrophoresis was because this buffer can be re-used and has a better buffering capacity (Sambrook *et al.*, 1989). However, the presence of borate ions within the buffer can be detrimental to enzymes used for DNA

isolation from the agarose. Therefore, for PCR products, a TAE DNA gel was used because these products could then be excised from the gel for possible cloning and sequencing.

7.2.5.2.1 TBE electrophoresis of genomic DNA

The method was adapted from Sambrook *et al.*, 1989. A 1% (w/v) agarose gel (50 ml) was prepared containing 0.5 x TBE (45 mM Tris, 45 mM EDTA, 1 mM EDTA, pH 8.0). A gel loading buffer was prepared containing bromophenol blue (0.25% w/v) and 40% (w/v) sucrose. To each DNA, 4 µl of gel loading buffer was added. Each DNA sample contained 10-20 µg of genomic DNA. The samples were run at 60 volts against a 0.5-10 kb DNA ladder (Sigma), in 100 ml of running buffer containing 0.5 x TBE until the bromophenol blue had migrated to within 1 cm from the end of the gel. The gels were stained with ethidium bromide (2µg/µl) overnight in 100 ml of DEPC treated water.

7.2.5.2.2 TAE electrophoresis of genomic DNA

The methodology for electrophoresis of the DNA was the same as above. However, the 1 % agarose gel was made up in 1 x TAE (40 mM Tris, 1 mM EDTA, 40 mM glacial acetic acid, pH 8.0). The DNA running buffer was also at the concentration of 1 x TAE.

The percentage concentration (w/v) of agarose was altered depending on the weight of the DNA. If the DNA products were of a similar size then a higher percentage agarose gel (about 3%) would be used to allow distinction between the similar sized DNA.

7.2.6 Restriction digest of genomic DNA

To determine which restriction enzymes should be used to cut the genomic DNA, a range of restriction enzymes were tried. The method was adapted from Sambrook *et al.*, (1989), with specific alterations in relation to the restriction enzyme used.

To 1µg of genomic DNA a given volume of water was added, to make up a final volume of 18µl.

Three restriction enzymes were then used to digest the DNA:

Hind III (concentration of 1unit/µl)

The Hind III (Sigma) enzyme recognises the sequence A/AGCTT .

The enzyme was supplied with a 10 x stock of restriction buffer, which had to be diluted to a 1 x concentration. Therefore, to the genomic DNA 2µl of 10 x restriction buffer was added to give the final concentration of a 1x buffer (250 mM NaCl, 0.1 mM EDTA, 1.0 mM dithierythritol, 50% (v/v) glycerol, 10 mM Tris/HCl pH 7.5). One unit of enzyme was required to completely digest 1µg of DNA, therefore 1µl of enzyme was added to the genomic DNA.

Sal I (concentration of 1 unit/µl)

The restriction enzyme Sal I (Sigma) recognises the sequence G/TCGAC . To the genomic DNA, 2 µl of 10 x restriction buffer was added to give a final concentration of 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 50 mM Tris/HCl pH 7.5. One unit of enzyme was added to digest 1 µg of DNA.

Eco RI (concentration 1 unit/1µl)

The enzyme Eco RI (Appligene) recognises the sequence GAATT/C. To the genomic DNA, 2 µl of 10 x restriction buffer was added to give a final

concentration of 100 mM NaCl, 10 mM MgCl2, 10 mM 2-Mercaptoethanol, 10 mM Tris/HCl pH 8.0. One unit of enzyme was added to digest 1 μg of DNA.

After the addition of the above restriction enzymes the sample of genomic DNA was incubated at 37°C overnight. After the overnight incubation, the reaction was stopped by addition of 10 mM EDTA. The digested DNA was run on a 1% TBE agarose gel at 60 volts for one hour, against a 0.5-10 kb DNA ladder and undigested genomic DNA.

7.2.7.1 Design of PCR primers

PCR primers were designed to enable isolation of the particular region of DNA that encodes the pig ileal Na^+/bile acid co-transport protein. Using the nucleotide sequences for the ileal Na^+ /bile acid co-transport protein obtained for hamster, rat and human, the homologous regions between these mammals were identified.

The nucleotide and amino acid sequences of the hamster, rat and human ileal Na^+ dependent bile acid transport protein were obtained from the EMBL database. Using CLUSTALW software, the amino acid sequence for the bile acid transport proteins from the above mammals were compared. Comparison of the amino acid sequences revealed four regions of particularly high homology between the transport proteins from the three species. Two regions were proximal to the N terminus of the protein and two regions were proximal to the C terminus of the protein.

Comparison of the nucleotide sequence for these conserved regions revealed that the nucleotide sequences which code for these conserved amino acids, altered slightly between species, due to different nucleotide triplets encoding the same

amino acid (Stryer, 1988). Therefore, for the primer design the nucleotides that were conserved between the majority of three mammals were used.

In order to produce a double stranded DNA template from PCR, forward and reverse primers are required. The forward primer anneals to the N terminus coding region of the DNA template whilst the reverse primer anneals to the C terminus coding region of the DNA template. To enable the amplification of both strands of DNA, the forward and reverse primers had to be complementary to either the sense or antisense DNA strands. The forward primer was designed to be complementary to the N terminus coding region of the 3'-5' antisense DNA, whilst the reverse primer was complementary to the C terminus of the 5'-3' sense DNA strand.

Using a range of internet primer design software sites (EMBL PcRimer; Info.Med.Yale.Edu, Biochem.UCL, Alkami Biosystems), the proposed primers were examined to determine if they were suitable for use in PCR. The annealing temperatures, GC ratios and ability to form hairpins or self anneal was analysed.

To ensure that both primers were able to anneal to the DNA within the same PCR reaction, their annealing temperatures needed to be between 55-75°C and within a range of 4-6°C. The annealing temperatures of the primers were calculated using the following equations (Alkami Biosystems, Inc):

For Primers below 20 bp in length : $[4(G+C) + 2(A+T)]-5^{\circ}C$

For Primers above 20 bp in length: $62.3^{\circ}C + 0.41^{\circ}C$ (%G-C) – 500/length – $5^{\circ}C$

By testing the proposed primers in this way, it was determined that the reverse primer (primer 4) temperature was too low. To produce sets of primers with similar temperature ranges, nucleotides can be added to the primer with the lower

annealing temperature. Therefore, to the reverse primers (primer 4), 3 adenosine nucleotides were added to the 3' region of the primer, which had the effect of increasing the annealing temperature (Info. Med.Yale.Edu).

The primers also had to have a G/C (guanine/cytosine) and a purine/pyrimidine ratio of 40-60%. For one of the reverse primers (primer 4) the purine/pyrimidine ratio was below 40%, so a string of adenosine (purines) were added to the 3' end of the reverse primer which also had the effect on increasing the purine/pyrimidine ratio (Info. Med.Yale.Edu).

To ensure that the PCR transcripts transcribed from the pig ileal DNA encoded a functional protein, the PCR primers were required a start codon (ATG) and a stop (ATT) codon. One of the reverse primers (primer 4) already contained a stop codon because this primer corresponded to the very end (3') of the C terminus. The conserved regions towards the N terminus of the protein did not extend to the 5' end of the protein reading frame, so therefore did not include the start codon. Therefore, forward primer 1 had an artificial start codon added to the primer sequence, which would allow the PCR product to be used in a expression system (Biochem.UCL).

The nucleotide sequences of the forward and reverse primers were then examined to ensure that the primers were not complementary to each and so would not anneal to each other. To prevent any self annealing of the primers, (primer/dimer formation), a GC clamp was added to the 5' end of all four primers, which consisted of GGCGC (Biochem.UCL).

7.2.7.2 Annealing temperature, concentration and sequence of primers

For Primers with the GC clamp

Primer 1: Forward primer

5' GGCGCATGGAGAGCAACTTCAAT 3'

Optical density: 12.31 Molecular weight: 7471.6 µg/µmole nmol: 46.7

Annealing temperature 52.7°C

Primer 2: Forward Primer

5' GGCGCATCCTGTTGGCCTTGGTG 3'

Optical density: 12.06 Molecular weight: 7452.6 µg/µmole nmol: 52.2

Annealing temperature 56.74°C

Primer 3: Reverse Primer

5'GGCGCTCCATGACATTTCTTGTA 3'

Optical density: 11.64 Molecular weight: 7379.6 µg/µmole nmol: 48.7

Annealing temperature 57.3°C

Primer 4: Reverse Primer

5' GGCGCCTACTTCTCATCTGGTTGAAA 3'

Optical density: 13.19 Molecular weight: 8362.2 µg/µmole nmol: 48.3

Annealing temperature 54.8°C

The primers were made by MWG Biotech and all four primers had to be reconstituted within a given volume of TE buffer (1 mM EDTA, 10 mM Tris/HCl pH 8.0) to give a desired stock concentration of 100 µM. Therefore, 466 µl, 482 µl, 522 µl and 487 µl of TE buffer was added to primers 1, 2, 3 and 4 respectively. After the addition of the TE buffer, the primers were allowed to dehydrate for 2 minutes and were then vortexed for 15 seconds. The primers could then be stored at –20°C for six months.

To determine if the dilution of the primers had resulted in the correct concentration, the optical densities of the primers were measured. To a final volume of 1000 µl of water, 5 µl of each primer was added and the absorbance was read at 260 nm. Using the absorbance reading at 260 nm and a mM extinction

coefficient for the each of the purine and pyrimidine bases, the final concentration for each of the diluted primers was calculated.

7.2.8 DNA amplification by the polymerase chain reaction (PCR)

Using the designed primers, PCR was carried out on total genomic DNA isolated from pig ileum. PCR was also carried out on plasmid DNA that encoded only for the hamster ileal Na^+/bile acid co-transport protein that was the positive control for the PCR reaction. A negative control for the PCR reaction was carried out, using plasmid DNA that encoded the liver Na^+ /bile acid co-transport protein.

A 'Ready To Go' PCR kit (Amersham Pharmacia Biotech) was used to amplify the pig DNA. The kit consisted of dried beads that contained all the PCR reagents pre-mixed and pre-dispensed into 0.5 ml PCR eppendorfs. PCR eppendorfs were thin walled for efficient heat transfer and did not contain any RNase, DNase, or pyrogens that could interfere with the PCR reaction.

To the PCR beads, 1 μg of genomic/plasmid DNA was added, followed by the addition of the forward and reverse primers (50 μM stock concentration). To the PCR reaction, distilled water was added to make up a final reaction volume of 25 μl. After dilution with water, each PCR reaction contained 1.5 units of Taq DNA polymerase, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl pH 9.0, and 200 μM dNTP. The final concentration of the primers within the PCR reaction (after the addition of water) was 5 μM for both the forward and reverse primer. After the addition of water, the PCR reaction was mixed and pelleted by brief microcentrifugation (Denver Instruments Microcentrifuge).

The PCR reaction was carried out in a FTS 400 capillary fast thermal sequencer (Corbett thermal cycler).

The PCR cycling times and temperature were as followed:

Step 1: 1 cycle at 95°C for 2 seconds

Step 2: A, 95°C for 10 seconds
B, 50°C for 10 seconds
C, 72°C for 30 seconds

Repeated for 40 cycles

Step 3: 1 cycle at 72°C for 2 seconds

Step 1 of the PCR reaction involved a 'hot start', where the PCR reaction was heated to 95°C. This 'hot start' denatured the double stranded DNA into single strands and more importantly denatured any proteins and proteases present in the PCR mixture.

Step 2 of the PCR reaction involved the continual denaturing, primer annealing and elongation/amplification of the PCR template. Heating the DNA to 95°C denatured the DNA and cooling to 50°C allowed the primers to anneal to the DNA. The section of double stranded DNA created by the primers binding to the single stranded DNA, provided the binding region for the Taq DNA polymerase. An increase in temperature to 72°C allowed the Taq DNA polymerase to elongate the DNA template. The process of repeating these 3 temperatures for 40 cycles, enabled the elongation and amplification of the specific region of DNA. The final step of PCR involved a 2 second incubation period at 72°C. This enabled the Taq DNA polymerase to complete elongation of the PCR templates and to add a single adenosine to the end of each PCR transcript. After the PCR reaction was completed, the PCR products were run on a 1% TAE gel at 60 volts for an hour. The PCR products were run against a 0.5-10 Kb (Sigma) DNA ladder.

7.2.9 Functional confirmation of the PCR product

To determine if the PCR product isolated from the pig ileum encoded the Na^+/bile acid co-transport protein, functional confirmation of the PCR product was required. Using the hybrid arrest procedure (section 7.2.4.2), 100 ng of either the pig PCR product or the hamster PCR product was added to 2 μg of pig ileal mRNA. The mRNA/DNA complex was injected into *Xenopus laevis* oocytes (method described in section 7.2.4.1 of the methods chapter) and incubated in Barth's solution for 5 days. Taurocholate transport into the oocytes was assayed in the presence of Na^+, K^+ and choline, as described in section 7.2.4.3 of the methods chapter.

7.2.10.1 Cloning and sequencing of the PCR product

For DNA sequencing, a sufficient concentration of the isolated PCR product was required. Therefore, the PCR product needed to be cloned into a plasmid vector and then inserted into *E.coli*. The PCR product added into the plasmid vector would then be under the control of the natural DNA replication system present in the *E.coli*.

The addition of the PCR product into the plasmid was carried out using the TOPO TA cloning kit from Invitrogen. This kit allowed the insertion of PCR products that had been amplified by Taq polymerase into a specific plasmid vector (pCR 2.1-TOPO) which had complementary ends to the PCR product.

Taq DNA polymerase has a non-template dependent terminal transferase activity which adds a single adensoine (A) to the 3' ends of the PCR products, as previously mentioned. The pCR 2.1-TOPO (see appendix for vector map) linearised vector has a single overhang 3' deoxythymidine (T) residue, which

allows the complementary ligation (A ligates to T) of the PCR product to the plasmid.

The PCR product was inserted into the pLac region of the plasmid which encoded for the Lac Z gene. This Lac Z gene, present in the lac operon, is involved in lactose utilisation with the Lac Z gene coding for the enzyme B-galactosidase. Insertion of the PCR product removed the Lac Z gene. This meant that the *E.coli* cells that contained the plasmid (with the PCR product) would not be able to produce the B-galactosidase gene. The lack of the B-galactosidase meant that these *E.coli* colonies would have a white appearance, which contrast with the blue colour of the *E.coli* colonies that contained the plasmid that had retained the pLac region.

To ensure that the PCR product (generated using the protocol described in section 7.2.8) contained an A overhang, the product was heated again, at 72°C for 30 minutes to allow the Taq DNA polymerase still present in the sample to add A overhangs to all the amplified PCR products.

To 4µl of the PCR product, 1µl of the pCR 2.1-TOPO vector (10 ng/µl plasmid DNA, 50% glycerol, 1 mM EDTA, 1 mM DDT, 0.1% Triton-X-100, 50 mM Tris/HCl pH 7.4, 100 ng/µl BSA, phenol red) was added and the volume made up to 6µl with sterile water. The reaction was gently mixed and incubated at room temperature for 5 minutes. From the PCR product/vector mix, 2µl was removed and added to a vial containing one shot chemically competent TOPO 10 *E.Coli* cells (200 mM NaCl, 10 mM $MgCl_2$) (Invitrogen).

The sample was incubated on ice for 5 minutes and the cells were then heat shocked for 30 seconds at 42°C. The samples were then transferred to ice and 250

µl of SOC medium (2 % trypton, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added. The samples were then shaken at 37°C for one hour.

To selective medium plates (1 % trypton, 0.5 % yeast extract, 1 % NaCl, 1.5 % agar, 50 µg/ml ampicillin, pH 7.0), 10 µl of the sample mixture was spread over the plates. The plates were then incubated at 37°C overnight and all the white colonies produced were harvested.

The single colonies were harvested, by using a sterile tooth pick, and were added to 5 ml of LB broth (1 % trypton, 0.5 % yeast extract, 1 % NaCl, 50 µg/ml ampicillin, pH 7.0) and incubated at 37°C overnight whilst being shaken to allow aeration of the samples.

7.2.10.2 DNA purification of plasmids containing the cloned PCR product

To enable sequencing of the cloned PCR product, the plasmids containing the inserted PCR product needed to be isolated from the *E .coli.* DNA purification was carried out using the Wizard plus SV mini prep kit (Promega).

5 ml of bacterial cultural was centrifuged at 10,000 xg for 5 minutes (Denver Instruments) which pelleted the *E.coli.* The supernatant was discarded and 250 µl of cell resuspension solution (10 mM EDTA, 100 µg/ml RNase A, 50 mM Tris/HCl pH 7.5) was added to the pellet. The pellet was resuspended by vortexing and 250 µl of cell lysis solution (200 mM NaOH, 1 % SDS) was added. The sample was mixed by inverting the tube and left at room temperature for 5 minutes. To the sample, 350 µl of neutralisation solution (4.09 M guanidine hydrochloride, 759 mM potassium acetate, 2.12 M glacial acetic acid, pH 7.2) was added, the tube was mixed by inverting and the sample was then spun at 14,000 xg

for 10 minutes (Denver Instruments). The clear supernatant (containing the plasmid lysate) was removed and added to a spin column. The column containing the lysate was then centrifuged at 14,000 xg for 1 minute to ensure that the lysate passed through the filter at the base of the column. The lysate that had passed through the column was discarded and the plasmid DNA retained on the column filter was washed with 750 µl of column wash solution (60 mM potassium acetate, 60% ethanol, 10 mM Tris/HCl pH 7.5). The column was spun at 14,000 xg for 1 minute and the lysate that passed through the column was discarded. The column was then transferred to a clean eppendorf and 100 µl of nuclease free water was added. This eluted the plasmid DNA, which was collected in an eppendorf. The eppendorf contained only the plasmid and PCR insert, at a concentration of about 100 ng/µl, which could be used for sequencing.

7.2.10.3 Isolation of PCR product from plasmid and determination of the PCR product size

To ensure that the plasmid contained the right sized PCR product, the PCR product needed to be isolated from the plasmid and its size determined by gel electrophoresis.

To isolate the PCR product from the plasmid, the plasmid was digested with Eco R1 which cut the plasmid at the Eco R1 restriction sites (see appendix for map). The plasmid DNA (100 ng) was digested with 1µl of Eco R1 (1 unit/µl) for 1 hour at 37°C. The undigested (100 ng) and digested (100 ng) plasmid DNA was then run on a 1x TBE gel (as described in section 7.2.5.2.1).

7.2.10.4 DNA sequencing

The plasmids which contained the right sized DNA insert were then sent to MWG Biotech (Milton Keynes) for sequencing. The company used the M13 forward and reverse primers present on the plasmid (see appendix) for sequencing.

7.2.11 Computer modelling of the protein

7.2.11.1 Conversion of nucleotide sequence to amino acid sequence

The nucleotide sequence was translated to the amino acid sequence using the software, PC gene (Version IV, IntelliGenetics.Inc). The software translated the nucleotide sequence to produce three variations of amino acid sequence, depending on which of the first three nucleotide bases were taken to represent the first of the gene sequence. Analysis of the three variations of amino acid sequences allowed the determination of which amino acid sequence appeared to encode the Na^+/bile acid co-transport protein.

7.2.11.2 Identification of the amino acid sequence

To identify the translated amino sequence, all three variations of amino acid sequence were analysed using the BLAST software available from the website: Expasy.ch/tools/. The amino acid sequence was added into the relevant section of the BLAST software (Altschul *et al.*, 1990) and a basic search was conducted. The software then searched the EMBL/Brookhaven databases of all known amino acid sequences to identify the amino acid sequence entered. The BLAST software compiled a list of the possible proteins that shared varying degrees of homology with the entered amino acid sequence. The degree of homology that the entered amino acid sequence shared with each protein was expressed as an E value; the smaller the E value the greater degree of homology between the two sequences.

7.2.11.3 Sequence alignment of the amino acid sequence

After the identification of the protein for which the amino acid sequence encoded, a sequence alignment between the derived amino acid sequence and other amino acid sequences encoding the same protein in different mammals was performed. The multiple sequence alignment was carried out using the CLUSTALW software (Thompson *et al.*, 1994) at EBI. The amino sequences of all the proteins were entered into the relevant section and the CLUSTALW software aligned each sequence to asses the conservation between species.

7.2.11.4 Determination of number and position of transmembrane regions

To determine how many transmembrane regions the pig ileal Na^+/bile acid co-transport protein had, the softwares TMpred, HMMTOP and TopPred2 were used. The amino acid sequences of ileal Na^+/bile acid co-transport protein from the 6 species for which the sequence was known were entered into the relevant sections for each software package and these softwares will then predict the amino acids involved in the transmembrane domains.

However, each software will suggest a different amino acid that starts and finishes each transmembrane domain because each software will use a different known protein (from its data base) to compare against the entered amino acid sequence. Therefore, the predictions of the number and positions of the transmembrane regions were compiled and averaged.

7.2.11.4.1 HMMTOP software

The HMMTOP software predicts membrane topology of proteins by using the Hidden Markov Model (Tusnady and Simon, 1998). The Markov model has special architecture which was developed to search for the possible locations of

transmembrane domains of the entered amino acid sequence, by comparing the transmembrane topology of proteins (programmed into the database) with known transmembrane domains.

The amino acid sequence is entered in a SWISS PLOT format and the software then calculates transmembrane domains which are between 20-22 amino acids long.

7.2.11.4.2 TMpred software

The TMpred software makes predications of the possible location of transmembrane domains and their orientation by using an algorithm based on the statistical analysis of TM base (Hofmann and Stoffel, 1993). TM base is a database of transmembrane domains and their predicated transmembrane domains. TMpred predicts the possible location of the transmembrane domains of the entered amino acid sequence, by comparing the amino acid sequence with the proteins stored in the data base. As with HMMTOP the amino acid sequence can be added in SWISS PLOT format.

7.2.11.4.3 TopPred 2 software

This software uses a combination of physicochemical properties, such as hydrophobicity analysis and the positive inside rule, to predict the transmembrane domains of the entered amino acid sequence (Von Heijne, 1992). The main physicochemical property used to predict possible transmembrane domain location, is by analysing the hydrophobicity of the amino acids. The average hydrophobicity of around 20 amino acids is calculated by the TopPred 2 software and compared to the hydrophobicity of known proteins entered into the database.

Using this date the software then predicts the possible location of each transmembrane domian.

7.2.11.5 Generation of a 2D model of the ileal Na^+/bile acid co-transport protein

To examine the 2D structure of the ileal Na^+/bile acid co-transport protein the software 2D MEM MAP (Antoniw and Mullins, unpublished) was used. The amino acid sequence of the transport protein was entered and the settings for the averaged positions and numbers of transmembrane domains were entered. The 2D model of the transport protein was produced by using the topology section of the 2D MEM MAP software.

CHAPTER EIGHT:

8. The purification and characterisation of pig intestinal brush border membrane vesicles (BBMV)

The Na^+/bile acid co-transport protein is present within the brush border membrane of intestinal enterocytes, so the isolation of intact brush border membrane from the rest of the enterocyte was required. BBMV were prepared from pig duodenum, jejunum and ileum, using cation precipitation and differential centrifugation, as described in the methods (section 7.2.1.1).

BBMV were then characterised in the following ways: -

1, Electron microscopy of BBMV, to determine if the brush border membranes had formed vesicles and also to assess whether the vesicles were right side out or inside out.

2, The purity of the prepared BBMV were examined, to ensure minimal contamination with other cellular organelles that are present in enterocytes. The activities of marker enzymes of the brush border membrane were compared to the activities of marker enzymes of the membranes of other cellular organelles (mitochondria, endoplasmic reticulum and Golgi body) in the original tissue homogenate.

3, Further characterisation of the BBMV was by examination of Na^+ dependent D-glucose transport (SGLT1), which occurs exclusively on the brush border membrane (Crane *et al.*,1962; Wright *et al.*,1992). The characterisation of the SGLT1 transport process provided a further test of the membrane origin and functional viability of the vesicles.

8.1 Electron microscopy of BBMV

The BBMV were resuspended in either a mannitol buffer or distilled water, to determine whether the mannitol buffer was destroying the BBMV by osmotic lysis.

As can be seen in figure 8.1, the brush border membrane had formed vesicles and the microvilli were pointing outwards (as demonstrated by the white edge on the BBMV). The formation of BBMV with the brush border membrane located on the outside of the vesicles, confirmed that the vesicles had formed correctly. In comparison, the brush border membrane resuspended in water (figure 8.2) had not formed vesicles due to osmotic lysis. This finding confirmed that resuspending brush border membranes in an isotonic mannitol buffer was essential for proper formation of BBMV.

Figure 8.1, Electron microscopy of BBMV resuspended in an isotonic mannitol buffer

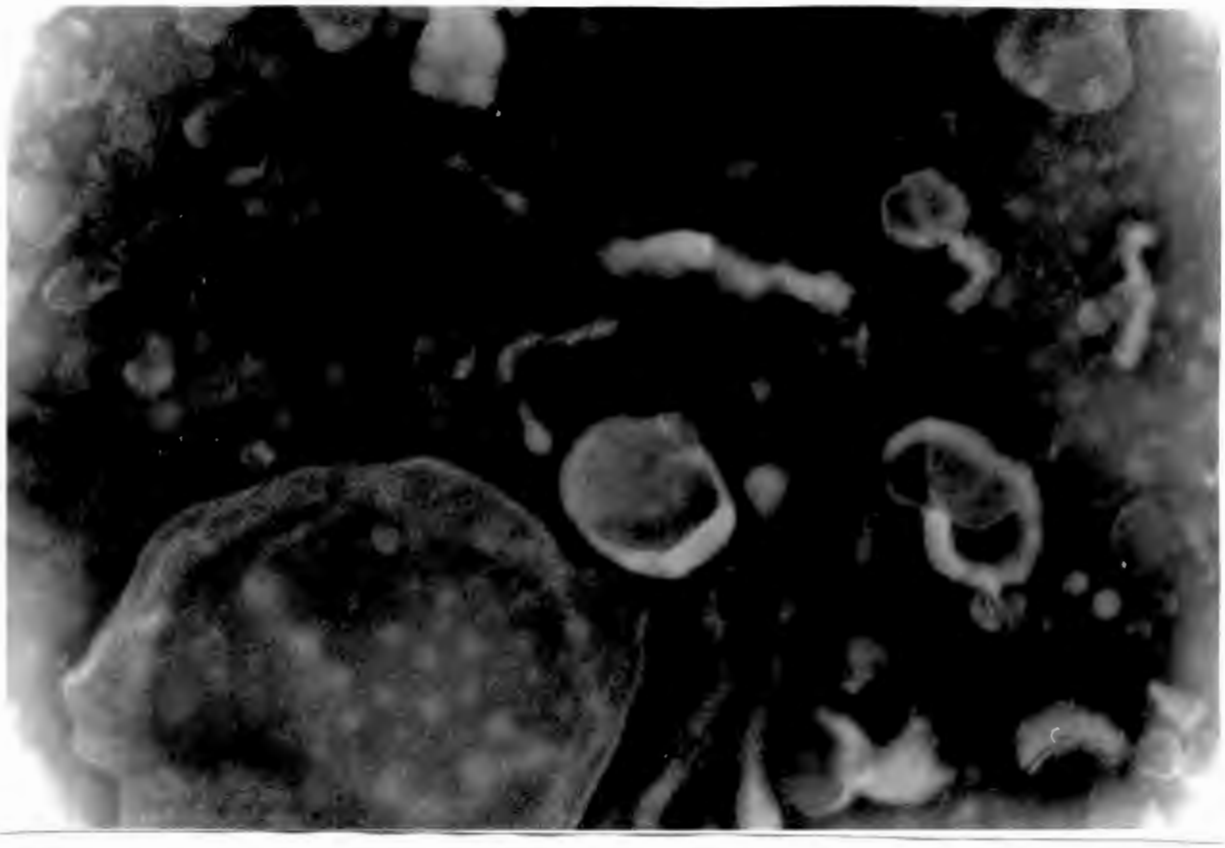
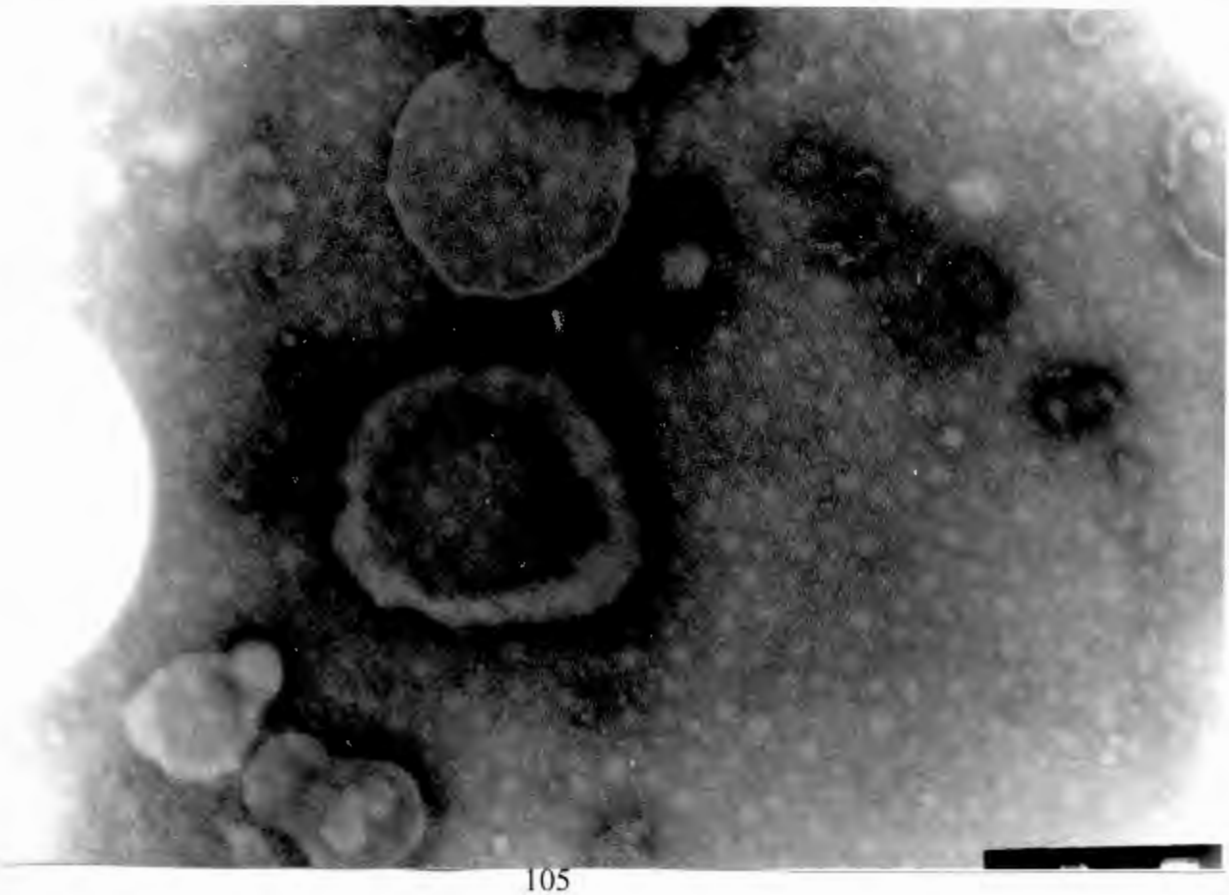


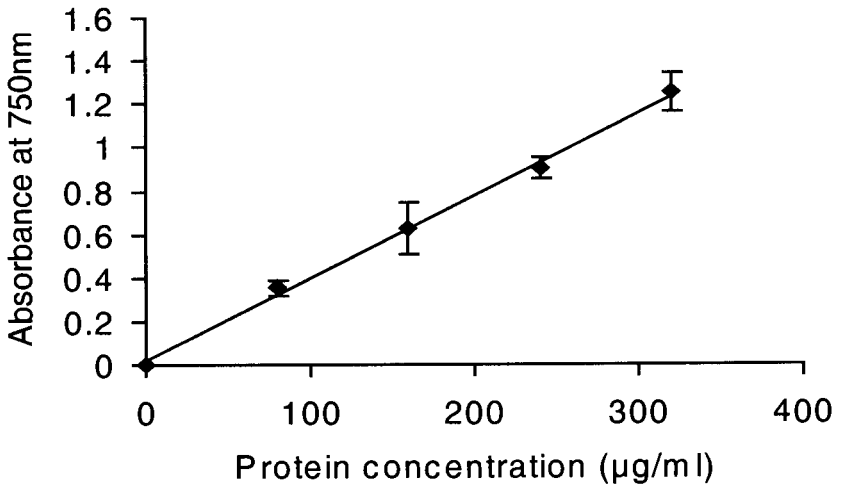
Figure 8.2, Electron microscopy of BBMV resuspended in water



8.2 Determination of homogenate and BBMV protein concentration

The protein concentrations of BBMV and homogenate were assayed by the Lowry method (1951) as described in the methods section.

Figure 8.3, Standard curve of protein concentration



A stock solution of bovine serum albumin (2 mg/ml) was serially diluted with water to achieve a standard curve of known protein concentrations (as described in the method section). Absorbance was read at 750 nm. Each point represents the mean ± S.D. for three assays (n=9). Only the liner region of standard curve is shown in this graph, because this was the region used for the protein determination

To determine the concentration of protein within the homogenate and BBMV, the absorbance values for the homogenate and BBMV were compared to the absorbance values of the standard curve.

8.3 Activities of marker enzymes

Previous work has shown that isolated BBMV should be enriched in brush border marker enzymes, whilst the activities of marker enzymes for the nucleus, mitochondria, endoplasmic reticulum and Golgi bodies should be depleted when

compared to the original intestinal homogenate (Kessler *et al.*,1978; Shirazi *et al.*,1981; Mullins *et al.*,1992).

The brush border enzymes, sucrase and alkaline phosphatase, were used as markers for the brush border membrane of the enterocytes, whilst succinnate dehydrogenase, α-mannosidase and α-glucosidase were used as membrane marker enzymes for mitochondria, Golgi bodies and endoplasmic reticulum respectively.

Table 8.1, The specific activity, enrichment and recovery of marker enzymes in BBMV prepared from pig small intestine

	Specific activity (μmol/min/mg prot)	% Recovery (Activity)	% Recovery (Protein)	Enrichment
Sucrase:-				
Homogenate	0.265 ± 0.04		100	1
BBMV	3.833 ± 0.09	26.0	2.2	14.46
Alkaline phosphatase:-				
Homogenate	0.056 ± 0.04		100	1
BBMV	0.885 ± 0.03	20.2	1.86	15.80
SDH:-				
homogenate	0.0181 ± 0.03		100	1
BBMV	0.0158 ± 0.06	1.6	2.16	0.71
α-glucosidase:-				
homogenate	0.0038 ± 0.00		100	1
BBMV	0.0024 ± 0.01	2.5	2.075	0.63
α-mannosidase:-				
homogenate	0.0119 ± 0.02		100	1
BBMV	0.0212 ± 0.01	2.8	1.783	1.72

* SDH = succinate dehydrogenase

BBMV were prepared from adult pig intestine as described in the methods section. Each value represents the mean ± S.D. for three assays(n=9).

The enrichments of specific activity were calculated as the ratio of specific enzyme activities in the final BBMV compared to the initial homogenate. Protein recovery in the final BBMV was calculated as the percentage of the total protein found in the initial

homogenate. The recovery of total activity of marker enzymes in the final BBMV was calculated as the percentage of the total activity of marker enzymes found in the initial homogenate.

The specific enzyme activities of the brush border marker enzymes (sucrase and alkaline phosphatase) were compared in BBMV and the original homogenate. BBMV displayed a 14-16 fold enrichment of these enzymes, with a recovery of 20-25% of the total enzyme activity (table 8.1). However, comparison of specific enzyme activities for succinate dehydrogenase, α-mannosidase and α-glucosidase showed that these enzymes were not enriched in BBMV and had only low levels of total enzyme recovery (1.5-3%).

These results showed that brush border membranes had been isolated from the enterocytes with little contamination from other cellular organelles, which corresponds to preparations of other workers (Mullins *et al.*,1992; Lin *et al.*,1993; Kramer *et al.*,1993). Therefore, the enzyme enrichment values for the isolated BBMV indicated that the BBMV were suitable for the functional characterisation of pig intestinal transport processes.

8.4 Characteristics of D- glucose transport in pig small intestinal BBMV

The active transport of sugars was one of the first intestinal transport mechanisms to be studied. Crane (1962) proposed that monosaccharides such as glucose and galactose were transported across the brush border membrane of intestinal enterocytes by a carrier mechanism, which co-transported Na^+.

Further work has shown that Na^+ dependent transport of D-glucose achieves its maximum rate in the jejunum, with lower rates of uptake observed in the duodenum and ileum (Kessler *et al.*, 1978). Passive diffusion of D-glucose occurs at a similar rate throughout the small intestine (Bluet,1986). The removal of the

Na^+ gradient resulted in only passive diffusion being observed (Aoshima *et al.*,1987).

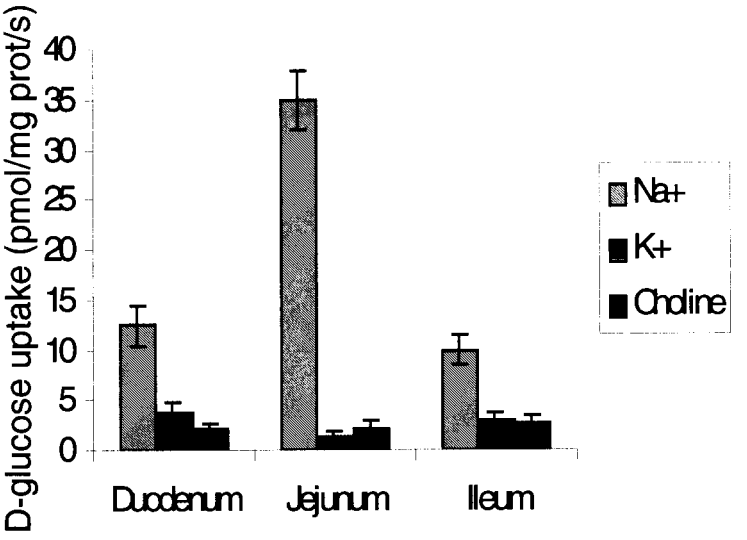
The SGLT1 D-glucose transport process has been shown to be an electrogenic process and required two monovalent cations to pass through the membrane for every glucose molecule (Murer and Hopfer, 1974; Okada, 1979; Stieger *et al.*,1984). Previous work has shown that electrogenic transport processes are sensitive to the anions that may be coupled to the cation. The transport of two Na^+ ions into the BBMV leads to the creation of a membrane potential (positive inside the BBMV) which is compensated by the transport of the anion (coupled to the cation) across the membrane. Certain anions have a higher permeability across the brush border membrane and will have the effect of altering the membrane potential of the BBMV. Therefore, the same anion, namely chlorine was used throughout the study of Na^+ dependent D-glucose transport.

8.4.1 Characterisation of BBMV integrity by D-glucose transport

Intestinal transport of D-glucose has now been widely characterised (Turk *et al.*, 1991; Wright,1993) and has been used in previous studies as a functional measure of the transport capacity of isolated BBMV (Shirazi-Beechey *et al.*,1990; Mullins *et al.*,1992).

In this study the secondary active uptake of D-glucose into intestinal BBMV isolated from pig duodenum, jejunum and ileum were compared in the presence of inwardly directed Na^+, K^+ or choline chloride gradients. The transport properties of the BBMV were measured using radiolabelled D-glucose and a rapid stop-filtration procedure.

Figure 8.4, Regional variation in D-glucose transport throughout the pig small intestine, in the presence of different cations



BBMV were prepared from the duodenum, jejunum and ileum of adult pigs as described in the methods sections. D-glucose transport was assayed in the presence of Na^+, K^+ and choline, with the incubation medium containing either 100 mM NaCl, KCl or choline chloride, 100 mM mannitol, 20 mM HEPES/Tris, pH 7.4 and 100 μM ^{14}C D-glucose (37 Kbq). The assays were carried out at 37° C. Upon the addition of 100 μl of incubation medium the reaction was allowed to proceed for 10 seconds, after which the uptake was stopped. Each point represents the mean ± S.D. for three assays (n=9).

As shown in figure 8.4, Na^+ dependent D-glucose transport occurred in BBMV isolated from all regions of adult pig intestine. However, the initial rates were appreciably higher in the jejunum. This indicated that the Na^+ dependent D-glucose co-transport protein (SGLT1) was present throughout the small intestine but was more abundant in the jejunum followed by the duodenum and ileum. This observation was consistent with previous findings (Bluet,1986) that Na^+ dependent D-glucose transport declines after the proximal small intestine, due to a lower number of transport proteins in the distal region.

The rate of D-glucose transport into the duodenal, jejunal and ileal BBMV was greatly reduced in the presence of K^+ and choline when compared to Na^+. This observation correlated with previous findings (Aoshima *et al.*, 1987) that secondary active D-glucose transport does not occur in the presence of K^+ or choline.

In order to investigate active transport of substrates, the passive diffusion rates of these substrates must be ascertained. Choline is a large bulky cation which should not be able to pass through the brush border co-transport proteins. Therefore, throughout this study, uptake in the presence of choline was taken to be representative of passive diffusion of the substrate.

8.5 Summary of the characterisation of pig intestinal BBMV

The electron microscopy of the isolated BBMV revealed that the brush border membranes had formed vesicles and that the microvilli were on the external surface. The characterisation of pig intestinal BBMV by the activity of brush border marker enzymes demonstrated a 14-16 fold enrichment in BBMV when compared to the original enterocyte homogenate. This indicated that the brush border membranes had been isolated from the enterocytes with virtually no contamination from other cellular organelles (Moe and Jackson, 1987).

The characterisation of pig BBMV by uptake of D-glucose displayed characteristics observed in similar studies (Shirazi-Beechey *et al.*, 1990; Turk *et al.*, 1991; Wright, 1993). The characterisation of pig BBMV showed that the enzyme activities and transport functions attributed to the brush border *in vivo* were maintained in the BBMV. Therefore, the BBMV were a suitable model for the study of intestinal bile acid transport.

CHAPTER NINE:

9. Bile acid transport in pig small intestine

Studies using *in vivo* methods, isolated epithelial cells and BBMV have shown that bile acids are reabsorbed by passive diffusion throughout the small intestine (Krag and Phillips,1974; Wilson, 1981; Marcus *et al.*, 1991, Stiehl *et al.*, 1995), whilst secondary active bile acid transport in most cases is localised to the distal ileum (Lack and Weiner, 1961; Holt *et al.*, 1964; Schiff *et al.*,1972). Furthermore, studies have shown that the transport of bile acids against their concentration gradient is dependent on energy derived from a Na^+ gradient inwardly directed across the brush border membrane (Wilson and Treanor, 1975; Lucke *et al.*,1978; Schwenk *et al.*,1983).

In this study, the properties of taurocholate transport in pig intestinal BBMV were measured by using radiolabelled ^3H taurocholate and a rapid stop-filtration procedure. The uptake of taurocholate into BBMV was routinely assayed in the presence of an inwardly directed NaCl, KCl or choline chloride gradient.

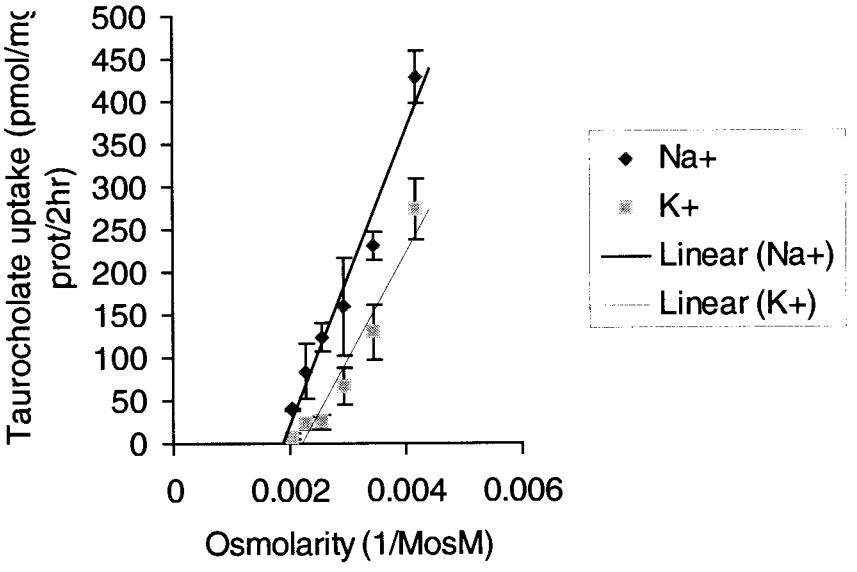
9.1 Effect of varying the intravesicular volume of pig ileal BBMV

In the study of a transport system using isolated membrane vesicles, it was necessary to investigate whether the observed uptake of the solute was actually due to its transport into the vesicles. A certain proportion of uptake maybe due to extravesicular binding of the solute to the membrane vesicles, therefore the osmotic potential of the BBMV were manipulated in order to increase or decrease the size of the vesicles. If the observed transport of the solute (in this case taurocholate) was due to transport into the BBMV, decreasing the intravesicular

volume would have led to a decrease in radiolabelled uptake of the substrate with the opposite trend observed when increasing the intravesicular space.

In this study, the intravesicular volume of the BBMV was altered by a 2 hour incubation in media which had varying amounts of mannitol. The osmotic potential of the incubation media was varied from 240-490 mosM. By extrapolating the relationship between osmolarity and uptake to infinite osmolarity (approaching zero intravesicular volume), the binding and membrane incorporated component of taurocholate uptake into BBMV could be estimated.

Figure 9.1, The effect of varying the external osmolarity of the incubation media on taurocholate uptake into BBMV



BBMV were prepared from pig ileum as described in the methods section. The BBMV were incubated at 37° C for 2 hours in 6 different incubation media varying from 240 to 490 mosM. The incubation media all contained 100 mM NaCl or KCl, 100 μM ^{3}H taurocholate (37 Kbq) and 20 mM HEPES/Tris pH 7.4, with 0, 50, 100, 150, 200 and 250 mM mannitol corresponding to osmolarity concentrations of 240, 290, 340, 390, 440

and 490 mosM respectively. Each point represents the mean ± S.D. for three assays (n=9).

Figure 9.1 showed that there was an inverse relationship between taurocholate uptake and osmolarity. The graphical extrapolation to infinite osmolarity (where the line crossed the Y axis) which was representative of zero intravesicular volume, indicated that no taurocholate uptake was observed. This established that the observed taurocholate uptake was due to transport of taurocholate into the BBMV and not due to extravesicular or intra-membrane binding of taurocholate to the BBMV. This data confirmed that the isolated pig intestinal BBMV were a suitable model for the study of bile acid transport through the brush border membrane.

9.2 Characteristics of taurocholate transport in pig small intestinal BBMV

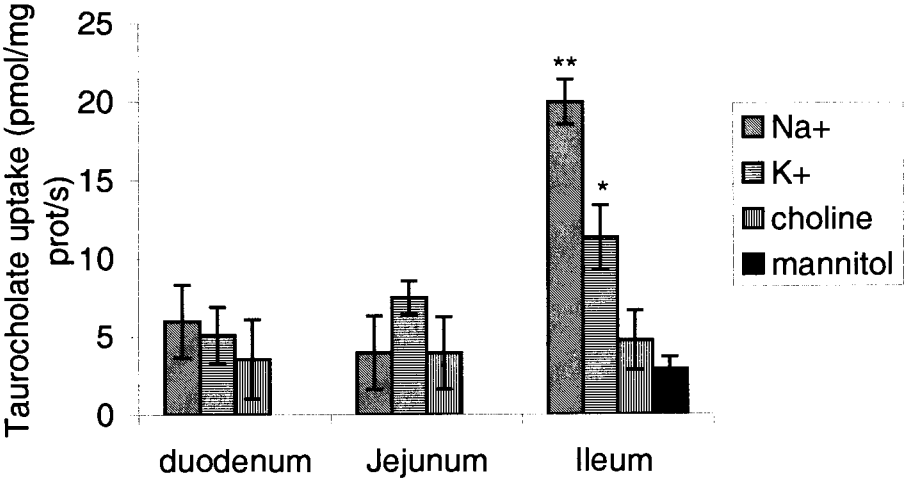
9.2.1 Distribution of taurocholate transport throughout pig small intestine

Previous studies have shown that the intestinal Na^+/bile acid co-transport protein is localised to the ileum (Wilson, 1981; Kramer *et al.*,1983; Mullins *et al.*, 1992; Sorscher *et al* ., 1992; Wong *et al.*,1994). BBMV isolated from the duodenum, jejunum and ileum were assayed for taurocholate uptake in the presence of different cations in order to determine which region of the small intestine contained the Na^+ /bile acid co-transport protein.

As shown in figure 9.2, there was elevated uptake of taurocholate into ileal BBMV (37 pmol/mg prot/s) in the presence of an inwardly directed Na^+ gradient when compared to taurocholate uptake into duodenal BBMV (6 pmol/mg prot/s) and jejunal BBMV (4 pmol/mg prot/s) under the same conditions. Furthermore,

the rates of duodenal and jejunal taurocholate transport in the presence of Na^+ were very similar to the rates of taurocholate uptake observed in the presence of choline (3 pmol/mg prot/s in the duodenum and 4 pmol/mg prot/s in the jejunum). These reduced rates were similar to rates of bile acid passive diffusion observed in previous studies (Krag and Phillips, 1974, Wilson 1981).

Figure 9.2, Taurocholate transport in pig duodenum, jejunum and ileum in the presence of different cations



*BBMV were prepared from the duodenum, jejunum and ileum of adult pigs as described in the methods section. Taurocholate transport was assayed in the presence of Na^+, K^+ and choline throughout the small intestine. These incubation media contained either 100 mM NaCl, KCl or choline chloride, 100 mM mannitol, 20 mM HEPES/Tris, pH 7.4 and 100 μM ^3H Taurocholate (37 Kbq). Taurocholate transport in pig ileum was also assayed in the presence of 300 mM mannitol, 20 mM HEPES/Tris, pH 7.4. The assays were carried out at 37° C. Upon the addition of 100 μl of incubation medium, the reaction was allowed to proceed for 10 seconds after which the uptake was stopped. Each point represents the mean ± S.D. for three assays (n=9). An Anova statistical test was carried out in excel, the rates of taurocholate uptake was compared between each cation in the duodenum , jejunum and ileum. 95% significance = *, 99 % significance = ***

The rate of taurocholate uptake in the presence of K^+ in the jejunum (8 pmol/mg prot/s) was slightly elevated when compared to taurocholate uptake in the presence of choline (4 pmol/mg prot/s) from the same region. The increased uptake of taurocholate in the presence of K^+ could have been due to a bile acid carrier mediated process identified by Amelsberg and colleagues (1996). Therefore, the rate of passive and secondary active bile acid transport in pig small intestine appeared to be consistent with previous studies, which have shown that Na^+ dependent bile acid transport is localised to the ileum, with passive bile acid diffusion occurring throughout the small intestine (Schiff *et al.*,1972; Krag and Phillips, 1974; Lack *et al.*, 1977) and with the possibility of an carrier mediated transport process present in the jejunum (Amelsberg *et al.*, 1996).

However, in ileal BBMV, the uptake of taurocholate in the presence of an inwardly directed K^+ gradient (12 pmol/mg prot/s) was elevated when compared to uptake in the presence of choline (3.4 pmol/mg prot/s). To ensure that taurocholate uptake in the presence of choline was representative of passive bile acid diffusion (as observed in D-glucose transport), taurocholate uptake was measured in the presence of 300 mM mannitol (representing the total removal of a cation gradient). The removal of all cations resulted in very similar rates of taurocholate transport when compared to taurocholate transport in the presence of choline (mannitol = 3.2 pmol/mg prot/s, choline = 3.4 pmol/mg prot/s.). Lack and colleagues (1977) had previously observed that taurocholate uptake in the presence of 100 mM mannitol gave transport rates of about 0.025 nmoles/mg protein which was taken to represent passive diffusion (Lack *et al.*, 1977). Likewise, in this study the similarly low levels of uptake rates in the presence of

choline and mannitol were taken to represent passive diffusion. This in turn gave rise to the notion that ileal taurocholate uptake observed in the presence of K^+, which was consistently 3-4 fold higher than choline, represented a transport process other than passive diffusion. Further work was required to determine if taurocholate uptake observed in the presence of K^+ was due to passive diffusion, a carrier mediated transport process, or a secondary active transport process.

9.2.2 Temperature dependence of taurocholate transport in the presence of different cations

A characteristic of active transport is its dependence on temperature. Previous *in vitro* work has shown, that at 25°C there is a 42.4% inhibition of cholate transport in rat ileum, when compared to transport at 37°C (Holt, 1964). At temperatures below the functional range of the Na^+/ bile acid co-transport protein only passive diffusion of bile acids are observed in BBMV (Kramer *et al.*, 1994). To determine if taurocholate uptake observed in the presence of Na^+ and K^+ were both due to a secondary active transport process, ileal BBMV were incubated at a range of temperatures and then assayed for taurocholate transport in the presence of inwardly directed Na^+, K^+ and choline gradients.

Table 9.1, Temperature dependence of taurocholate uptake into ileal BBMV in the presence of different cations

Cation	15°C	25°C	37°C
Na^+	2.22 ± 2.08	4.44 ± 1.37	37.5 ± 20.7
K^+	2.07 ± 1.85	3.38 ± 1.70	12.96 ± 2.9
Choline	1.51 ± 1.51	3.59 ± 2.56	3.78 ± 1.23

BBMV were isolated from pig ileum as described in the methods section. Ileal BBMV

were incubated at either 15° C, 25° C, or 37° C. Taurocholate uptake (pmol/mg prot/s) was measured for 10 seconds after the addition of 100 µl incubation media, which contained 100 µM ^{3}H taurocholate (37 Kbq), 100 mM mannitol, 20 mM HEPES/Tris pH 7.4 and 100 mM NaCl, KCl or choline chloride. Each point represents the mean ± S.D. for three assays (n=9).

Table 9.1 shows that taurocholate uptake in the presence of both Na^+ and K^+ was greatly reduced at 15°C and 25°C when compared to uptake at 37°C, while uptake in the presence of choline (passive diffusion) was not affected by temperature. The temperature sensitivity of bile acid transport in the presence of Na^+ has been demonstrated in previous studies, with the inference that such sensitivity is indicative of an active transport process (Holt, 1964; Kramer *et al.*,1994). Therefore, the observation that taurocholate uptake in the presence of K^+ was also temperature dependent gave an indication that secondary active transport of taurocholate was probably occurring in the presence of K^+.

9.2.3 Concentrative accumulation of taurocholate into ileal BBMV

Another functional characteristic of secondary active bile acid transport is the concentrative accumulation of bile acids. In intestinal enterocytes, the flow of Na^+ down its electrochemical gradient provides sufficient energy to enable the Na^+ /bile acid co-transport protein to transport conjugated bile acids against their concentration gradient.

In this study the concentrative accumulation of taurocholate was examined in 2 ways:-

1, The elimination of an inwardly directed taurocholate gradient by pre-loading ileal BBMV with 100 µM taurocholate. This ensured that the taurocholate

concentration was in equilibrium between the external medium and the intravesicular space of the BBMV.

2, The time course of taurocholate uptake into ileal BBMV over a time period of 9 hours.

Table 9.2, Effect of dispelling the taurocholate gradient on taurocholate transport

Cation	Uptake into unloaded BBMV (pmol/mg prot/s)	Uptake into taurocholate – loaded BBMV (pmol/mg prot/s)
Na^+	20.26 ± 3.11	17.08 ± 4.44
K^+	13.18 ± 2.96	7.69 ± 1.00
Choline	3.82 ± 0.104	2.45 ± 1.19

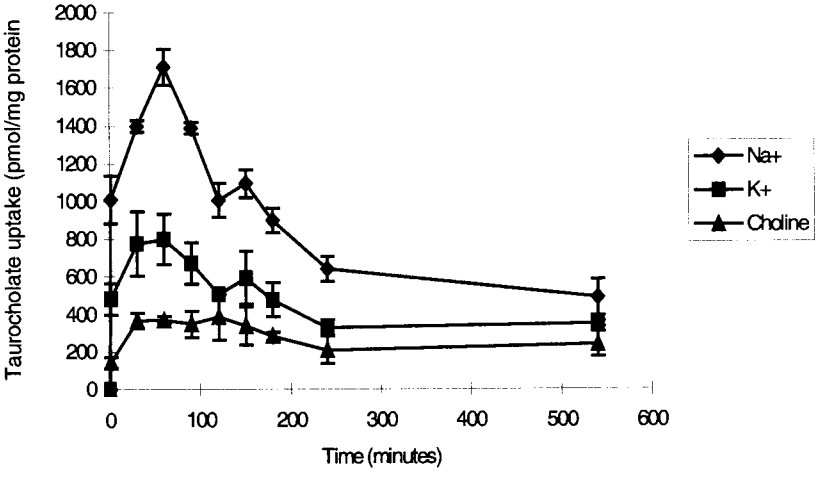
Unloaded ileal BBMV were prepared in an isotonic mannitol buffer as described in the methods section. Taurocholate-loaded BBMV were re-suspended at the last stage of the BBMV preparation in a buffer containing 300 mM mannitol, 20 mM HEPES/Tris pH 7.4 and 0.1 mM $MgSO_4$ with the addition of 100 µM taurocholate. Both sets of BBMV were then assayed for 10 seconds in 100 mM mannitol, 20 mM HEPES/Tris pH 7.4, 100 µM ^{3} H taurocholate (37 Kbq) and either 100 mM NaCl, KCl or choline chloride. Each point represents the mean ± S.D. for three assays (n=9). An anova test was carried out, but none of the data was found to be significant.

As shown in table 9.2, the removal of an inwardly directed taurocholate gradient by pre-loading BBMV, did not result in a significant decrease in taurocholate uptake in the presence of any of the three cations.

Taurocholate uptake in loaded BBMV produced similar uptake rates in the presence of Na^+ (17.08 pmol/mg prot/s) to those observed in non-loaded BBMV (Na^+ = 20.26 pmol/mg prot/s). There was reduced uptake of taurocholate in the presence of K^+ when loaded to non-loaded BBMV were compared (13.18 pmol/mg prot/s, compared to 7.69 pmol/mg prot/s), but in both cases the rate of

taurocholate uptake was still above passive diffusion levels (loaded = 3.82 pmol/mg prot/s compared to 2.45 pmol/mg prot/s for unloaded BBMV). The rates of taurocholate uptake into loaded and non-loaded BBMV in the presence of Na^+ and K^+ were both above the rates of passive diffusion. This indicated that the movement of both Na^+ and K^+ down their concentration gradients into taurocholate loaded BBMV brought about the concentrative co-transport of taurocholate. These findings provided more evidence that secondary active transport of taurocholate could occur in the presence of both Na^+ and K^+.

Figure 9.3 A, Time course of taurocholate uptake by pig ileal BBMV in the presence of different cations

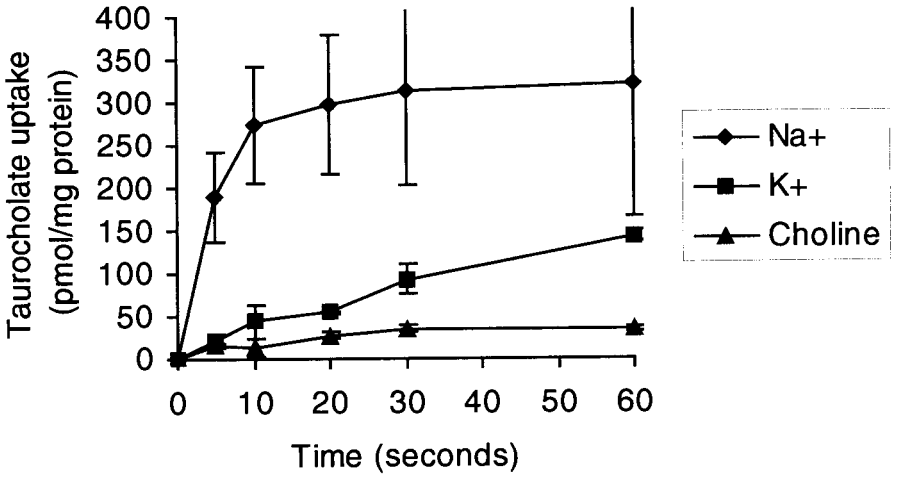


BBMV were prepared from pig ileum as described in the methods section. The uptake of taurocholate was measured in the presence of either 100 mM NaCl, KCl and choline chloride. The assay conditions were as described in the methods section. The uptake reaction was stopped after either 0, 5, 10, 20, 30 and 60 seconds, and after 30, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420, 480 and 540 minutes. Each point represents the mean +/- S.D for three assays (n=9).

As can be seen in figure 9.3 A, there was an "overshoot" of taurocholate uptake into pig ileal membrane vesicles in the presence of Na^+ and K^+ after 30-60

minutes, with the accumulation of taurocholate in the presence of Na^+ (1700 pmol/mg prot.) being 2 fold higher, at the peak of the overshoot, when compared to K^+ (800 pmol/mg prot.).

Figure 9.3B, Time course of taurocholate uptake by pig ileal brush border membrane vesicles in the presence of different cations, from 0-60 seconds



After reaching its peak at 60 minutes, the taurocholate concentration slowly began to fall within the BBMV, in the presence of both Na^+ or K^+. This reduction was probably due to a slow efflux of taurocholate out of BBMV, eventually reaching equilibrium with the external concentration of taurocholate. However, there is a possibility that this reduction of concentrative accumulation after one hour could have been due to the bi-directional transport of taurocholate out of the BBMV, as previously observed in the hamster ileal Na^+/bile acid co-transport protein (Craddock *et al.*, 1998). The reduced rate of taurocholate into the BBMV in the presence of choline was representative of passive diffusion.

The observation in this study of an overshoot in the presence of Na^+ corresponded to previous findings and is recognised as a characteristic of secondary active

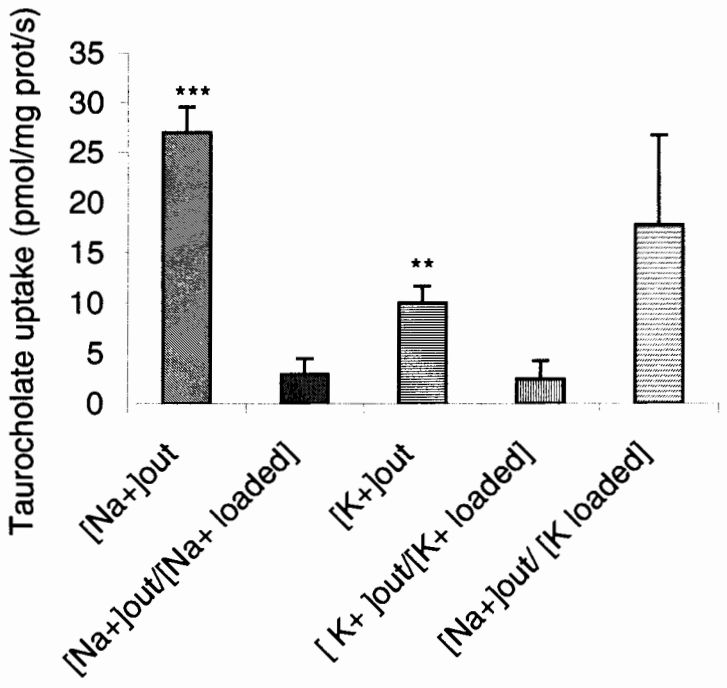
transport (Lucke *et al.*,1978; Rouse and Lack, 1979; Gong *et al.*, 1991; Kramer *et al.*,1992 ;1993). However, concentrative accumulation in the presence of K^+ has not previously been observed. The observation of concentrative accumulation in the presence of K^+ gave a further indication that the secondary active transport of bile acids was occurring in the presence of K^+.

9.2.4 The effect of eliminating the inwardly directed cation gradient

Na^+ is pumped out of intestinal enterocytes across the basolateral membrane by a Na^+-K^+ ATPase (Clausen, 1998). This energy requiring process ensures that there is always an inwardly directed Na^+ gradient, which in turn enables continuous active transport of bile acids by the intestinal co-transport protein.

In BBMV, an inwardly directed Na^+ gradient is artificially induced, but because the basolateral membrane is not present there is no active removal of Na^+ from the BBMV by Na^+-K^+ ATPase. Preloading BBMV with Na^+ will abolish the inwardly directed cation gradient, which in turn should stop active bile acid transport. Likewise, if the secondary active transport of taurocholate was coupled to an inwardly directed K^+ gradient, the removal of this gradient would result in only passive diffusion being observed. Therefore, ileal BBMV were preloaded with either 100 mM Na^+ or K^+, to equal the concentration of the respective cation outside of the brush border membrane (present in the uptake media) in order to abolish the inwardly directed cation gradient.

Figure 9.4, The effect of dispelling the cation gradient



KEY:
Out = presence of a cation in the uptake media, therefore outside of the BBMV
Loaded = presence of a cation inside the BBMV

*BBMV were isolated from pig ileum, as described in the methods section. For preloaded BBMV, 100 mM of the cation was added to 300 mM mannitol, 0.1mM $MgSO_4$, 20 mM HEPES/Tris pH 7.4, which the BBMV were resuspended in. The uptake of taurocholate was measured for 10 seconds in the presence of either 100 mM NaCl or KCl with the rest of the assay conditions as described in the methods section. Each point represents the mean $\pm$ S.D. for nine determinations. An anova statistical test was carried, comparing the difference in the rate of taurocholate was between unloaded and loaded BBMV in the presence of Na^+ or K^+, 99 % significance =**, 99.9 % significance = ***.*

The removal of a cation gradient had the effect of reducing the rates of taurocholate transport to levels of passive diffusion in the presence of both cations (figure 9.4). Uptake rates were reduced from 10.2 pmol/mg prot/s to 2.4 pmol/mg prot/s for K^+ and 27 pmol/mg prot./s to 2.9 pmol/mg prot/s for Na^+. These results

showed that inwardly directed Na^+ and K^+ gradients were essential for secondary active taurocholate transport, which again indicated that secondary active taurocholate transport was coupled to the flow of either Na^+ or K^+. Figure 9.4 also showed that loading the BBMV with K^+, which should have reduced the negative electrochemical potential inside the BBMV, had no significant effect on active taurocholate transport in the presence of Na^+. This showed that the concentration of other cations on the inside of the membrane was not a significant factor in affecting Na^+ dependent bile acid transport. This finding also gave an indication that taurocholate transport in the presence of Na^+ appeared to be an electronetural transport process because the reduction in electrochemical potential within the BBMV did not have a significant effect on secondary active transport.

9.3 Summary of results

From the results observed using pig ileal BBMV, it was concluded that the BBMV could be used to study the active transport bile acids. The transport characteristics of taurocholate uptake in the presence of Na^+ corresponded to previous findings using BBMV and isolated epithelial cells (Lack and Weiner, 1961; Holt, 1964; Mullins *et al*,. 1992; Kramer *et al*,. 1993), which indicated that the ileal BBMV contained the Na^+/ bile acid co-transport protein.

The observation that taurocholate transport in the presence of K^+ was elevated when compared to passive bile acid diffusion had not been discussed in previous investigations into secondary active bile acid transport. As in the presence of Na^+, taurocholate transport in the presence of K^+ was temperature sensititive and demonstrated concentrative accumulation. The functional characteristics of taurocholate transport in the presence of K^+ were very similar to those observed

with the secondary active transport process in the presence of Na^+. However, transport rates in the presence of K^+ were consistently 2-3 fold lower when compared to Na^+. From these results it was proposed that secondary active taurocholate transport occurred in the presence of both Na^+ and K^+.

CHAPTER TEN:

10. Characterisation of the ileal Na^+/bile acid co-transport protein in the presence of Na^+ and K^+

Having shown that secondary active bile acid transport could occur in the presence of both Na^+ and K^+, it was important to determine if it was the same transport protein that was involved in both secondary active transport processes. Characterisation of this process involved inhibition studies and Michealis Menton kinetics using ileal BBMV.

10.1 Competitive inhibition of ileal taurocholate uptake by taurodeoxycholate

Secondary active bile acid transport in the ileum has long been established as a multi-specific transport process that allows the absorption of bile acids, whether dihydroxylated or trihydroxylated (Krag and Philips, 1974; Schiff *et al.*, 1972; Wilson,1981). Studies using BBMV have revealed that the addition of another bile acid can lead to concentration-dependent inhibition of the bile acid being studied. This has been rationalised as competition between the bile acids for the Na^+ /bile co-transport protein, with dihydroxylated bile acids being the most potent competitive inhibitors (Burckhardt *et al.*,1983b; Kramer *et al.*, 1992; Mullins *et al.*, 1992).

In this study, the uptake of taurocholate into ileal BBMV was measured in the presence of either 100μM ^{3}H taurocholate on its own or 100 μM ^{3}H taurocholate and 1mM taurodeoxycholate. Taurocholate transport was measured in the presence of both Na^+ and K^+ in order to determine if transport in the presence of these two cations demonstrated similar patterns of inhibition.

Table 10.1, Inhibition of taurocholate uptake by taurodeoxycholate, in the presence of different cations

Cation	Taurocholate uptake (pmol/mg prot./s)	Taurocholate uptake in the presence of taurodeoxycholate (pmol/mg prot./s)	Percentage inhibition of taurocholate uptake by taurodeoxycholate
Na^+	30 ± 10	8.5 ± 2.7	71.7
K^+	11 ± 3.03	3.03 ± 0.88	72.5
Choline	5.6 ± 3.78	7.16 ± 2.21	N/A

BBMV were prepared from pig ileum as described in the methods section. The incubation media contained either NaCl, KCl or choline chloride, 100 mM mannitol, 20 mM HEPES/Tris, pH 7.4 and 100 µM ^{3}H taurocholate (37 Kbq). In addition, taurocholate uptake was also measured in the presence of 100 µM taurocholate and 1 mM taurodeoxycholate. Each point represents the mean ± S.D. for three assays (n=9).

Table 10.1 shows that taurocholate uptake was inhibited to a similar extent (between 71-73%) in the presence of taurodeoxycholate, irrespective of whether Na^+ or K^+ was the driving cation. In the presence of choline, taurocholate uptake was not significantly inhibited because both bile acids were diffusing into the vesicles and not utilising the bile acid transport protein.

The levels of inhibition in this study were consistent with those previously observed for Na^+-dependent taurocholate uptake displayed by the Na^+/bile acid co-transport protein (Wilson and Treanor, 1975; Burckhardt *et al.*,1983b; Mullins *et al.*, 1992; Kramer *et al.*,1992; Wong *et al.*,1994). This demonstrated that Na^+ dependent bile

acid transport observed in this study was a result of the Na^+/bile acid co-transport protein.

The observation that K^+ dependent taurocholate uptake exhibited similar inhibition profiles to Na^+ dependent taurocholate uptake, which had not been discussed in previous studies, gave an indication that the same intestinal bile acid co-transport protein was probably employing both cations for active transport.

10.2 Kinetic analysis of taurocholate transport

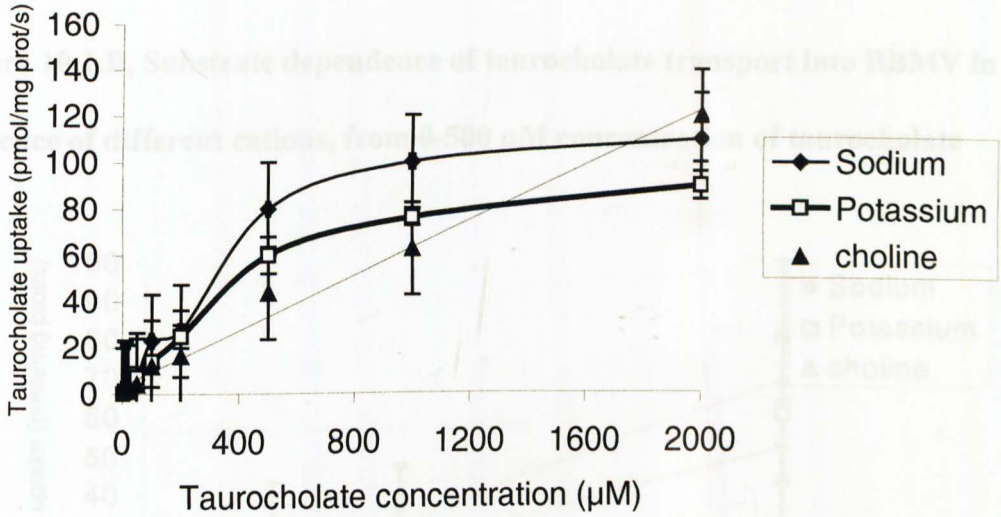
As a method of further characterising whether the same bile acid transport protein was able to utilise both cations, the substrate and cation dependency of the transport process was investigated.

Secondary active bile acid transport in the ileum has been shown to exhibit Michaelis-Menton kinetics, which is a further characteristic of carrier mediated transport (Wilson and Dietschy, 1974; Wilson and Treanor, 1979; Marcus *et al.*, 1991).

10.2.1 Substrate dependence of taurocholate transport in the presence of Na^+, K^+ and choline

In this study, analysis of the substrate dependence of taurocholate uptake by ileal BBMV was undertaken by varying the concentration of taurocholate over the range of 10 – 2000 μM.

Figure 10.1.A, Substrate dependence of taurocholate transport into BBMV in the presence of different cations

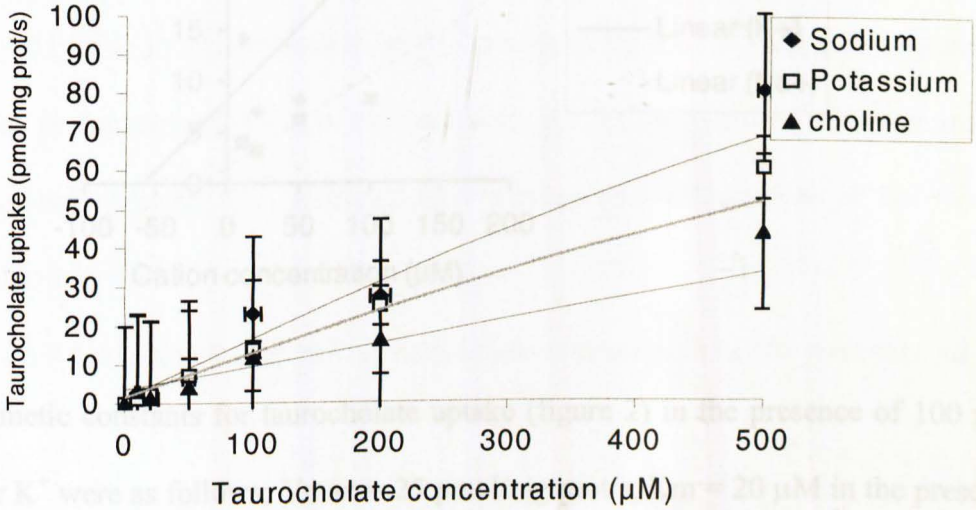


BBMV were prepared from pig ileum. Uptake assays were carried out in the presence of Na^+, K^+ and choline as described in the methods section. The incubation medium contained either 100 mM NaCl, KCl or choline chloride, 100 mM mannitol, and 20 mM HEPES/Tris, pH 7.4 in addition to ^3H taurocholate (37 Kbq) at 10, 20, 50, 100 , 200 500, 1000 and 2000 μ M. The initial uptake rates were measured after 10 seconds. The data is presented as v vs [S] relationships (Fig. 1A &B) and as Hanes-Woolf plots ([S]/v vs [S]), though the data is only shown to 200 μM (Fig. 2). Each point represents the mean ± S.D. for three assays (n=9).

As shown in Figure 10.1A & B, the lower taurocholate concentrations (10-500 μM) resulted in a rapid linear increase of taurocholate transport in the presence of both Na^+ and K^+, reaching a peak at 500 μM. At concentrations above 500 μM, there was very little further increase in the rate of taurocholate uptake, which is representive of a saturable transport process, as shown in previous studies (Mullins *et al.*,1992; Kramer *et al.*,1992). From this data it can be concluded that above 500 μM the Na^+/bile acid

co-transport protein has reached its saturation point and is not able to actively transport bile acids at a faster rate, even if the concentration of bile acid molecules is increased.

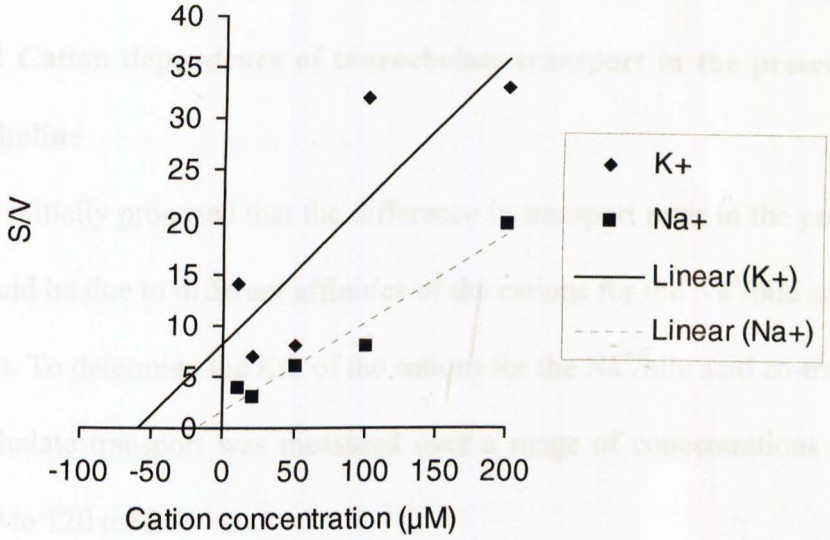
Figure 10.1.B, Substrate dependence of taurocholate transport into BBMV in the presence of different cations, from 0-500 µM concentration of taurocholate



In this study, taurocholate uptake in the presence of choline was not saturable (figure, 10.1A), rising linearly with the concentration of taurocholate. This was indicative of passive diffusion of bile acids and made the calculation of Michaelis-Menten kinetic constants impossible.

The uptake rates due to passive diffusion were subtracted from the combined uptake due to passive and active transport processes in order to obtain results for Na^+ and K^+ dependent transport processes. These results were plotted as a Hanes-Woolf plot (figure, 10.2) and the kinetic constants calculated.

Figure 10.2, Hanes-Woolf plot of taurocholate transport in ileal BBMV in the presence of different cations



The kinetic constants for taurocholate uptake (figure 2) in the presence of 100 mM Na^+ or K^+ were as follows; Vmax = 25 pmol/mg prot/s, Km = 20 μM in the presence of Na^+,Vmax = 8.5 pmol/mg prot/s, Km = 60 μM in the presence of K^+. These results showed that taurocholate transport in the presence of Na^+ and K^+ did not occur with greatly different transport affinities, and the Km values observed in this study were similar to those previously observed for Na^+ (Kramer *et al.*, 1992; Mullins *et al.*, 1992).

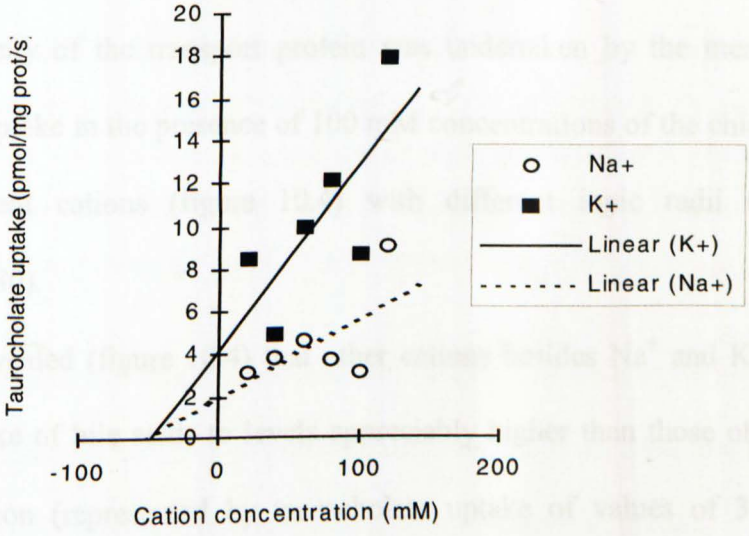
Furthermore, in this study there was a close relationship observed between maximal velocities and affinities. The Vmax for taurocholate uptake in the presence of Na^+ was approximately 3 times higher than in the presence of K^+, and the Km for taurocholate transport in the presence of Na^+ was one third that in the presence of K^+.

This close association between transport velocity and affinity in the presence of the two cations strongly suggested that Na^+-dependent and K^+-dependent bile acid transport was mediated by the same transport system.

10.2.2 Cation dependence of taurocholate transport in the presence of Na^+, K^+ and choline

It was initially proposed that the difference in transport rates in the presence Na^+ and K^+ could be due to different affinities of the cations for the Na^+/bile acid co-transport protein. To determine the Km of the cations for the Na^+/bile acid co-transport protein, taurocholate transport was measured over a range of concentrations of Na^+ or K^+ from 0 to 120 mM.

Figure 10.3, Hanes Woolf plot of taurocholate transport in the presence of Na^+ and K^+



BBMV were isolated from the ileum as described in the methods section. Uptake assays were carried out in the presence of Na^+ and K^+ as described in the methods section. Taurocholate uptake was assayed in the presence of either Na^+ or K^+ at concentrations of 0, 20, 40, 60, 80,

100 and 120mM. The results are presented with passive diffusion already subtracted. Each point represents the mean ± S.D. for three assays(n=9).

The Hanes-Woolf plot (figure 10.3) revealed a Km for Na^+ dependent and K^+ dependent taurocholate uptake of approximately 55 mM for both cations. The Vmax was again higher in the presence of Na^+ (27.5 pmol/mg prot/s), than for K^+ (12.7 pmol/mg prot/s).

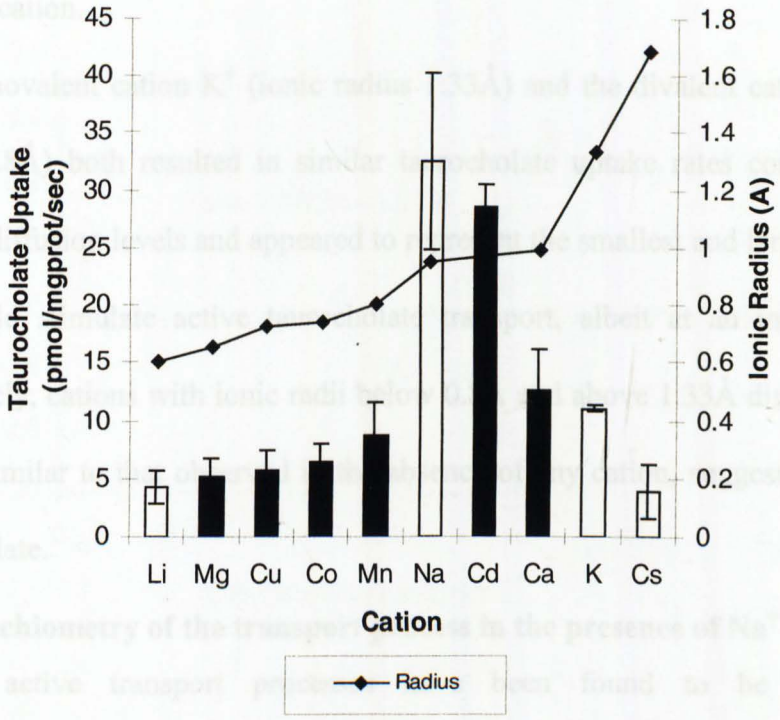
These findings showed that both Na^+ and K^+ had the same Km values, so the affinity of the transport process for each cation was the same.

10.3 Taurocholate uptake in the presence of a range of cations

The observation that the Na^+/bile acid co-transport protein was able to utilise both Na^+ and K^+, led to investigation of whether the transport protein could use other cations to actively transport bile acids. Further investigation of the more general cation specificity of the transport protein was undertaken by the measurement of taurocholate uptake in the presence of 100 mM concentrations of the chloride salts of several different cations (figure 10.4) with different ionic radii (Cotton and Wilkinson, 1980).

The results revealed (figure 10.4) that other cations besides Na^+ and K^+ could also stimulate uptake of bile acids to levels appreciably higher than those observed with passive diffusion (represented by taurocholate uptake of values of 3-6 pmol/mg prot/s).

Figure 10.4, Rate of taurocholate transport in ileal brush border membrane vesicles in the presence of a range of cations of varying ionic radius



BBMV were prepared from pig ileum as described in the methods section. Taurocholate uptake was measured in the presence of the uptake buffer described in the methods section, with all cations at 100 mM concentration. Initial rates of taurocholate uptake were in the presence of monovalent cations (indicated in white) and divalent cations (indicated in black). Each point represents the mean +/- S.D for three assays (n=9).

It appeared that both monovalent and divalent cations could cause enhanced levels of taurocholate uptake above those of passive diffusion. This indicated that the charge of the cation was only important in relation to it being positively charged.

In addition, all the cations that were able to stimulate taurocholate transport above passive diffusion possessed ionic radii within a discrete range from 0.8 – 1.33Å. The highest transport rates were observed with cations that have an ionic radius of around

1Å, namely Na^+, Cd^{2+} and Ca^{2+}. The most notable finding from a physiological perspective was that taurocholate uptake was stimulated in the presence of calcium, a divalent cation.

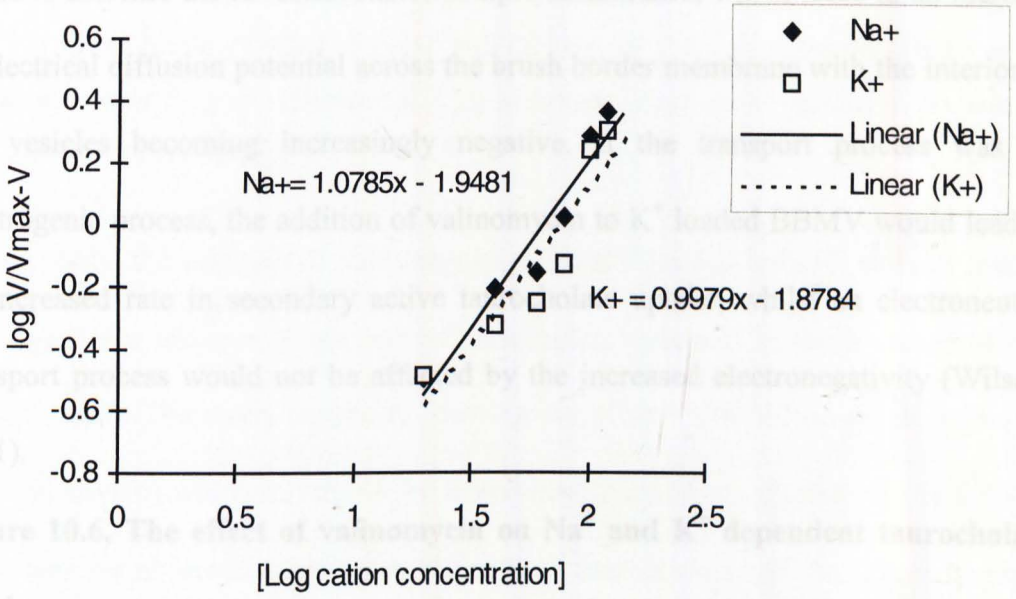
The monovalent cation K^+ (ionic radius 1.33Å) and the divalent cation Mn^{2+} (ionic radius 0.8Å) both resulted in similar taurocholate uptake rates consistently above passive diffusion levels and appeared to represent the smallest and largest ionic radius that could stimulate active taurocholate transport, albeit at an intermediate rate. Conversely, cations with ionic radii below 0.8Å and above 1.33Å displayed levels of uptake similar to that observed in the absence of any cation, suggesting diffusion of taurocholate.

10.4 Stoichiometry of the transport process in the presence of Na^+ or K^+

Certain active transport processes have been found to be dependent on electronegativity within the cell, such as Na^+ dependent D-glucose transport which is an electrogenic process (Kauntiz *et al.*, 1982; Umbach *et al.*, 1990; Mullins *et al.*,1992). However, for bile acid transport there have been conflicting observations as to whether bile acid transport is electrogenic (Lucke *et al.*, 1978; Craddock *et al.*, 1998, Weinman, 1998) or electroneutral (Rouse and Lack, 1979; Barnard and Ghishan, 1987; Mullins *et al.*, 1992).

In this study, the stoichiometry of Na^+ and K^+ dependent taurocholate transport was investigated by a Hill transformation plot, uptake studies involving valinomycin and the substitution of different anions.

Figure 10.5, Hill Plot of taurocholate transport in ileal brush border membrane vesicles in the presence of different concentrations of Na^+ or K^+

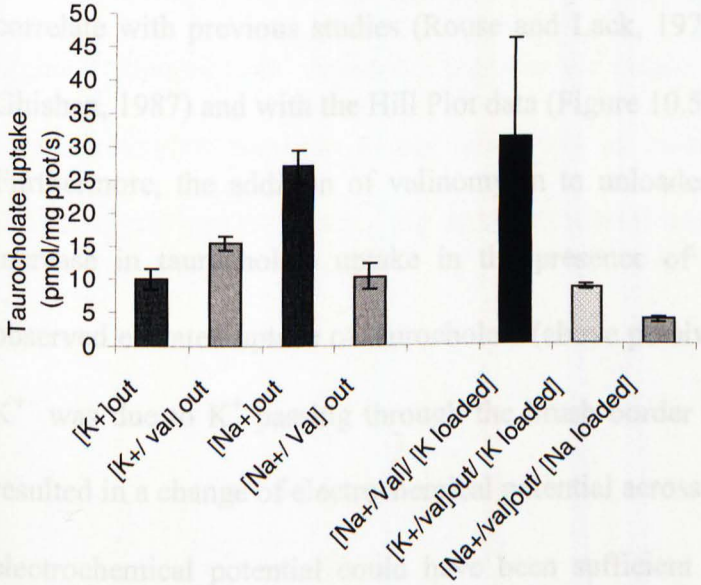


BBMV were isolated from the ileum as described in the methods section. Uptake assays were carried out in the presence of Na^+ and K^+ as described in the methods section. Taurocholate uptake was assayed in the presence of either Na^+ or K^+ at concentration of 120, 100, 80, 60, 40, 20 and 0mM. The results are presented with passive diffusion already subtracted. Each point represents the mean $\pm$ S.D. for three assays(n=9).

The Hill transformation plot (figure, 10.5) revealed a slope of 1.08 for Na^+ compared to 1.00 for K^+, which strongly indicated a 1:1 cation/taurocholate coupling ratio for both Na^+ and K^+. Therefore, from the Hill plot it appeared that the movement of one Na^+ or K^+ ion down its concentration gradient was sufficient to allow the transport of one taurocholate molecule, which indicated an electroneutral process.

The stoichiometry of the pig intestinal bile acid transport process was confirmed by the addition of valinomycin (an ionophore specific to K^+). Valinomycin has been found to increase the K^+ conductance of lipid membranes, which leads to an increase in electrical diffusion potential across the brush border membrane with the interior of the vesicles becoming increasingly negative. If the transport process was an electrogenic process, the addition of valinomycin to K^+ loaded BBMV would lead to an increased rate in secondary active taurocholate uptake, whilst an electroneutral transport process would not be affected by the increased electronegativity (Wilson, 1981).

Figure 10.6, The effect of valinomycin on Na^+ and K^+ dependent taurocholate uptake



KEY =
Out = Presence of a cation or valinomycin in the uptake media, on the outside of the BBMV
Loaded = Presence of a cation or valinomycin in the interior of the BBMV, therefore the BBMV were preloaded

BBMV were isolated from pig ileum, as described in the methods section. For the preloaded BBMV, 100 mM of the cation was added to 300 mM mannitol, 0.1 mM $MgSO_4$, 20 mM HEPES/Tris pH 7.4 and the BBMV were resuspended within the fore mentioned buffer. Valinomycin was added to the uptake buffer (100 mM mannitol, 100 mM of Na^+ or K^+, 100 μM ^{3} H taurocholate (37Kbq), 20 mM HEPES/Tris pH 7.4) or preloaded BBMV at the concentration of 1 μg of valinomycin/ 1 μg of protein. Each point represents the mean ± S.D. for nine determinations.

In this study, the addition of valinomycin to BBMV loaded with K^+ did not lead to any significant increase in the rate of taurocholate transport in the presence of Na^+ (figure, 10.6). Therefore, despite K^+ passing out of the BBMV (via the pore created by valinomycin) which increased the electronegativity of the interior of the BBMV, there was no observed increase in the rate of taurocholate uptake. These findings supported an electroneutral mechanism for taurocholate transport in pig ileum and correlate with previous studies (Rouse and Lack, 1979; Wilson, 1981; Barnard and Ghishan, 1987) and with the Hill Plot data (Figure 10.5).

Furthermore, the addition of valinomycin to unloaded BBMV did not result in an increase in taurocholate uptake in the presence of K^+. It was possible that the observed elevated uptake of taurocholate (above passive diffusion) in the presence of K^+ was due to K^+ passing through the brush border membrane K^+ channel, which resulted in a change of electrochemical potential across the membrane. This change in electrochemical potential could have been sufficient to induce elevated uptake of taurocholate. However, figure 10.5 showed that an increase in membrane potential did not stimulate taurocholate uptake in the presence of K^+ to any great degree. This

indicated that it was the process of K^+ passing through the ileal Na^+/ bile acid co-transport protein that stimulated secondary active transport of taurocholate.

To confirm that bile acid transport was an electroneutral process, taurocholate transport was investigated in the presence of a range of anions. If the bile acid transport mechanism was an electrogenic process, the presence of more permeable anions would have led to an increase in Na^+ dependent taurocholate uptake (Mullins, 1992).

Table 10.2 (below), showed that altering the anion coupled to the cation did not result in any significant difference in taurocholate uptake in the presence of either Na^+ or K^+. The ratio of the rate of Na^+ to K^+ dependent taurocholate uptake, in the presence of the different anions, was on average 1.8 which corresponded to previous findings in this study, that the rate of Na^+ dependent taurocholate uptake was 2-3 fold higher compared to K^+ dependent taurocholate uptake. Therefore this data confirmed that taurocholate transport in pig ileum was an electroneutral transport process as shown in previous work using pig small intestine (Mullins *et al.*, 1992).

Table 10.2, The effect of different anions on secondary active taurocholate transport

Anion	Na^+ (pmol/mgprot/s)	K^+ (pmol/mgprot/s)	Ratio of the rate of uptake between Na^+ and K^+
Chloride	29 ± 0.5	12.5 ± 1.2	2.32
Glutamate	21 ± 1.2	13.3 ± 5.6	1.57
Thiocyanate	19 ± 6.9	16.9 ± 7.9	1.12
Nitrate	23.5 ± 6.4	12.8 ± 5.3	1.83
Isethionate	21.9 ± 6.9	12 ± 6.6	1.83

BBMV were isolated from pig ileum as described in the methods section. Taurocholate uptake was measured in the presence of 100 μM ^3H taurocholate (37 Kbq), 100 mM mannitol, 20 mM HEPES/Tris pH 7.6 and 100 mM of the respective cation salt. Uptake was measured over 10 seconds at 37°C and the reaction was stopped with the addition of 1 ml ice cold solution. Each point represents the mean ± S.D. for nine determinations.

10.5 Bi-directional transport of taurocholate

Computer modelling of the ileal Na^+/bile acid co-transport protein has revealed that the potential cation and bile acid binding sites present on the transport protein occur on both the intracellular and extracellular sides of the transport protein (Antoniw and Mullins, unpublished data). Therefore, it was proposed that the pig ileal Na^+/bile acid co-transport protein might be able to transport bile acids into and out of the cell depending on the direction of the cation gradient.

Table's 10.3-10.5, Bi-directional transport of taurocholate in the presence of either Na^+, K^+ or choline

Na^+	Before the addition of the uptake media	After addition of Na^+ to the outside of the BBMV	Amount of ^3H TC transported out of the BBMV (pmol/mg prot/s)	After addition of mannitol to the outside of BBMV	Amount of ^3H TC transported out of the BBMV (pmol/mg prot/s)
Amount of ^3H TC left inside the pre-loaded BBMV (pmol/mg prot)	152 ± 20	127 ± 20	2.5	96 ± 10	5.6
Amount of ^3H TC left inside the transported BBMV (pmol/mg prot)	59 ± 15	47 ± 15	1.2	25 ± 12	3.4

K^+	Before the addition of the uptake media	After addition of Na^+ to the outside of the BBMV	Amount of ^3H TC transported out of the BBMV (pmol/mg prot/s)	After addition of mannitol to the outside of BBMV	Amount of ^3H TC transported out of the BBMV (pmol/mg prot/s)
Amount of ^3H TC left inside the pre-loaded BBMV (pmol/mg prot)	61 ± 18	47 ± 18	1.4	32 ± 6	2.9
Amount of ^3H TC left inside the transported BBMV (pmol/mg prot)	22 ± 3	18 ± 3	0.4	5 ± 0.4	1.8

Choline	Before the addition of the uptake media	After addition of Na^+ to the outside of the BBMV	Amount of ^3H TC transported out of the BBMV (pmol/mg prot/s)	After addition of mannitol to the outside of BBMV	Amount of ^3H TC transported out of the BBMV (pmol/mg prot/s)
Amount of ^3H TC left inside the pre-loaded BBMV (pmol/mg prot)	48 ± 12	45 ± 12	0.3	40 ± 10	0.8
Amount of ^3H TC left inside the transported BBMV (pmol/mg prot)	15 ± 0.5	8 ± 0.5	0.7	10 ± 1.2	0.5

For the 'pre-loaded BBMV', the BBMV were preloaded with either 100 mM NaCl, KCl or choline chloride and 100 μM ^{3}H taurocholate (37 Kbq), 100 mM mannitol, 0.1 mM $MgSO_4$, 20 mM HEPES/Tris pH 7.4. The BBMV were preloaded by resuspension of the freshly isolated brush border membrane pellet in the respective cation solution. After resuspension, the brush border membranes were snap frozen in liquid nitrogen to produce BBMV which contained 100 mM of the respective cation and 100 μM of ^{3}H taurocholate.

For the 'transported BBMV', the BBMV were placed at 37°C in 20μl of 100 μM ^{3}H taurocholate (37 Kbq), 100 mM mannitol, 20 mM HEPES/Tris pH 7.4 in the presence of either 100 mM NaCl, KCl or choline chloride. The reaction was allowed to proceed for 1 hour to allow the cations and taurocholate to be transported into the BBMV.

To both sets of BBMV, 100 μl of uptake buffer was added which contained either 100 mM of mannitol, which resulted in an outwardly directed cation gradient or 100 mM of the same cation present inside the BBMV , which resulted in no cation gradient. The uptake buffer also contained 100 μm of non radioactive taurocholate, to limit the passive diffusion of taurocholate out of the BBMV.

The reaction was allowed to proceed for 10 seconds, after which the reaction was stopped by the addition of 1ml of ice cold solution. The experiment then continued in the standard procedure as described in the methods section 7.2.1. Each point represents the mean ± S.D. for nine determinations.

Table's 10.3-5 showed, that in the presence of all three cations there was some transport of taurocholate out of the BBMV, when compared to the amount of ^{3}H taurocholate initially present in the BBMV before the addition of the uptake media.

In the absence of an outwardly directed cation gradient there was only a low rate of taurocholate transport out of the BBMV, which was most likely to represent passive diffusion of taurocholate. In the presence of an outwardly directed cation gradient, there was a greater rate of taurocholate transport out of the BBMV but only in the

presence of either a Na^+ or K^+ gradient. This observation indicated that the presence of an outwardly directed Na^+ or K^+ gradient was essential for the bi-directional transport of taurocholate. However, the rate of taurocholate transport out of the BBMV was greatly reduced when compared to taurocholate transport into the BBMV, which is normally around 25 pmol/mg prot/s for Na^+ and 13 pmol/mg prot/s for K^+.

The observation of bi-directional transport of bile acids in the presence of Na^+ corresponds to previous findings (Craddock *et al.*, 1998), though the use of K^+ for bi-directional taurocholate transport has not been observed before.

10.6 Summary

From the findings of this study, it can be proposed that secondary active intestinal bile acid transport in the presence of Na^+ and K^+ occurs by the same intestinal bile acid co-transport protein.

Values obtained from the inhibition and kinetics data were similar to previous observations of the Na^+/bile acid co-transport protein, with the exception of the consistent observation of stimulated transport in the presence of K^+. This finding indicated that the ileal Na^+/bile acid co-transport protein appeared to be able to utilise K^+ in secondary active transport of bile acids.

However, K^+ dependent transport was 2-3 fold slower when compared to Na^+. This reduced rate in the presence of K^+ appeared not to be due to the affinity of the cations for the transport protein or a difference in the stoichiometry of the transport process. Transport rates in the presence of a range of cations appeared to be dependent on the size of the cation being transported. Cations that had a radius of 1Å stimulated

maximal rate of secondary active bile acid uptake, though cations which had radii between 0.8 – 1.33Å still stimulated taurocholate uptake but to a lesser degree.

From these transport studies, it was concluded that rather than being a strictly Na^+ dependent process, the pig ileal Na^+/bile acid co-transport merely displays a strong preference for Na^+ and can also utilise other cations (such as K^+) as a driving force. Furthermore, the ileal Na^+/bile acid co-transport protein can transport taurocholate into and out of the BBMV, in a bi-directional manner.

CHAPTER ELEVEN:

11. The expression of the Na^+/bile acid co-transport protein in *Xenopus laevis* oocytes

In this study, *Xenopus laevis* oocytes were used to functionally characterise the mRNA that codes for the Na^+/bile acid co-transport protein. The oocytes were used as a protein expression system in order to investigate the functional characteristics of the Na^+/bile acid co-transport protein. Previous studies have used the oocyte expression system to study intestinal D-glucose transport (Wright *et al.*,1992), a renal ascorbic acid transporter (Dyer *et al.*, 1994), intestinal bile acid transport (Mullins *et al.*,1992; Sorscher *et al.*,1992) and a Na^+/H^+ exchanger (Busch *et al.*,1995).

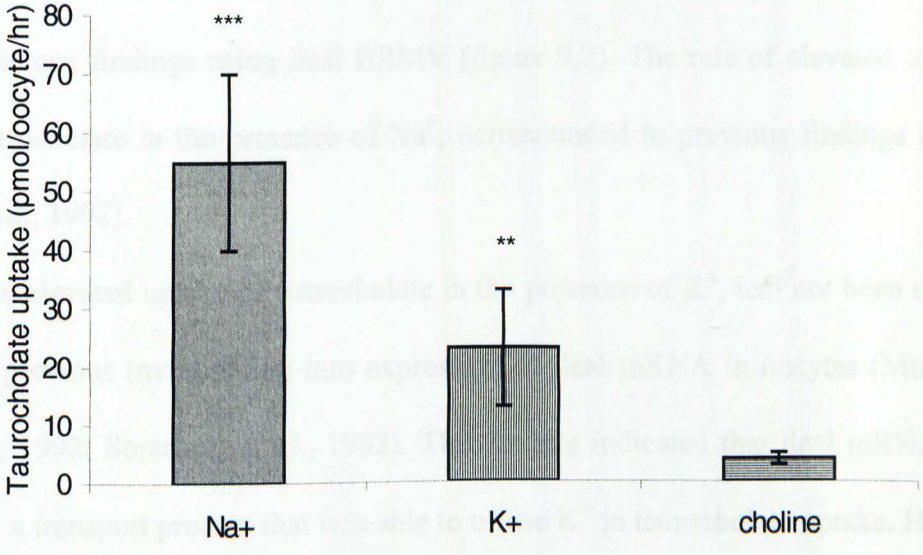
11.1 Isolation of RNA and mRNA from pig small intestine

In order to investigate the functional characteristics of the Na^+/bile acid co-transport protein by expression in *Xenopus laevis* oocytes, undegraded mRNA needed to be isolated from the rest of the RNA, DNA and proteins present in intestinal enterocytes. The RNA was isolated using phenol/chloroform extraction (as described in section 7.2.3.2 of the methods chapter) and mRNA was then isolated from the total RNA by oligo(dT) cellulose (as described in section 7.2.3.3 of the methods chapter). Electrophoresis of the isolated RNA and mRNA determined whether the RNA and mRNA had been isolated from the pig intestine in an intact form.

11.2 Expression of the ileal Na^+/bile acid co-transport protein in *Xenopus laevis* oocytes

Ileal mRNA was injected into *Xenopus laevis* oocytes, to determine if the translation of ileal mRNA resulted in the elevated uptake of taurocholate in the presence of Na^+ and K^+. If elevated uptake of taurocholate, in the presence of Na and K^+, occurred in oocytes injected with ileal mRNA, this would show that the ability of a transport process that utilised both cations was encoded by ileal mRNA.

Figure 11.1 Taurocholate transport into *Xenopus laevis* oocytes injected with ileal mRNA



To each Xenopus laevis oocyte, 40-50 ng of ileal mRNA (1 µg/µl) was injected, with 46 nl of water injected into oocytes to act as a control. The oocytes were maintained for 4-5 days in a modified Barths solutions (89 mM NaCl, 2.4 mM $NaHCO_3$,15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$,0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) as described in the methods section. After a 5 day incubation the oocytes were incubated at 30° C for 1 hour in a choline chloride based Barths solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6) in order to remove any Na^+ or K^+ inside the oocytes. Upon removal

of the choline chloride Barths solution, the corresponding modified Barths solutions containing either Na^+ (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$), K^+ (89 mM KCl, 2.4 mM $KHCO_3$, 15 mM HEPES/1M KOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$), or choline (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6) along with 100 μM ^3H taurocholate, (37 Kbq) was added to each group of oocytes.
*For each group, 20 mRNA injected and 10 water injected oocytes were incubated for 1 hour in the corresponding Barths solution and then the reaction stopped by the addition of ice cold stop solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6, 1 mM taurocholate). An anova statistical test was carried, with the rate of taurocholate uptake in the presence of Na^+ and K^+ compared to the rate of taurocholate transport in the presence of choline. 99% significance = **, 99.9% significance = ****

As can be seen in figure 11.1, there was elevated uptake of taurocholate in the presence of Na^+ and K^+, when compared to choline, which correlated to the previous findings using ileal BBMV (figure 9.2). The rate of elevated uptake of taurocholate in the presence of Na^+, corresponded to previous findings (Mullins *et al.*, 1992).

The elevated uptake of taurocholate in the presence of K^+, had not been observed in previous investigation into expression of ileal mRNA in oocytes (Mullins *et al.*, 1992; Sorscher *et al.*, 1992). This finding indicated that ileal mRNA coded for a transport process that was able to utilise K^+ in taurocholate uptake. However, the observation of elevated uptake of taurocholate, in the presence of Na^+ and K^+, did not indicate whether one or more than one transport process was being expressed.

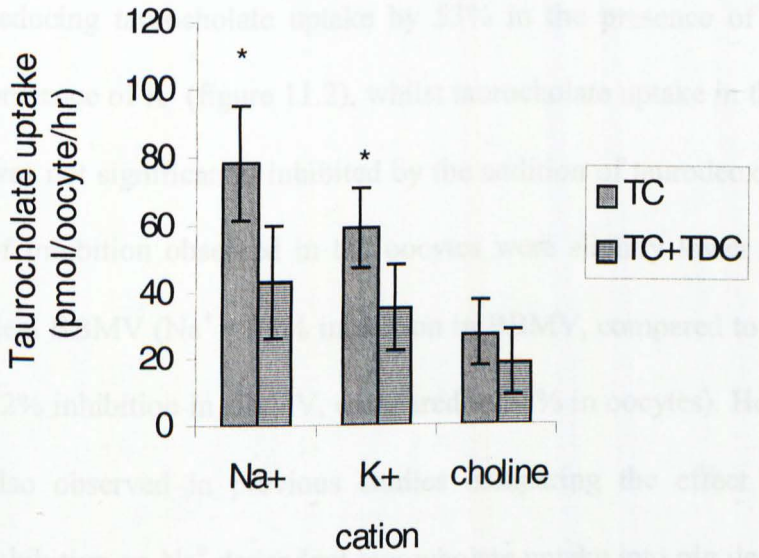
11.3 Further characterisation of taurocholate transport by *Xenopus laevis* oocytes

To determine if more than one bile acid transport process was translated from the ileal mRNA, further investigation of the functional characteristics of taurocholate transport in the presence of Na^+ and K^+ was required.

11.3.1 Inhibition of taurocholate transport by taurodeoxycholate

In this study, the uptake of taurocholate into oocytes was measured in the presence of either 100 μM ^3H taurocholate, or 100 μM ^3H taurocholate and 1 mM taurodeoxycholate. Taurocholate transport was measured in the presence of either Na^+, K^+ or choline, in order to determine if taurocholate transport in the attendance of Na^+ or K^+ demonstrated similar patterns of inhibition observed in the BBMV work (chapter 10).

Figure 11.2, Inhibition of taurocholate uptake by taurodeoxycholate



40-50 ng of ileal mRNA (1 μg/μl) was injected into each oocyte, with 46 nl of water injected into control oocytes. The oocytes were maintained for 4-5 days in a modified

*Barths solutions (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) as described in the methods section. After 5 days the oocytes were incubated at 30° C for 1 hour in a choline chloride based Barths solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6) in order to remove any Na^+ or K^+ within the oocytes. Upon removal of the choline chloride Barths solution, the corresponding modified Barths solutions containing either Na^+ (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$), K^+ (89 mM KCl, 2.4 mM $KHCO_3$, 15 mM HEPES/1M KOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) or choline (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6) was added to each group of 20 mRNA injected oocytes and 10 water injected oocytes. Oocytes were then assayed in the presence of either 100 µM ^3H taurocholate (37 Kbq) or 100 µM ^3H taurocholate (37 Kbq) and 1 mM taurodeoxycholate for 1 hour, after which the reaction was stopped by the addition of ice cold stop solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6, 1 mM taurocholate). An anova test was carried, comparing the rate of taurocholate inhibition by the addition of taurodeoxycholate, in the presence of either Na^+,K^+ or choline, 95% signifiance = *.*

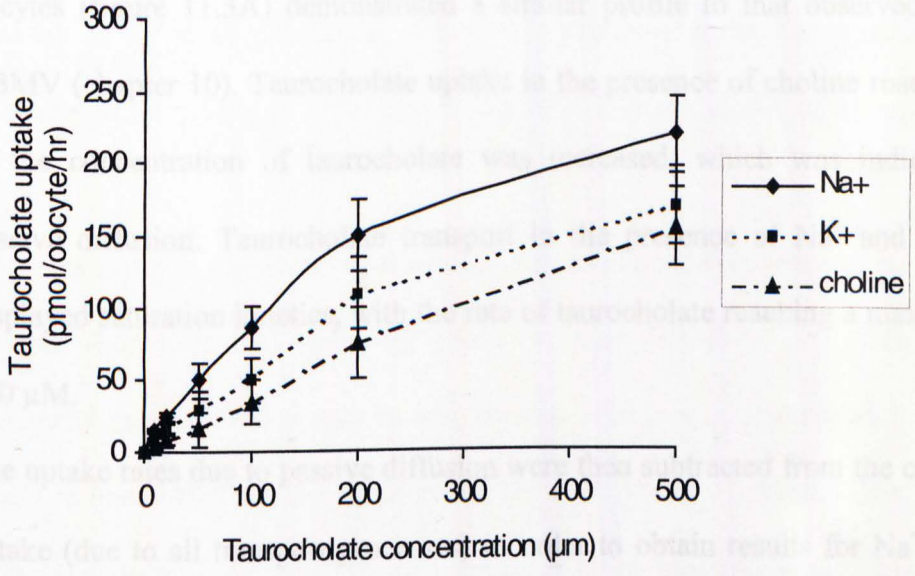
The addition of another bile acid, in this case taurodeoxycholate, had the effect of reducing taurocholate uptake by 53% in the presence of Na^+ and 58% in the presence of K^+ (figure 11.2), whilst taurocholate uptake in the presence of choline was not significantly inhibited by the addition of taurodeoxycholate. These levels of inhibition observed in the oocytes were slightly lower than that observed in ileal BBMV (Na^+ = 71% inhibition in BBMV, compared to 53% in oocytes, K^+ = 72% inhibition in BBMV, compared to 58% in oocytes). However, this trend was also observed in previous studies comparing the effect of taurodeoxycholate inhibition on Na^+ dependent taurocholate uptake into pig ileal BBMV and oocytes injected with pig ileal mRNA (Mullins,1992).

The similar levels of taurocholate inhibition observed in the presence of both Na^+ and K^+ supported the indications of the work in BBMV, that the same transport process appeared to be able to utilise both Na^+ and K^+ for active bile acid transport.

11.3.2 Kinetic analysis of taurocholate transport.

The previous chapter of results (chapter 10) reporting substrate kinetics of the Na^+/bile acid co-transport protein in ileal BBMV, showed that at taurocholate concentrations above 500 µM, the co-transport protein had reached saturation point. Therefore, in the oocyte protein expression experiments, analysis of the substrate dependence of taurocholate uptake by ileal mRNA injected oocytes was undertaken by varying the concentration of taurocholate over a range of 10 – 500 µM.

Figure 11.3A, Substrate dependence of taurocholate transport in the presence of Na^+, K^+ and choline.



40-50 ng of ileal mRNA (1 µg/µl) was injected into each of 400 oocytes, with 46 nl of

water injected into a further 210 oocytes. The oocytes were maintained for 4-5 days in a modified Barths solutions (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) as described in the methods section. After 5 days the oocytes were incubated at 30° C for 1 hour in a choline chloride based Barths solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6) in order to remove any Na^+ or K^+ inside the oocytes. Upon removal of the choline chloride Barths solution, the corresponding modified Barths solutions containing either Na^+ (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$), K^+ (89 mM KCl, 2.4 mM $KHCO_3$, 15 mM HEPES/1M KOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) or choline (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6) was added to each group of oocytes. Furthermore, there was the addition of ^3H taurocholate at concentrations of 10, 20, 50, 100 , 200 or 500 μM. For each concentration of taurocholate, 20 mRNA injected and 10 water injected oocytes were incubated for 1 hour in the presence of Na^+, K^+ or choline. The reaction was stopped by the addition of ice cold stop solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6, 1 mM taurocholate)

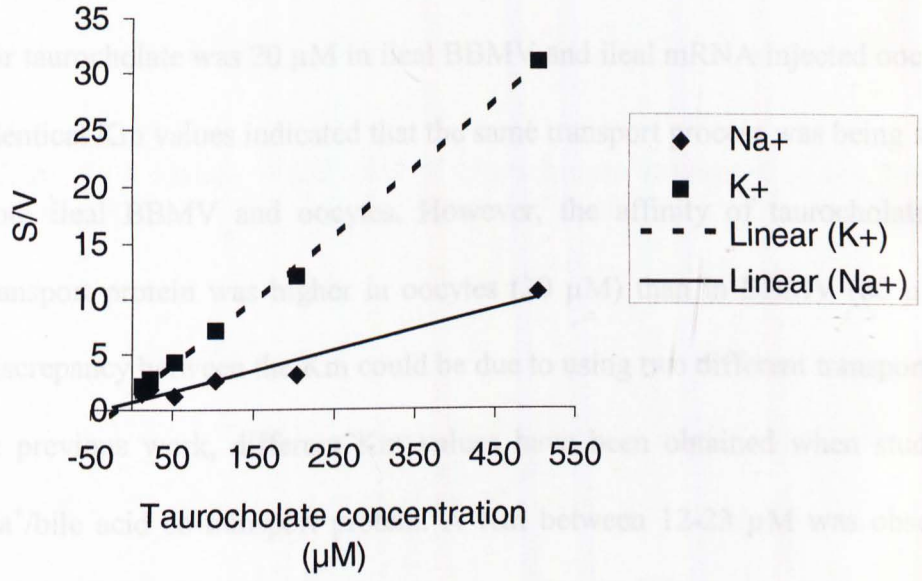
The data is presented as V vs [S] relationships (Fig.11. 3A) and as Hanes-Woolf plots [S]/v vs [S] (Fig. 11.3B).

The substrate dependence of taurocholate transport in ileal mRNA injected oocytes (figure 11.3A) demonstrated a similar profile to that observed in ileal BBMV (chapter 10). Taurocholate uptake in the presence of choline rose linearly as the concentration of taurocholate was increased, which was indicative of passive diffusion. Taurocholate transport in the presence of Na^+ and K^+ both displayed saturation kinetics, with the rate of taurocholate reaching a maximum at 500 μM.

The uptake rates due to passive diffusion were then subtracted from the combined uptake (due to all transport processes) in order to obtain results for Na^+ and K^+

dependent transport processes. These results were plotted as a Hanes-Woolf plot (figure, 11.3B) and the kinetic constants calculated.

Figure 11.3B, Hanes-Woolf plot of taurocholate transport in the presence of Na^+ and K^+



The kinetic constants for taurocholate uptake (figure 11.3B) in the presence of 100 mM Na^+ or K^+ were as follows ; Vmax = 35 pmol/oocyte/hr, Km = 20 μM in the presence of Na^+, Vmax= 16.6 pmol/oocyte/hr, Km = 20 μM in the presence of K^+.

The Vmax of Na^+ dependent taurocholate transport in the presence of oocytes was 35 pmol/oocyte/hr (25 pmol/mg prot/s in ileal BBMV). The Vmax of K^+ dependent taurocholate uptake in oocytes was 16.6 pmol/oocyte/hr (8.8 pmol/mg prot/s in ileal BBMV). The relationship between Vmax of taurocholate transport in the presence of the two cations corresponded to previous results observed in BBMV studies, with the Vmax for Na^+ dependent taurocholate transport being 2-3

fold higher than K^+ dependent taurocholate transport. This observation was a further indication that the same transport protein could utilise both Na^+ and K^+ for secondary active transport of taurocholate.

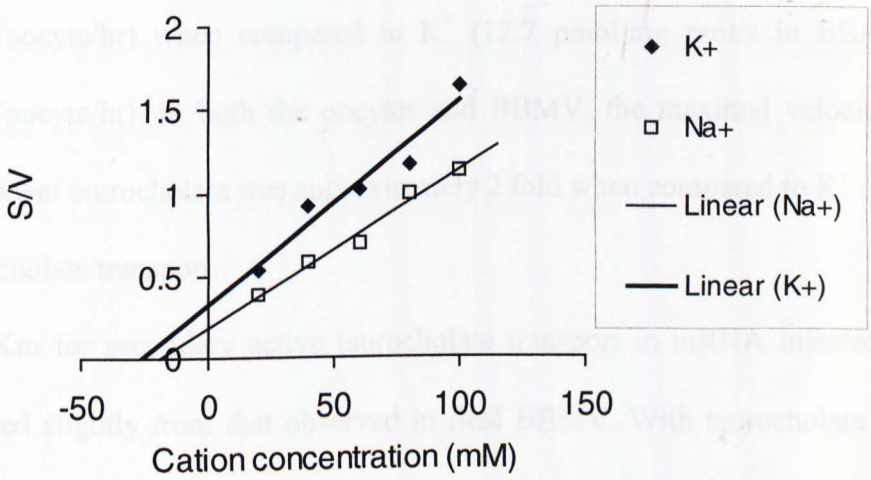
The affinity data for taurocholate uptake into oocytes in the presence of Na^+ corresponded to the previous data observed in ileal BBMV (chapter 10), the Km for taurocholate was 20 μM in ileal BBMV and ileal mRNA injected oocytes. The identical Km values indicated that the same transport process was being studied in both ileal BBMV and oocytes. However, the affinity of taurocholate for the transport protein was higher in oocytes (20 μM) than in BBMV (60 μM). This discrepancy between the Km could be due to using two different transport models. In previous work, different Km values have been obtained when studying the Na^+/bile acid co-transport protein. A Km between 12-23 μM was observed for Na^+ dependent uptake in perfused rat intestine (Schiff *et al.*,1972), and a Km of 33μM was demonstrated in COS cells, expressing the hamster Na^+/bile acid co-transport protein in the presence of Na^+ (Wong *et al.*,1994). Furthermore, previous investigation into the kinetics of the pig Na^+/bile acid co-transport protein revealed a Km of 48μM in oocytes compared to 40μM in BBMV (Mullins *et al.*, 1992).

Though there were some discrepancies in Km between BBMV and expression studies, the observation of identical Km's for both Na^+ and K^+ dependent taurocholate uptake into oocytes provided further evidence that the same transport process was able to utilise both cations for active transport.

11.3.3 Cation dependency of taurocholate transport in the presence of Na^+, K^+ and choline.

The effect of varying the cation concentrations of Na^+ and K^+ was investigated over a 0-100 mM range. The cation concentration could not be investigated above 100 mM because this would have meant the removal of all the HEPES within the solution, which would have affected the osmolarity and pH of the solution.

Figure 11.4 Hanes Woolf plot of taurocholate transport in the presence of Na^+ and K^+



40-50 ng of ileal mRNA (1 µg/µl) was injected into oocytes, with 46 nl of water injected into a further 120 oocytes. The oocytes were maintained for 4-5 days in a modified Barths solutions (89 mM NaCl, 2.4 mM $NaHCO_3$,15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) as described in the methods section. After 5 days the oocytes were incubated at 30° C for 1 hour in a choline chloride based Barths solution (92.9 mM choline chloride, 15mM HEPES/Tris pH 7.6) in order to remove any Na^+ or K^+ within the oocytes. Upon removal of the choline chloride Barths solution, the corresponding modified Barths solutions containing either Na^+ or K^+ was added. Taurocholate uptake was assayed in the presence of 0, 20, 40, 60, 80 and 100 mM of either NaCl or KCl and 15 mM HEPES/Tris pH 7.6. Varying the concentrations of

choline chloride maintained the standard osmolarity of 107.93 mM. To each group of oocytes 100 μM ^{3}H taurocholate (37 Kbq) was also added.

For each cation concentration, 20 mRNA injected and 10 water injected oocytes were incubated for 1 hour in the presence of Na^+, K^+ or choline. The reaction was stopped by the addition of ice cold stop solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6, 1 mM taurocholate). The rates of passive bile acid diffusion were subtracted from each data point.

The cation dependence data for taurocholate transport in ileal mRNA injected oocytes (figure 11.4) demonstrated a similar kinetic profile to that observed in ileal BBMV. The maximal velocity of secondary active bile acid transport was elevated in the presence of Na^+ (27.5 pmol/mg prot/s in BBMV; 100 pmol/oocyte/hr) when compared to K^+ (12.7 pmol/mg prot/s in BBMV; 59.5 pmol/oocyte/hr). In both the oocytes and BBMV, the maximal velocity of Na^+ dependent taurocholate was approximately 2 fold when compared to K^+ dependent taurocholate transport.

The Km for secondary active taurocholate transport in mRNA injected oocytes differed slightly from that observed in ileal BBMV. With taurocholate transport into oocytes the Km was 20 mM in the presence of Na^+ and 25 mM in the presence of K^+ compared to 55 mM in ileal BBMV in the presence of both cations. These discrepancies in Km between oocytes and BBMV were also observed in the previous taurocholate substrate kinetics (section 11.3.2), and may be due to differences in membrane composition. As previously mentioned, other studies has shown that the affinity of the transport process can vary between different transport models (Wilson, 1981). Therefore, the difference in Km between saturation kinetics in the BBMV and oocytes, was not sufficient to

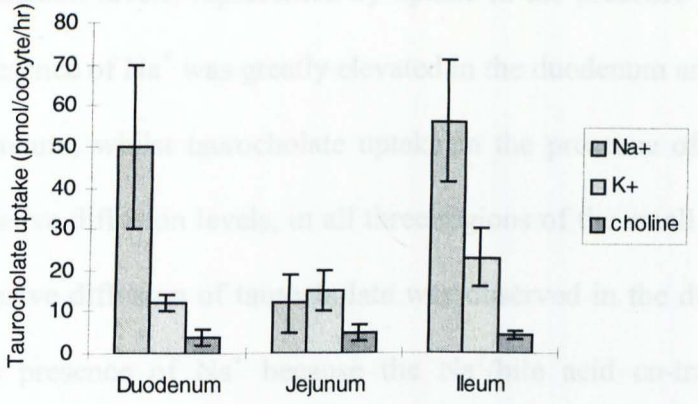
indicate two different transport processes, so it can be concluded that the same transport protein was being investigated.

Therefore, further investigation into the functional characteristics of the ileal mRNA coding for taurocholate uptake in the presence of Na^+ and K^+, indicated that the same transport process was using both cations for secondary active taurocholate uptake and that this transport process is conducted by the ileal Na^+/bile acid co-transport protein.

11.3.4 Regional distribution of mRNA encoding the Na^+/bile acid co-transport protein

To determine whether taurocholate transport in oocytes displayed a similar regional distribution profile to Na^+/ bile acid co-transport observed in BBMV, isolated pig duodenal, jejunal and ileal mRNA's were injected into *Xenopus laevis* oocytes.

Figure 11.5, Distribution of taurocholate transport in *Xenopus laevis* oocytes injected with intestinal mRNA



To each Xenopus laevis oocytes, 40-50 ng of either duodenal, jejunal or ileal mRNA (1 µg/µl) was injected into each oocyte, with 46 nl of water injected into oocytes to act as a control. The oocytes were maintained for 4-5 days in a modified Barths solutions (89 mM NaCl, 2.4 mM $NaHCO_3$,15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) as described in the methods section. After 5 days incubation the oocytes were incubated at 30° C for 1 hour in a choline chloride based Barths solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6) in order to remove any Na^+ or K^+ inside the oocytes. Upon removal of the choline chloride Barths solution, the corresponding modified Barths solutions containing either Na^+ (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$), K^+ (89 mM KCl, 2.4 mM $KHCO_3$, 15 mM HEPES/1M KOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$), or choline (92.9 mM choline chloride, 15mM HEPES/Tris pH 7.6) along with 100 µM ^3H taurocholate (37 Kbq), was added to each group of oocytes.

For each group, 20 mRNA injected and 10 water injected oocytes were incubated for 1 hour in the corresponding Barths solution and then the reaction stopped by the addition of ice cold stop solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6, 1mM taurocholate).

Figure 11.5 showed that the expression of mRNA isolated from all three regions of the small intestine resulted in elevated uptake of taurocholate above passive diffusion levels, represented by uptake in the presence of choline. Uptake in the presence of Na^+ was greatly elevated in the duodenum and ileum but less so in the jejunum, whilst taurocholate uptake in the presence of K^+ was elevated, above passive diffusion levels, in all three regions of the small intestine. In BBMV only passive diffusion of taurocholate was observed in the duodenum and jejunum in the presence of Na^+ because the Na^+/bile acid co-transport protein was not expressed in these regions of the small intestine. However, the finding that Na^+ dependent taurocholate transport occurs in oocytes upon injection of pig duodenal, jejunal or ileal mRNA has been observed in previous studies using pig mRNA isolated from the three regions of the small intestine. In that study, it was proposed that the mRNA encoding the Na^+/bile acid co-transport protein was present throughout the small intestine, but was only translated into a functional protein in the ileal enterocytes (Mullins *et al.*, 1992).

The elevated uptake of taurocholate in the presence of K^+ observed in the oocytes injected with ileal mRNA corresponded to the findings observed in the ileal BBMV and figure 11.1 of this chapter. These results suggest that ileal taurocholate transport in the presence of K^+ is occurring by the Na^+/bile acid co-transport protein.

However, in this study the rate of taurocholate uptake in the jejunum, in the presence of Na^+, was reduced compared to the previous work investigating regional distribution of mRNA encoding the Na^+/bile acid co-transport protein (Mullins *et al.*,1992). Therefore, it was possible that in this study the jejunal

uptake of taurocholate was due to another transport process and not the expression of the Na^+/bile acid co-transport protein. This observation was supported by the increased rate of jejunal taurocholate transport in the presence of K^+, which was above the rate of uptake in the presence of Na^+. This elevated uptake of jejunal taurocholate transport in the presence of K^+ had also been observed in the BBMV (figure 9.2). Therefore, the elevated uptake of taurocholate in the jejunum observed in this study could be due to a bile acid carrier mediated process, which has been observed in other studies, using guinea pigs and rats (Lewis and Root, 1990; Amelsberg *et al.*, 1995).

11.4 Investigation of whether the same gene codes for Na^+ dependent and K^+ dependent bile acid transport processes.

The results obtained using ileal BBMV and ileal mRNA injected oocytes indicated that the active transport of taurocholate could occur in the presence of both Na^+ and K^+. The kinetic and inhibition studies gave a good indication that the same transport process, namely the ileal Na^+/bile acid co-transport protein, was responsible for bile acid transport in the presence of both Na^+ and K^+. However, these observations could not definitely be attributed to the activity of a specific transport protein. In order to further determine if only one specific transport protein was using both Na^+ and K^+ for secondary active bile acid transport, the gene encoding the transport protein was investigated by means of translation hybrid arrest using a known cDNA to block the translation of mRNA.

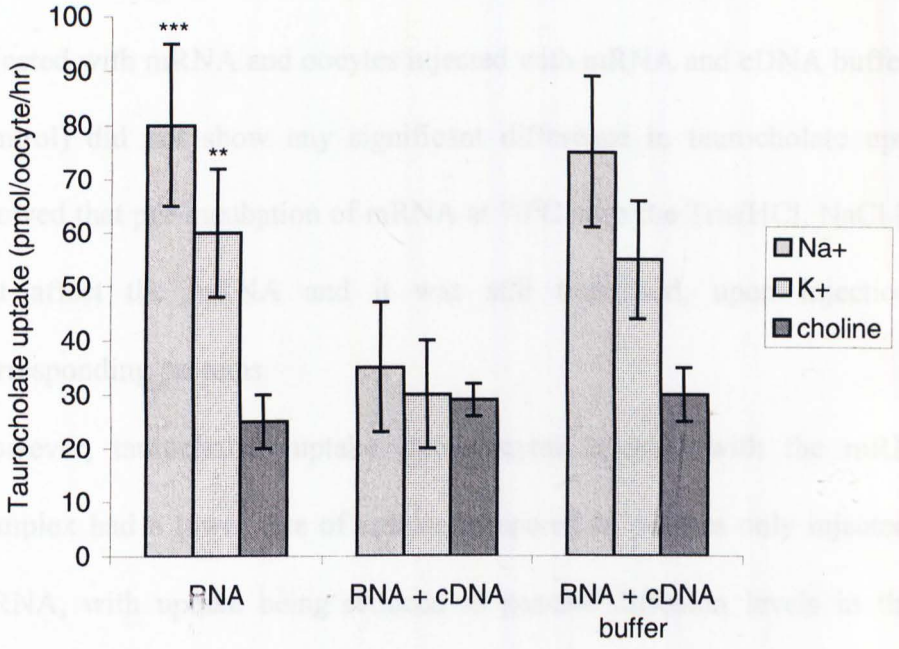
11.4.1 Translation hybrid arrest of the pig intestinal bile acid co-transport protein by a cDNA encoding the hamster Na^+/bile acid co-transport protein

Previous work had shown that pre-incubation of mRNA with a specific cDNA clone encoding a type I iodothyronine 5'-deiodinase from rat liver, inhibited translation of 5'-deiodinase activity in oocytes, with the cDNA hybridising to a 1.9 Kb RNA (St Germain *et al.*, 1990).

In this study, pig ileal mRNA was pre-incubated with a cDNA clone that encoded the hamster Na^+/ bile acid co-transport protein (a kind gift from Paul Dawson), which had been proposed to be totally dependent on a Na^+ gradient (Wong *et al.*, 1994; Dawson, 1994).

If the cDNA probe annealed to the gene encoding the pig Na^+/bile acid co-transport protein, then that gene would not be translated into its corresponding protein. The absence of the Na^+/ bile acid co-transport protein would result in only passive diffusion levels of taurocholate uptake into the oocytes. Pre-incubation of mRNA with the hamster cDNA would not affect the rates of passive diffusion, so taurocholate transport in the presence of choline would not be affected .

Figure 11.6, Effect of a cDNA encoding the hamster ileal bile acid co-transport protein, on pig ileal taurocholate transport



2 µg of ileal mRNA (1 µg/µl) was pre-incubated with 4 µg of cDNA in 10 mM Tris/HCl, 100 µM NaCl for 70 °C for 5 minutes and then 55°C for 20 minutes. The mRNA and cDNA were rapidly cooled on ice. In addition, for a positive control, 2 µg of mRNA was pre-incubated with only 10 mM Tris/HCl, 100 µM NaCl (no cDNA).

*To 60 Xenopus laevis oocytes, 40-50 ng of ileal mRNA (1 µg/µl) was injected into each oocyte, with 46 nl of water injected into a further 30 oocytes for the standard negative control. For hybrid arrest studies, 40-50 ng of ileal mRNA /cDNA complex was injected into 60 oocytes, with 46 nl of water injected into 30 oocytes. For the positive control, 60 Xenopus laevis oocytes were injected, with 40-50 ng of ileal mRNA and cDNA buffer (no cDNA), with 46 nl of water injected into 30 oocytes. Experiments in the presence of Na^+, K^+ and choline were carried out as previously described. Statistical analysis was carried using the excel anova test. The difference in taurocholate uptake between oocytes only injected with mRNA and oocytes injected with mRNA/cDNA were compared, in the presence of all three cations. Furthermore, the statistical difference between taurocholate uptake into oocytes injected with mRNA and those injected with mRNA and cDNA buffer was compared. 99% signifiance = **, 99.9% signifiance = ****

As shown in figure 11.6, oocytes injected with ileal mRNA again exhibited increased taurocholate uptake in the presence of Na^+ and K^+, when compared to taurocholate uptake in the presence of choline. Comparison between oocytes injected with mRNA and oocytes injected with mRNA and cDNA buffer (positive control) did not show any significant difference in taurocholate uptake. This showed that pre-incubation of mRNA at 70°C with the Tris/HCl, NaCl buffer, did not affect the mRNA and it was still translated, upon injection, to the corresponding proteins.

However, taurocholate uptake into oocytes injected with the mRNA/cDNA complex had a lower rate of uptake compared to oocytes only injected with the mRNA, with uptake being reduced to passive diffusion levels in the oocytes injected with the mRNA/cDNA complex. This finding, supports the hypothesis that translation arrest of the ileal Na^+/bile acid co-transport protein has occurred by the addition of the hamster cDNA and that taurocholate uptake in the presence of Na^+ and K^+ are both affected.

However, the standard deviation between the group of occytes only injected with mRNA and the group of oocytes injected with the mRNA/cDNA complex overlaps and therefore can not be determined to be significant. In order to determine if the difference in uptake between the mRNA injected oocytes and the mRNA/cDNA injected oocytes was significant, statistical analysis of the data was performed, using a anova test. The anova test showed that in the presence of Na^+ and K^+ there is a significant difference between taurocholate uptake into oocytes injected with just mRNA and oocytes injected with the mRNA/cDNA complex. The difference in taurocholate uptake in the presence of choline between the two

sets of oocytes are not significantly different, shown by the P value for the data being higher that 0.05% This findings shows that the addition of the hamster cDNA coding for the Na^+/bile acid co-transport protein has bound to and prevented the translation of the corresponding region of mRNA coding for the pig ileal Na^+/bile acid co-transport protein. Furthermore, the abolishment of taurocholate transport in the presence of Na^+ and K^+, to that of passive diffusion levels, again indicates that the same transport protein utilise both these cations for secondary active transport.

However, this experiment did not take into account the possibility that the hamster cDNA was binding to, so thereby preventing translation, of more than one gene coding for proteins involved in the transport of bile acids. There is a slight possibility that two or more different transport proteins could be responsible for secondary active transport in the presence of either Na^+ or K^+ , but have high sequence homology and so have been inactivated by the presence of the hamster ileal cDNA probe.

Therefore, from the hybrid arrest data it appeared that Na^+ and K^+ dependent taurocholate uptake occurred via the Na^+/ bile acid co-transport protein, which previous studies had determined to be strictly dependent on Na^+. This provided further evidence that the pig intestinal Na^+/bile acid co-transport does not have a strict dependence on Na^+.

11.4.2 Effect of a cDNA encoding the hamster ileal bile acid co-transport protein on methyl-α-D-glucopyranoside transport

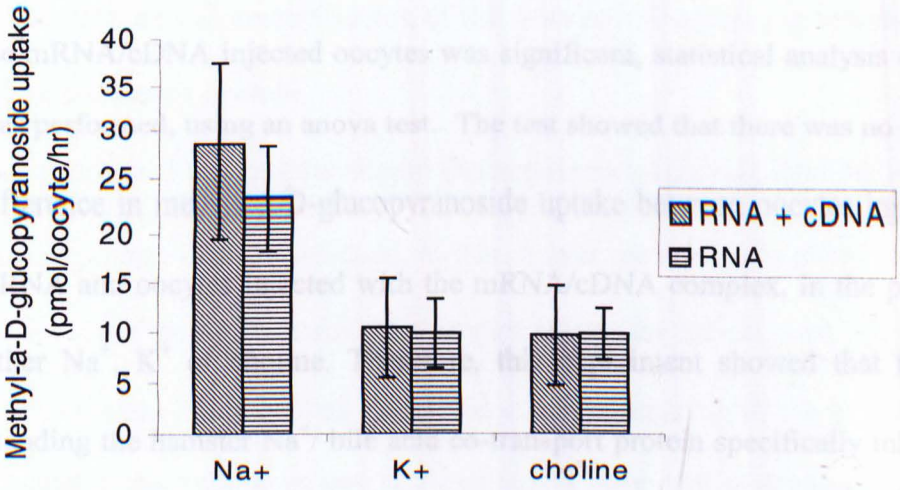
To determine that the hamster cDNA was not rendering all the genes that were coded by the mRNA inactive, another transport process was investigated. Pre-

incubated pig ileal mRNA/cDNA was injected into oocytes, which were then assayed for methyl -α-D-glucopyranoside transport and compared to methyl -α-D-glucopyranoside transport into oocytes injected only with pig ileal mRNA.

Methyl-α-D-glucopyranoside was used for the characterisation of the Na^+ dependent D-glucose transport system by the SGLT1 protein. Methyl-α-D-glucopyranoside was used in the expression system instead of D-glucose because Methyl-α-D-glucopyranoside is a non metabolised hexose which can be transported by the Na^+ dependent D-glucose co-transport protein, SGLT1 (Hediger *et al.*,1987). If D-glucose was used in the oocyte expression system, the sugar would become rapidly hydrolysed and undetectable.

Therefore, any elevated Na^+ dependent transport of methyl-α-D-glucopyranoside observed should be entirely due to the activity of the Na^+ dependent D-glucose co-transport protein (SGLT1).

Figure 11.7, Methyl-α-D-glucopyranoside transport in the presence of Na^+, K^+ and choline



2 μg of ileal mRNA (1 μg/μl) was pre-incubated with 4 μg cDNA in 10 mM Tris/HCl, 100 μM NaCl for 70 °C for 5 minutes and then 55°C for 20 minutes. The mRNA and cDNA were rapidly cooled on ice. In addition, as a positive control 2μg of mRNA was pre-incubated with only 10 mM Tris/HCl, 100 μM NaCl (no cDNA).

To 60 Xenopus laevis oocytes, 40-50 ng of ileal mRNA (1 μg/μl) was injected into each oocyte, with 46 nl of water injected into a further 30 oocytes. For hybrid arrest studies, 40-50 ng of ileal mRNA/cDNA complex was injected into 60 Xenopus laevis oocytes with 46 nl of water injected into 30 oocytes. The oocytes were maintained for 4-5 days and then assayed as previously described, with 100 μM ^{14}C Methyl-α-D-glucopyranoside added to each group. For each group, 20 mRNA injected and 10 water injected oocytes were incubated for one hour and the reaction stopped by the addition of ice cold stop solution (92.9 mM choline chloride, 15 mM HEPES/Tris pH 7.6, 1 mM Methyl-α-D-glucopyranoside).

Figure 11.7 showed that Na^+ dependent methyl-α-D-glucopyranoside transport occurred in mRNA and mRNA/DNA injected oocytes. The replacement of Na^+, by K^+ or choline resulted in a decrease in methyl-α-D-glucopyranoside transport to passive diffusion levels. This data corresponded to previous work (Hediger *et*

al., 1987 a & b) and the results observed in this study using pig ileal BBMV (chapter 8).

To determine if this difference in uptake between the mRNA injected oocytes and the mRNA/cDNA injected oocytes was significant, statistical analysis of the data was performed, using an anova test. The test showed that there was no significant difference in methyl-α-D-glucopyranoside uptake between oocytes injected with mRNA and oocytes injected with the mRNA/cDNA complex, in the presence of either Na^+, K^+ or choline. Therefore, this experiment showed that the cDNA encoding the hamster Na^+/ bile acid co-transport protein specifically inhibited the translation of the gene encoding the pig bile acid co-transport protein.

11.5 Summary

Further investigation of secondary active bile acid transport using *Xenopus laevis* oocytes confirmed the previous findings in pig ileal BBMV, that secondary active bile acid transport could occur in the presence of Na^+ and K^+. The translation hybrid arrest of the ileal Na^+/bile acid co-transport protein gave further indication that the same transport protein was involved in utilising both Na^+ and K^+ for secondary active transport. Furthermore, it gave an initial indication that only one gene coded for the Na^+/bile acid co-transport protein and by preventing the translation of this gene into its corresponding protein had the affect of abolishing secondary active taurocholate transport in the presence of Na^+ and K^+.

CHAPTER TWELVE:

12. Isolation and characterisation of the gene encoding the pig intestinal Na^+/bile acid co-transport protein

In the chapters 9 to11 the functional characteristics of the pig ileal Na^+/bile acid co-transport protein were investigated. In this chapter the molecular characteristics of the gene encoding the Na^+/bile acid co-transport protein are investigated.

Previous studies have reported the isolation and sequencing of the genes that encode the hamster, rat, rabbit, mouse and human Na^+/bile acid co-transport protein. The nucleotide sequence for the Na^+/bile acid co-transport protein gene has been found to be around 1044 bases (348 amino acids long) with a molecular weight of 38kDa for all five species (Wong *et al.*, 1994; Dawson and Oelkers, 1995; Dawson *et al.*, 1995; Seaki *et al.*, 1999).

12.1 Isolation of the DNA which encodes the pig intestinal Na^+/bile acid co-transport protein

To allow the molecular characterisation of the DNA that encodes the Na^+/bile acid co-transport protein, the particular sequence of DNA which encodes the protein needed to be isolated from the rest of the pig ileal DNA.

The Na^+/bile acid co-transport protein has previously been sequenced in five other mammals and it has been determined that the amino acid sequences for the protein in each of the mammals, displayed a large degree of homology between the species. This high sequence homology was utilised in this study. It was proposed that the DNA

which encoded the pig ileal Na^+/bile acid co-transport protein could be isolated by using PCR primers designed around regions of sequence homology observed in the previously sequenced genes from the hamster, rat and human. These three mammals where chosen to be used for comparison of sequence homology because the rabbit sequence varies slightly at the start and end of the sequence and the mouse sequence was not available on EMBL at this stage of the study.

12.1.1 Design of PCR primers used for the isolation of specific DNA encoding the Na^+/bile acid co-transport protein in pig ileum

In order to design primers that would be complementary to the DNA sequence which encodes the pig Na^+/bile acid co-transport protein, the nucleotide and amino acid sequences of the Na^+/bile acid co-transport protein in hamster, rat and human were compared by sequence alignment.

Figure 12.1 Amino acid sequence of the Na^+/bile acid co-transport protein from the human, rabbit, mouse, rat and hamster

```
ntcihuman       -MNDPNSCVDNATVCSGASCVVPESNFNNILSVVLSTVLTILLALVMFSMGCNVEIKKFL 59
ntcirabbit      MSNLTVGCLANATVCEGASCVAPESNFNAILSVVLSTVLTILLALVMFSMGCNVEIKKFL 60
ntcimouse       -MDNSSVCPPNATVCEGDSCVVPESNFNAILNTVMSTVLTILLAMVMFSMGCNVEVHKFL 59
ntcirat         -MDNSSVCSPNATFCEGDSCLVTESNFNAILSTVMSTVLTILLAMVMFSMGCNVEINKFL 59
ntcihamster     -MDNSSICNPNATICEGDSCIAPESNFNAILSVVMSTVLTILLALVMFSMGCNVELHKFL 59
                  : .  *  ***.*.* **:..***** **..*:*********:**********::***

ntcihuman       GHIKRPWGICVGFLCQFGIMPLTGFILSVAFDILPLQAVVVLIIGCCPGGTASNILAYWV 119
ntcirabbit      GHIRRPWGIFIGFLCQFGIMPLTGFVLAVAFGIMPIQAVVVLIMGCCPGGTASNILAYWV 120
ntcimouse       GHIKRPWGIFVGFLCQFGIMPLTGFILSVASGILPVQAVVVLIMGCCPGGTGSNILAYWI 119
ntcirat         GHIKRPWGIFVGFLCQFGIMPLTGFILSVASGILPVQAVVVLIMGCCPGGTGSNILAYWI 119
ntcihamster     GHLRRPWGIVVGFLCQFGIMPLTGFVLSVAFGILPVQAVVVLIQGCCPGGTASNILAYWV 119
                **::***** :***************:*:** .*:*:******* *******.*******:

ntcihuman       DGDMDLSVSMTTCSTLLALGMMPLCLLIYTKMWVDSGSIVIPYDNIGTSLVALVVPVSIG 179
ntcirabbit      DGDMDLSVSMTTCSTLLALGMMPLCLYVYTKMWVDSGTIVIPYDNIGTSLVALVVPVSIG 180
ntcimouse       DGDMDLSVSMTTCSTLLALGMMPLCLFVYTKMWVDSGTIVIPYDSIGISLVALVIPVSFG 179
ntcirat         DGDMDLSVSMTTCSTLLALGMMPLCLFIYTKMWVDSGTIVIPYDSIGISLVALVIPVSIG 179
ntcihamster     DGDMDLSVSMTTCSTLLALGMMPLCLFIYTKMWVDSGTIVIPYDSIGTSLVALVIPVSIG 179
                ************************** :*********:******.** ******:***:*

ntcihuman       MFVNHKWPQKAKIILKIGSIAGAILIVLIAVVGGILYQSAWIIAPKLWIIGTIFPVAGYS 239
ntcirabbit      MFVNHKWPQKAKIILKVGSIAGAVLIVLIAVVGGILYQSAWIIEPKLWIIGTIFPMAGYS 240
ntcimouse       MFVNHKWPQKAKIILKIGSITGVILIVLIAVIGGILYQSAWIIEPKLWIIGTIFPIAGYS 239
ntcirat         MFVNHKWPQKAKIILKIGSIAGAILIVLIAVVGGILYQSAWIIEPKLWIIGTIFPIAGYS 239
ntcihamster     MYVNHKWPQKAKIILKIGSIAGAILIVLIAVVGGILYQSAWTIEPKLWIIGTIYPIAGYG 239
                *:***************:***:*.:*******:********* * *********:*:***.

ntcihuman       LGFLLARIAGLPWYRCRTVAFETGMQNTQLCSTIVQLSFTPEELNVVFTFPLIYSIFQLA 299
ntcirabbit      LGFFLARIAGQPWYRCRTVALETGMQNTQLCSTIVQLSFSPEDLTYVFTFPLIYSIFQIA 300
ntcimouse       LGFFLARLAGQPWYRCRTVALETGMQNTQLCSTIVQLSFSPEDLNLVFTFPLIYTVFQLV 299
ntcirat         LGFFLARLAGQPWYRCRTVALETGMQNTQLCSTIVQLSFSPEDLNLVFTFPLIYTVFQLV 299
ntcihamster     LGFFLARIAGQPWYRCRTVALETGLQNTQLCSTIVQLSFSPEDLNLVFTFPLIYSIFQIA 299
                ***:***:** *********:***:***************:**:*. ********::**:.

ntcihuman       FAAIFLGFYVAYKKCHGKNKAEIPESKENGTEPESSFYKANGGFQPDEK 348
ntcirabbit      FAAIFLGIYVAYRKCHGKNDAEFPDIKDTKTEPESSFHQMNGGFQPE-- 347
ntcimouse       FAAVILGIYVTYRKCYGKNDAEFLEKTDNEMDSRPSFDETNKGFQPDEK 348
ntcirat         FAAIILGMYVTYKKCHGKNDAEFLEKTDNDMDPMPSFQETNKGFQPDEK 348
ntcihamster     FAAILLGAYVAYKKCHGKNNTELQEKTDNEMEPRSSFQETNKGFQPDEK 348
                ***::** **:*:**:***.:*: : .:.  :. .** : * ****:
```

The multiple sequence alignment was carried out using the CLUSTERW software at EBI. This is described in more details in the methods section (7.2.12.3).

The amino acid sequence alignment from the 5 mammals revealed 4 regions of homology that were used to design PCR primers (figure 12.1). In the design of the primers only the human, rat and hamster was used, but the rabbit and mouse sequence has been included in figure 12.1, to indicate the high degree of homology between the five species.

Even though the amino acid sequences for these regions of the transport proteins were conserved throughout the majority of the three mammals, the nucleotide sequences differed. The difference in nucleotide sequence between the three species were due to the fact that each amino acid is coded by a variety of triplet codons which have different nucleotide sequences.

Therefore, the nucleotide sequence of the related conserved regions for each of the three mammals were compared, as shown in tables 12.1 A-D

Table 12.1.A Nucleotide sequences from the 1st region of conserved amino acids

Amino acid Position	Hamster	Rat	Human	Non conserved nucleotide
E (23)	GAG	GAA	GAG	A in rat
S (24)	AGC	AGC	AGC	All conserved
N (25)	AAC	AAC	AAT	T in human
F (26)	TTC	TTC	TTC	All conserved
N (27)	AAC	AAT	AAT	C in hamster
A (28)	GCC	GCC	N = AAC	Different amino acid in human

Table 12.1.B Nucleotide sequences from the 2nd region of conserved amino acids

Amino acid Position	Hamster	Rat	Human	Non conserved nucleotide
I (40)	ATC	ATT	ATC	T in rat
L (41)	CTG	CTT	CTG	G in rat
L (42)	CTA	CTA	TTG	T & G in human
A (43)	GCC	GCC	GCC	All conserved
L (44)	TTG	M = ATG	TTG	Different amino acid in rat
V (45)	GTG	GTG	GTC	All conserved

Table 12.1.C Nucleotide sequences from the 3rd region of conserved amino acids

Amino acid Position	Hamster	Rat	Human	Non conserved nucleotide
Y(308)	TAC	TAC	TAC	All conserved
K (309)	AAG	AAG	AAG	All conserved
K (310)	AAA	AAA	AAA	All conserved
C (311)	TGT	TGT	TGC	C in Human
H (312)	CAT	CAT	CAT	All conserved
G (313)	GGA	GGA	GGA	All conserved

Table 12.1.D Nucleotide sequences from the 4th region of conserved amino acids

Amino acid Position	Hamster	Rat	Human	Non conserved nucleotide
F (343)	TTT	TTT	TTT	All conserved
Q (344)	CAA	CAA	CAA	All conserved
P (345)	CCA	CCA	CCT	T in human
D (346)	GAT	GAT	GAC	C in human
E (347)	GAG	GAG	GAA	A in human
K (348)	AAG	AAA	AAG	A in rat

Comparison of the nucleotide sequences from the 4 regions of conserved amino acids revealed some differences in nucleotide sequences encoding the same amino acid. In these cases, the nucleotides which were conserved between the majority of the three species were used for the final primer sequence.

The 2 conserved regions of nucleotides located near to the N terminus of the protein (shown in tables 12.1 A and B) were used to design both forward primers. The conserved regions of nucleotides located at the C terminus of the protein were used to design both reverse primers (shown in tables 12.1 C and D).

As described in the methods chapter (section 7.2.7.1) in order to isolate both the sense and anti-sense strands of the double stranded DNA, each set of forward and reverse primers needed to have one primer complementary to the sense DNA strand and one

primer complementary to the anti-sense DNA strand. Therefore, the nucleotide sequences of the reverse primers were designed to be complementary to the nucleotide sequences shown in table 12.1 C&D, which meant these reverse primers would bind to the sense strand of DNA. The forward primers had the identical nucleotide sequence to the conserved nucleotides shown in table 12.1A and B and would therefore bind to antisense DNA strand.

Table 12.2 Nucleotide sequences of the 4 primers designed to isolate the DNA that encodes the pig Na^+/bile acid co-transport protein.

Primer name	Nucleotide sequence of primers
Forward 1	5' GGCGCATGGAGAGCAACTTCAAT 3'
Forward 2	5' GGCGCATCCTGTTGGCCTTGGTG 3'
Reverse 3	5'GGCGCTCCATGACATTTCTTGTA 3'
Reverse 4	5' GGCGCCATCTTCTCATCTGGTTGAAA 3'

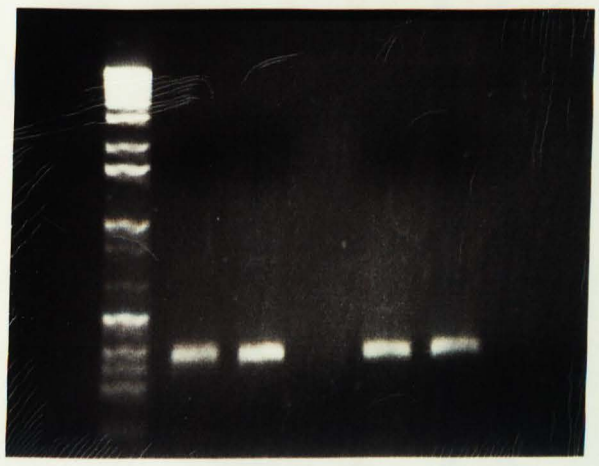
The reverse primer 4 was designed around the very end of the C-terminus region of the protein. This meant that primer needed a stop codon added to the end of the nucleotide sequence in order to produce a PCR transcript which encoded a single functional protein. Throughout the three species the stop codon TAG was conserved, so the nucleotide sequence ATC was added to the 5' end of the reverse primer 4. The forward primer 1 encodes a conserved region close to the start of the protein sequence, so a start codon (ATG) was added to the 5' end of the primer sequence to ensure that the PCR transcript isolated from the pig encoded a single protein. To all of

the 4 primers a GC clamp (GGCGC) was added to prevent the primers from annealing to each other.

12.1.2 Isolation by PCR of the DNA that encode the Na^+/bile acid co-transport protein by PCR

For the isolation of the DNA that encoded the pig Na^+/bile acid co-transport protein, primers 1 and 4 were used together in one PCR reaction and primers 2 and 3 were used together. PCR was carried out on pig ileal genomic DNA, a cDNA which encoded the hamster ileal Na^+/bile acid co-transport protein and a cDNA which encoded the hamster liver Na^+/bile acid co-transport protein. To determine if a PCR product had been produced by the two sets of primers, the PCR products were run on a 1% agarose gel and stained with ethidium bromide.

Figure 12.2 Electrophoresis of PCR products



KEY:

Lane 1: 0.5-10 Kb ladder

Lane 2: PCR reaction using cDNA encoding the hamster ileal Na^+/bile acid co-transport protein, with primers 1 & 4

Lane 3: PCR reaction using pig ileal DNA, with primers 1 & 4

Lane 4: PCR reaction using cDNA encoding the liver bile acid transport protein, with primers 1 & 4

Lane 5: PCR reaction using cDNA encoding the hamster ileal Na^+/bile acid co-transport protein, with primers 2 & 3

Lane 6: PCR reaction using pig ileal DNA, with primers 2 & 3

Lane 7: PCR reaction using cDNA encoding the liver bile acid transport protein, with primers 2 & 3

For each PCR reaction, 1 µg of genomic or plasmid DNA was used. Each PCR reaction also contained 1.5 units of Taq DNA polymerase, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl pH 9.0, and 200 µM dNTP and 5 µM for both the forward and reverse primers. The PCR reaction had: 1 cycle at 95° C for 2 seconds and then 40 cycles at 95° C for 10 seconds, 50° C for 10 seconds followed by 72° C for 30 seconds. After 40 cycles the PCR reaction finished with an extension phase of 2 seconds at 72° C. The PCR samples were run on a 1% TAE agarose gel and the gel was run at 60 volts for one hour. For each sample, 10 µl of the PCR product was run, against a 0.5-10 kb ladder. The gel was stained overnight with ethidium bromide and then visualised under UV light.

Figure 12.2 showed that both sets of primers isolated a PCR product from both the hamster and the pig. The PCR products produced using the hamster cDNA and pig genomic DNA were both 1-1.2 Kb in size.

The observation that PCR products of the same size were isolated from the hamster cDNA and the pig genomic DNA, indicated that both the PCR products encoded the Na^+/bile acid co-transport protein. The cDNA from the hamster only consisted of

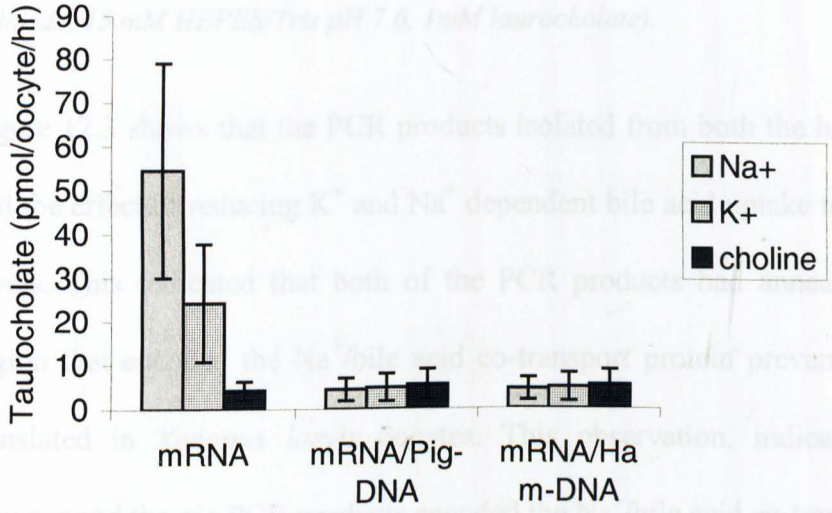
1044 nucleotides coding for the Na^+/bile acid co-transport protein, so if the primers did not correspond to regions within this nucleotide sequence then a PCR product would not have been produced. The hamster cDNA therefore acted as a positive control in the PCR experiments.

The cDNA encoding the liver bile acid transport protein did not produce a PCR product, which corresponded with previous findings (Hagenbuch *et al.*, 1996) that the liver Na^+/bile acid co-transport protein only shares limited homology with the ileal Na^+/bile acid co-transport protein. Therefore, the production of a PCR product from pig ileal DNA, which was the correct size of 1-1.2 Kb, indicated that the PCR product probably encodes the Na^+/bile acid co-transport protein.

12.2 Functional investigation of the PCR product, proposed to encode for the Na^+/bile acid co-transport protein by hybrid translation arrest

To investigate whether the isolated PCR product from the pig ileum did encode the Na^+/bile acid co-transport protein, translation hybrid arrest of the pig ileal mRNA by the PCR product was carried out. If the PCR product coded for the pig ileal Na^+/bile acid co-transport protein then the PCR product should anneal to the complementary region of mRNA, blocking its translation upon expression of the mRNA in *Xenopus laevis* oocytes.

Figure 12.3 Translation hybrid arrest of the mRNA encoding the Na^+/bile acid co-transport protein, by PCR products derived from pig intestinal genomic DNA and hamster cDNA



2 μg of ileal mRNA (1 μg/μl) was pre-incubated with 100 ng of pig or hamster DNA in a 10 mM Tris/HCl, 100 μM NaCl for 70 °C for 5 minutes and then 55°C for 20 minutes. The mRNA and DNA were rapidly cooled on ice.

To 60 Xenopus laevis oocytes, 40-50 ng of ileal mRNA (1 μg/μl) was injected into each oocyte, with 46 nl of water injected into a further 30 oocytes. For hybrid arrest studies, 46nl of ileal mRNA /DNA complex was injected into 60 Xenopus laevis oocytes, with 46 nl of water injected into 30 oocytes.

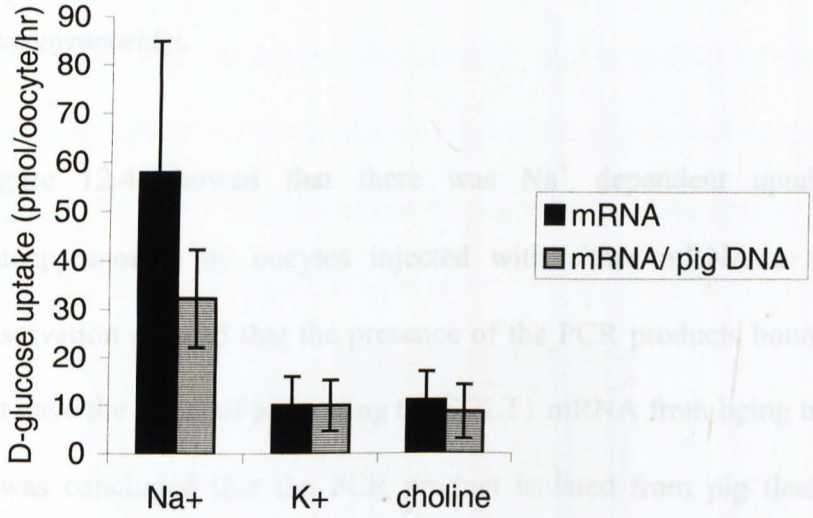
The oocytes were maintained for 4-5 days in a modified Barths solutions (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) as described in the methods section. After 5 days the oocytes were incubated at 30°C for 1 hour in a choline chloride based Barths solution (92.93 mM choline chloride, 15 mM HEPES/Tris pH 7.6).Upon removal of the choline chloride Barths solution, the corresponding modified Barths solutions containing either Na^+ (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$,0.41 mM $CaCl_2$, 0.82 mM

$MgSO_4$), K^+ (89 mM KCl, 2.4 mM $KHCO_3$, 15 mM HEPES/1M KOH pH 7.6, 0.3 mM $CaNO_3$ 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) or choline (92.93 mM choline chloride, 15 mM HEPES/Tris pH 7.6) was added to each group of oocytes, with 100 μM ^3H taurocholate. For each group, 20 mRNA injected and 10 water injected oocytes were incubated for one hour and the reaction stopped by the addition of ice cold stop solution (92.93 mM choline chloride, 15 mM HEPES/Tris pH 7.6, 1mM taurocholate).

Figure 12.3 shows that the PCR products isolated from both the hamster and the pig had the effect of reducing K^+ and Na^+ dependent bile acid uptake to passive diffusion levels. This indicated that both of the PCR products had annealed to the mRNA region that encoded the Na^+/bile acid co-transport protein preventing it from being translated in *Xenopus laevis* oocytes. This observation, indicated that both the hamster and the pig PCR products encoded the Na^+/bile acid co-transport protein.

To ensure that the PCR products were only annealing to the region of mRNA that encoded for the Na^+/bile acid co-transport protein, the mRNA/DNA complex was expressed in *Xenopus laevis* oocytes and then assayed for D-glucose uptake. As described in the previous chapter (chapter 11), the presence of the SGLT1 in oocytes was detected by using the substrate methyl-α-D-glucopyranoside.

Figure 12.4 Methyl-α-D-glucopyranoside transport in the presence of Na^+, K^+ and choline



2 μg of ileal mRNA (1 μg/μl) was pre-incubated with 100 ng of pig or hamster DNA in a 10 mM Tris/HCl, 100 μM NaCl for 70 °C for 5 minutes and then 55°C for 20 minutes. The mRNA and cDNA were rapidly cooled on ice.

To 60 Xenopus laevis oocytes, 40-50 ng of ileal mRNA (1 μg/μl) was injected into each oocyte, with 46 nl of water injected into a further 30 oocytes. For hybrid arrest studies, 46 nl of ileal mRNA and cDNA was injected into 60 Xenopus laevis oocytes with 46 nl of water injected into 30 oocytes. The oocytes were maintained for 4-5 days in a modified Barths solutions (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) as described in the methods section. After 5 days the oocytes were incubated at 30°C for 1 hour in a choline chloride based Barths solution (92.93 mM choline chloride, 15 mM HEPES/Tris pH 7.6).Upon removal of the choline chloride Barths solution, the corresponding modified Barths solutions containing either Na^+ (89 mM NaCl, 2.4 mM $NaHCO_3$, 15 mM HEPES/1M NaOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$), K^+ (89 mM KCl, 2.4 mM $KHCO_3$, 15 mM HEPES/1M KOH pH 7.6, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$) or choline (92.93 mM choline

chloride, 15 mM HEPES/Tris pH 7.6) was added to each group of oocytes, with 100 μM ^{14}C Methyl-α-D-glucopyranoside. For each group, 20 mRNA injected and 10 water injected oocytes were incubated for one hour and the reaction stopped by the addition of ice cold stop solution (92.93 mM choline chloride, 15 mM HEPES/Tris pH 7.6, 1 mM Methyl-α-D-glucopyranoside).

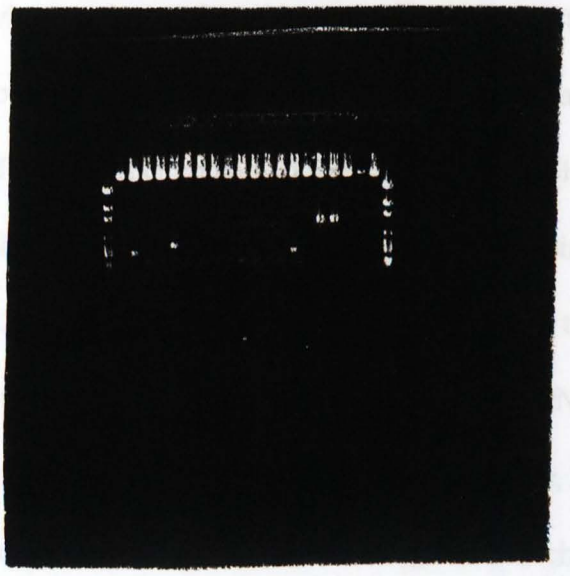
Figure 12.4 showed that there was Na^+ dependent uptake of methyl-α-D-glucopyranoside by oocytes injected with either mRNA or mRNA/DNA. This observation showed that the presence of the PCR products bound to the mRNA did not have the effect of preventing the SGLT1 mRNA from being translated. Therefore, it was concluded that the PCR product isolated from pig ileal DNA appeared to encode only the Na^+/bile acid co-transport.

12.3 Cloning and sequencing of the PCR product

To enable sequencing of the PCR product copied from pig ileal DNA, 400 ng of this product was required. However, the PCR reaction was unable to provide a sufficient quantity. To ensure that the DNA template was not the problem with the PCR reaction, RT-PCR was carried out. However, after the production of cDNA from the mRNA, no PCR product could be detected. After the alteration of a variety of environmental conditions, such as increasing the concentration of Taq polymerase, dNTP and $MgCl_2$ and varying cycling time and temperature, the amount of PCR product was still too low. Therefore, it was concluded that another method was required to produce sufficient amount of the PCR product for sequencing.

To ensure sufficient concentration of the PCR product to be used for sequencing, the PCR product was cloned into *E.coli* cell by the use of a plasmid vector. The cloning was carried out as described in the methods chapter (section 7.2.11.1-3). The pig PCR product that was cloned into the plasmid, was generated by using primers 1 and 4 because this PCR product contained most of the pig nucleotide sequence.

Figure, 12.5 Gel electrophoresis of the digested plasmid to determined which plasmids contained the PCR inserts



KEY:
Lane 1: DNA ladder
Lane 2- 21: DNA inserts
Lane 22: DNA ladder

The DNA digests were run on a 1% TBE agarose gel and the gel was run at 60 volts for one hour. For each sample, 10µl of the DNA digest was run, against a 0.5-10 kb ladder. The gel was stained over 2 hours with ethidium bromide and then visualised with UV light.

Figure 12.5 showed that the majority of the PCR products cloned into the plasmids were very small, with molecular weights of about 0.2-0.5 kb. The production of the large number of small DNA inserts by the TA cloning may be explained by the observation that the method of TA cloning preferentially clones small DNA inserts (Promega, 1996).

However, the lanes 5, 17 and 18 all had DNA inserts larger than 0.5 Kb. The DNA insert in lane 5 had been identified in a previous DNA purification experiment in this study, where only 10 plasmids were isolated from the agar plates that contained the *E.coli* colonies with the DNA inserts. Sequence analysis of this DNA insert revealed that the PCR product contained the forward primer 1 and reverse primer 4 but did not encode the pig ileal Na^+/bile acid co-transport protein. Using the software PC gene to convert the nucleotide sequence to amino acids and then EMBL to identify the amino acid sequence, the software indicated that the DNA insert encoded a membrane glyco-protein and not a transport protein. However, this finding indicated that other membrane proteins have nucleotide regions which share close homology to the regions of the Na^+/bile acid co-transport protein gene selected for primer design.

The two DNA inserts in lanes 17 and 18 were the same size as the previously isolated PCR product (figure 12.2). Therefore, these two DNA inserts appeared to be the PCR product isolated from pig ileum. The isolation of these two DNA inserts indicated that the PCR product had been successfully cloned and was in sufficient concentration to be sequenced.

The PCR product was sent to MWG Biotech for sequencing, using the M13 forward and reverse sites on the plasmid as sequencing primers (see appendix for plasmid map).

12.4 Summary

Comparison of the amino acid and nucleotide sequences for 3 mammals enabled the design of two sets of primers which were used to isolated a region of pig DNA by PCR. The PCR product isolated from the pig DNA was the same size, 1-1.2 Kb, as the positive control, which was a cDNA only encoding the hamster Na^+/bile acid co-transport protein. The isolation of only one PCR product from the pig genomic DNA confirmed previous findings in this study, that only one gene appeared to encode the pig ileal Na^+/bile acid co-transport protein

Hybrid translation arrest of the gene encoding the pig Na^+/bile acid co-transport protein, by the PCR product isolated from pig DNA, indicated that the PCR encoded the pig ileal Na^+/bile acid co-transport protein. Furthermore, this finding confirmed that the one coded for the Na^+/bile acid co-transport protein and that secondary active taurocholate transport in the presence of both Na^+ and K^+ was abolished when the translation of the Na^+/bile acid co-transport protein was prevented.

The cloning of the PCR product into a plasmid provided sufficient concentration of the pig PCR product. Restriction digest of the clones produced from the cloning, revealed that two clones contained the correct size DNA insert. One of these clones were then sequenced.

CHAPTER THIRTEEN:

13. Molecular modelling of the pig ileal Na^+/bile acid co-transport protein

13.1 Conversion of the nucleotide sequence into an amino acid sequence

To enable the identification and modelling of the protein encoded by the isolated PCR product, the nucleotide sequence (supplied by MWB Biotech) needed to be converted into an amino acid sequence. The conversion was carried out using PC gene as described in the methods section (7.2.12.1) and shown in figure 13.1.

13.2 Identification of the protein encoded by the amino acid sequence

To determine which protein the converted amino acid sequence encoded, the three variations of amino acid sequence were used to conduct a BLAST search as described in the methods section (7.2.12.2). Using the BLAST search it was determined that the first sequence of amino acids (shown in italics in figure 13.1) encoded the Na^+/bile acid co-transport protein. Furthermore, the first five proteins ascertained to share the greatest degree of homology with the amino acid sequence were all ileal Na^+/bile acid co-transport proteins. This provided confirmation that the isolated PCR product did encode the pig ileal Na^+/bile acid co-transport protein, which had not previously been isolated and sequenced. Furthermore, using the BLAST search it was also determined that the pig ileal Na^+/bile acid co-transport protein shared the greatest degree of homology with the hamster Na^+/bile acid co-transport protein followed by the rat > mouse > rabbit > human.

Figure 13.1 Amino acid sequence of the cloned PCR product

```
          10          20          30          40          50
          |           |           |           |           |
CGCATGGAGA GCAACTTCAA TGCCATCCTC AGCGTGGTGA TGAGCACCGT

 R  M  E  S  N  F  N  A  I  L  S  V  V  M  S  T  V
 A  W  R  A  T  S  M  P  S  S  A  W  -  -  A  P
 H  G  E  Q  L  Q  C  H  P  Q  R  G  D  E  H  R

          60          70          80          90          100
          |           |           |           |           |
GCTCACAATC CTCCTAGCCT TGGTGATGTT TTCCATGGGG TGCAATGTGG

 L  T  I  L  L  A  L  V  M  F  S  M  G  C  N  V
 C  S  Q  S  S  -  P  W  -  C  F  P  W  G  A  M  W
 A  H  N  P  P  S  L  G  D  V  F  H  G  V  Q  C  G

          110         120         130         140         150
          |           |           |           |           |
AACTCCACAA GTTTCTGGGA CACCTAAGGC GGCCATGGGG CATCGTCGTG

 E  L  H  K  F  L  G  H  L  R  R  P  W  G  I  V  V
 N  S  T  S  F  W  D  T  -  G  G  H  G  A  S  S  -
 T  P  Q  V  S  G  T  P  K  A  A  M  G  H  R  R

          160         170         180         190         200
          |           |           |           |           |
AGCTTCCTCT GTCAGTTTGG AATCATGCCT CTCACAGGTT TCGTCCTGTC

 S  F  L  C  Q  F  G  I  M  P  L  T  G  F  V  L  S
 A  S  S  V  S  L  E  S  C  L  S  Q  V  S  S  C
E  L  P  L  S  V  W  N  H  A  S  H  R  F  R  P  V

          210         220         230         240         250
          |           |           |           |           |
CGTGGCCTTT GGCATCCTCC CAGTGCAAGC TGTGGTGGTG CTGATCCAGG

 V  A  F  G  I  L  P  V  Q  A  V  V  V  L  I  Q
P  W  P  L  A  S  S  Q  C  K  L  W  W  C  -  S  R
 R  G  L  W  H  P  P  S  A  S  C  G  G  A  D  P  G

          260         270         280         290         300
          |           |           |           |           |
GTTGCTGCCC TGGAGGAACT GCCTCCAATA TCCTAGCCTA TTGGGTAGAT

 G  C  C  P  G  G  T  A  S  N  I  L  A  Y  W  V  D
 V  A  A  L  E  E  L  P  P  I  S  -  P  I  G  -  M
  L  L  P  W  R  N  C  L  Q  Y  P  S  L  L  G  R

          310         320         330         340         350
          |           |           |           |           |
GGCGACATGG ACCTCAGCGT TAGCATGACC ACCTGCTCCA CGCTGCTTGC

 G  D  M  D  L  S  V  S  M  T  T  C  S  T  L  L  A
  A  T  W  T  S  A  L  A  -  P  P  A  P  R  C  L
W  R  H  G  P  Q  R  -  H  D  H  L  L  H  A  A  C

          360         370         380         390         400
          |           |           |           |           |
CCTTGGAATG ATGCCCCTTT GCCTCTTCAT CTATACCAAG ATGTGGGTTG

 L  G  M  M  P  L  C  L  F  I  Y  T  K  M  W  V
P  L  E  -  C  P  F  A  S  S  S  I  P  R  C  G  L
 P  W  N  D  A  P  L  P  L  H  L  Y  Q  D  V  G  -
```

```
         410         420         430         440         450
          |           |           |           |           |
ACTCAGGGAC GATTGTGATT CCTTATGACA GCATTGGCAC TTCTCTGGTT

D  S  G  T  I  V  I  P  Y  D  S  I  G  T  S  L  V
 T  Q  G  R  L  -  F  L  M  T  A  L  A  L  L  W  L
  L  R  D  D  C  D  S  L  -  Q  H  W  H  F  S  G

         460         470         480         490         500
          |           |           |           |           |
GCTCTTGTTA TTCCTGTTTC CATTGGAATG TATGTGAATC ACAAATGGCC

A  L  V  I  P  V  S  I  G  M  Y  V  N  H  K  W  P
 L  L  L  F  L  F  P  L  E  C  M  -  I  T  N  G
C  S  C  Y  S  C  F  H  W  N  V  C  E  S  Q  M  A

         510         520         530         540         550
          |           |           |           |           |
CCAAAAAGCA AAGATCATAC TTAAAATTGG ATCCATCGCA GGTGCAATTC

  Q  K  A  K  I  I  L  K  I  G  S  I  A  G  A  I
P  K  K  Q  R  S  Y  L  K  L  D  P  S  Q  V  Q  F
 P  K  S  K  D  H  T  -  N  W  I  H  R  R  C  N  S

         560         570         580         590         600
          |           |           |           |           |
TCATTGTTCT CATCGCTGTG GTTGGAGGAA TACTGTACCA AAGTGCCTGG

L  I  V  L  I  A  V  V  G  G  I  L  Y  Q  S  A  W
 S  L  F  S  S  L  W  L  E  E  Y  C  T  K  V  P  G
  H  C  S  H  R  C  G  W  R  N  T  V  P  K  C  L

         610         620         630         640         650
          |           |           |           |           |
ACCATTGAAC CCAAGCTGTG GATTATAGGA ACCATATATC CTATAGCTGG

T  I  E  P  K  L  W  I  I  G  T  I  Y  P  I  A  G
  P  L  N  P  S  C  G  L  -  E  P  Y  I  L  -  L
D  H  -  T  Q  A  V  D  Y  R  N  H  I  S  Y  S  W

         660         670         680         690         700
          |           |           |           |           |
CTACGGCCTG GGGTTTTTCC TGGCTAGAAT TGCTGGTCAA CCCTGGTACA

  Y  G  L  G  F  F  L  A  R  I  A  G  Q  P  W  Y
A  T  A  W  G  F  S  W  L  E  L  L  V  N  P  G  T
 L  R  P  G  V  F  P  G  -  N  C  W  S  T  L  V  Q

         710         720         730         740         750
          |           |           |           |           |
GGTGCCGAAC AGTTGCCTTG GAAACCGGGT TGCAGAACAC TCAGCTGTGT

R  C  R  T  V  A  L  E  T  G  L  Q  N  T  Q  L  C
 G  A  E  Q  L  P  W  K  P  G  C  R  T  L  S  C  V
  V  P  N  S  C  L  G  N  R  V  A  E  H  S  A  V

         760         770         780         790         800
          |           |           |           |           |
TCCACCATTG TGCAGCTTTC CTTCAGCCCT GAGGACCTCA ACCTTGTGTT

S  T  I  V  Q  L  S  F  S  P  E  D  L  N  L  V  F
  P  P  L  C  S  F  P  S  A  L  R  T  S  T  L  C
F  H  H  C  A  A  F  L  Q  P  -  G  P  Q  P  C  V
```

```
          810         820         830         840         850
           |           |           |           |           |
CACCTTCCCC CTCATCTACA GCATCTTCCA GATCGCCTTT GCAGCAATAC

  T  F  P  L  I  Y  S  I  F  Q  I  A  F  A  A  I
S  P  S  P  S  S  T  A  S  S  R  S  P  L  Q  Q  Y
 H  L  P  P  H  L  Q  H  L  P  D  R  L  C  S  N  T

          860         870         880         890         900
           |           |           |           |           |
TATTAGGAGC TTATGTCGCA TACAAGAAAT GTCATGGAAA AAATAATACT

L  L  G  A  Y  V  A  Y  K  K  C  H  G  K  N  N  T
 Y  -  E  L  M  S  H  T  R  N  V  M  E  K  I  I  L
  I  R  S  L  C  R  I  Q  E  M  S  W  K  K  -  Y

          910         920         930         940         950
           |           |           |           |           |
GAGCTACAAG AGAAAACAGA CAATGAAATG GAGCCCAGGT CATCATTTCA

E  L  Q  E  K  T  D  N  E  M  E  P  R  S  S  F  Q
  S  Y  K  R  K  Q  T  M  K  W  S  P  G  H  H  F
-  A  T  R  E  N  R  Q  -  N  G  A  Q  V  I  I  S

          960
           |
GGAGACAAAC AAAGGA

  E  T  N  K  G
R  R  Q  T  K
 G  D  K  Q  R
```

The conversion of nucleotides to amino acids were carried out using the software 'PC gene'. The software gave the three possible amino acid sequences depending on which amino acid was taken to represent the beginning of the gene sequence.

13.3 Sequence alignment of the pig ileal Na^+/bile acid co-transport protein with ileal Na^+/bile acid co-transport proteins sequenced from other mammals

To determine the degree of homology shared between the pig ileal Na^+/bile acid co-transport protein and the Na^+/bile acid co-transport proteins from the other five mammals, from which it had been sequenced, a sequence alignment was conducted as described in the methods section (7.2.12.3). Due to the design of the original primers, the amino acid sequence of the pig ileal Na^+/bile acid co-transport protein was lacking the first 20 amino acids. However, the lack of the first 20 amino acids from the pig did not detrimentally affect the sequence alignment. As can be seen in figure 13.2, the pig shared a high number of conserved amino acids with all the other species. However, as previously mentioned the pig ileal Na^+/bile acid co-transport protein shared the greatest number of conserved amino acids with the hamster ileal Na^+/bile acid co-transport protein. The degree of homology between the pig and hamster transport proteins was 92.2%, which corresponded to previous findings of homology between the hamster and rat (Dawson and Wong, 1995).

13.4 Determination of the number of transmembrane regions (TM)

To determine which parts of the amino acid sequence would be expected to be present in the brush border membrane and which would be expected to be present on the outside or inside of the membrane, the number and position of the

Figure 13.2 Sequence alignment of the ileal Na^+/bile acid co-transport protein, using amino acid sequences from the pig, hamster, rabbit, mouse, rat and human

```
ntcipig          ----------------------RMESNFNAILSVVMSTVLTILLALVMFSMGCNVELHKFL 39
ntcihamster      -MDNSSICNPNATICEGDSCIAPESNFNAILSVVMSTVLTILLALVMFSMGCNVELHKFL 59
ntcihuman        -MNDPNSCVDNATVCSGASCVVPESNFNNILSVVLSTVLTILLALVMFSMGCNVEIKKFL 59
ntcirabbit       MSNLTVGCLANATVCEGASCVAPESNFNAILSVVLSTVLTILLALVMFSMGCNVEIKKFL 60
ntcimouse        -MDNSSVCPPNATVCEGDSCVVPESNFNAILNTVMSTVLTILLAMVMFSMGCNVEVHKFL 59
ntcirat          -MDNSSVCSPNATFCEGDSCLVTESNFNAILSTVMSTVLTILLAMVMFSMGCNVEINKFL 59
                                       ***** **..*:*********:**********::***

ntcipig          GHLRRPWGIVVSFLCQFGIMPLTGFVLSVAFGILPVQAVVVLIQGCCPGGTASNILAYWV 99
ntcihamster      GHLRRPWGIVVGFLCQFGIMPLTGFVLSVAFGILPVQAVVVLIQGCCPGGTASNILAYWV 119
ntcihuman        GHIKRPWGICVGFLCQFGIMPLTGFILSVAFDILPLQAVVVLIIGCCPGGTASNILAYWV 119
ntcirabbit       GHIRRPWGIFIGFLCQFGIMPLTGFVLAVAFGIMPIQAVVVLIMGCCPGGTASNILAYWV 120
ntcimouse        GHIKRPWGIFVGFLCQFGIMPLTGFILSVASGILPVQAVVVLIMGCCPGGTGSNILAYWI 119
ntcirat          GHIKRPWGIFVGFLCQFGIMPLTGFILSVASGILPVQAVVVLIMGCCPGGTGSNILAYWI 119
                 **::***** :.**************:*:** .*:*:******* *******.*******:

ntcipig          DGDMDLSVSMTTCSTLLALGMMPLCLFIYTKMWVDSGTIVIPYDSIGTSLVALVIPVSIG 159
ntcihamster      DGDMDLSVSMTTCSTLLALGMMPLCLFIYTKMWVDSGTIVIPYDSIGTSLVALVIPVSIG 179
ntcihuman        DGDMDLSVSMTTCSTLLALGMMPLCLLIYTKMWVDSGSIVIPYDNIGTSLVALVVPVSIG 179
ntcirabbit       DGDMDLSVSMTTCSTLLALGMMPLCLYVYTKMWVDSGTIVIPYDNIGTSLVALVVPVSIG 180
ntcimouse        DGDMDLSVSMTTCSTLLALGMMPLCLFVYTKMWVDSGTIVIPYDSIGISLVALVIPVSFG 179
ntcirat          DGDMDLSVSMTTCSTLLALGMMPLCLFIYTKMWVDSGTIVIPYDSIGISLVALVIPVSIG 179
                 ************************** :*********:******.** ******:***:*

ntcipig          MYVNHKWPQKAKIILKIGSIAGAILIVLIAVVGGILYQSAWTIEPKLWIIGTIYPIAGYG 219
ntcihamster      MYVNHKWPQKAKIILKIGSIAGAILIVLIAVVGGILYQSAWTIEPKLWIIGTIYPIAGYG 239
ntcihuman        MFVNHKWPQKAKIILKIGSIAGAILIVLIAVVGGILYQSAWIIAPKLWIIGTIFPVAGYS 239
ntcirabbit       MFVNHKWPQKAKIILKVGSIAGAVLIVLIAVVGGILYQSAWIIEPKLWIIGTIFPMAGYS 240
ntcimouse        MFVNHKWPQKAKIILKIGSITGVILIVLIAVIGGILYQSAWIIEPKLWIIGTIFPIAGYS 239
ntcirat          MFVNHKWPQKAKIILKIGSIAGAILIVLIAVVGGILYQSAWIIEPKLWIIGTIFPIAGYS 239
                 *:***************:***:*.:*******:********* * *********:*:***.

ntcipig          LGFFLARIAGQPWYRCRTVALETGLQNTQLCSTIVQLSFSPEDLNLVFTFPLIYSIFQIA 279
ntcihamster      LGFFLARIAGQPWYRCRTVALETGLQNTQLCSTIVQLSFSPEDLNLVFTFPLIYSIFQIA 299
ntcihuman        LGFLLARIAGLPWYRCRTVAFETGMQNTQLCSTIVQLSFTPEELNVVFTFPLIYSIFQLA 299
ntcirabbit       LGFFLARIAGQPWYRCRTVALETGMQNTQLCSTIVQLSFSPEDLTYVFTFPLIYSIFQIA 300
ntcimouse        LGFFLARLAGQPWYRCRTVALETGMQNTQLCSTIVQLSFSPEDLNLVFTFPLIYTVFQLV 299
ntcirat          LGFFLARLAGQPWYRCRTVALETGMQNTQLCSTIVQLSFSPEDLNLVFTFPLIYTVFQLV 299
```

The multiple sequence alignment was carried out using the CLUSTERW software at EBI, as described in the methods section (7.2.12.3).

Tables 13.1-13.8 Toplogy of the ileal Na^+/bile acid co-transport protein

TM1		software	
Mammal	**HMMTOP**	**Top Pred2**	**TM pred**
Hamster	29-53 (o-i)	31-51(o-i)	29-47(o-i)
Human	29-53 (o-i)	31-51(o-i)	29-47(o-i)
Mouse	35-59 (o-i)	32-55(o-i)	35-50(o-i)
Pig	26-60 (o-i)	31-51(o-i)	29-47(o-i)
Rabbit	2-22 (o-i)	3-23(o-i)	3-21(o-i)
Rat	28-52 (o-i)	31-51(o-i)	32-50(o-i)

TM2		software	
Mammal	**HMMTOP**	**Top Pred2**	**TM pred**
Hamster	66-53(o-i)	75-95(o-i)	73-103(o-i)
Human	66-90 (o-i)	68-98(o-i)	66-86(o-i)
Mouse	72-96(o-i)	66-86(o-i)	65-86(o-i)
Pig	73-97 (o-i)	75-95(o-i)	56-83(o-i)
Rabbit	30-54 (o-i)	32-52(o-i)	30-48(o-i)
Rat	65-89 (o-i)	66-86(o-i)	65-86(o-i)

TM3		software	
Mammal	**HMMTOP**	**Top Pred2**	**TM pred**
Hamster	98-119 (o-i)	98-119 (o-i)	92-119(o-i)
Human	98-119 (o-i)	92-112(o-i)	85-115(o-i)
Mouse	105-126 (o-i)	89-109(o-i)	88-111(o-i)
Pig	98-119 (o-i)	99-119(o-i)	92-119(o-i)
Rabbit	67-91 (o-i)	79-96(o-i)	66-92(o-i)
Rat	98-119 (o-i)	89-109(o-i)	88-111(o-i)

TM4		software	
Mammal	**HMMTOP**	**Top Pred2**	**TM pred**
Hamster	129-148 (o-i)	129-149(o-i)	131-148(o-i)
Human	125-149 (o-i)	129-149(o-i)	129-149(o-i)
Mouse	136-155(o-i)	129-149(o-i)	129-149(o-i)
Pig	136-155 (o-i)	129-149(o-i)	129-149(o-i)
Rabbit	99-118 (o-i)	99-119(o-i)	130-150(o-i)
Rat	129-148(o-i)	129-149(o-i)	129-149(o-i)

TM5		software	
Mammal	**HMMTOP**	**Top Pred2**	**TM pred**
Hamster	158-182 (o-i)	164-184(o-i)	164-182(o-i)
Human	158-182 (o-i)	164-184(o-i)	164-182(o-i)
Mouse	165-189 (o-i)	164-184(o-i)	156-182(o-i)
Pig	158-182 (o-i)	164-184(o-i)	164-182(o-i)
Rabbit	158-182 (o-i)	126-146(o-i)	155-183(o-i)
Rat	159-183 (o-i)	164-184(o-i)	164-182(o-i)

TM6		software	
Mammal	**HMMTOP**	**Top Pred2**	**TM pred**
Hamster	192-216 (o-i)	196-216 (o-i)	196-216 (o-i)
Human	192-216 (o-i)	196-216 (o-i)	196-216 (o-i)
Mouse	199-223 (o-i)	196-216 (o-i)	196-216 (o-i)
Pig	199-223 (o-i)	196-216 (o-i)	196-215 (o-i)
Rabbit	192-217 (o-i)	165-185 (o-i)	197-217 (o-i)
Rat	159-183 (o-i)	196-216 (o-i)	196-216 (o-i)

TM7		software	
Mammal	**HMMTOP**	**Top Pred2**	**TM pred**
Hamster	226-245 (o-i)	225-245(o-i)	228-245(o-i)
Human	226-250 (o-i)	225-245(o-i)	218-241(o-i)
Mouse	223-252(o-i)	225-245(o-i)	227-244(o-i)
Pig	223-252(o-i)	225-245(o-i)	228-245(o-i)
Rabbit	193-217 (o-i)	197-217(o-i)	228-245(o-i)
Rat	226-245 (o-i)	225-245(o-i)	228-245(o-i)

TM8		software	
Mammal	**HMMTOP**	**Top Pred2**	**TM pred**
Hamster	257-276 (o-i)	285-305(o-i)	291-311(o-i)
Human	258-279 (o-i)	290-310(o-i)	292-310(o-i)
Mouse	264-283(o-i)	285-305(o-i)	292-310(o-i)
Pig	277-246 (o-i)	285-305(o-i)	291-311(o-i)
Rabbit	277-246 (o-i)	224-246(o-i)	293-311(o-i)
Rat	285-309 (o-i)	290-310(o-i)	291-311(o-i)

KEY: TM = Transmembrane

(o-i) = Outside of the enterocyte (luminal membrane)- inside of the enterocyte (cytoplasmic membrane)

The topology of the protein was determined by entering the transporter amino acid sequence for each mammal into each of the programmes. These software's then determined the number of transmembrane regions for each amino acid sequence and the position of the amino acids that started and finished each of these regions.

transmembrane regions for all six mammals were predicted using three different types of software as described in the methods section (7.2.12.4). As shown in table's 13.1-13-8 all three pieces of software had variations in the start and end positions of the transmembrane regions, though a general trend could be observed (table 13.9). The determination of the start of the transmembrane regions for the rabbit ileal Na^+/bile acid co-transport protein was vastly different compared to the other five species. The beginning of transmembrane region 1 (TM1) started with amino acid 2 compared to 29 for the others, which had the effect of changing the predictions of the other transmembrane regions. Therefore, the results for the rabbit were discarded and not used to determine the general topology of the transporter.

Table 13.9 General topology of the ileal Na^+/bile acid co-transport protein

Transmembrane domain	TM1	TM2	TM3	TM4	TM5	TM6	TM7	TM8
Amino acid number	29-53	65-90	92-119	129-149-	158-184	196-216	225-245	285-311

The analysis showed that ileal Na^+/bile acid co-transport protein contained eight transmembrane regions (table 13.9), which corresponded to previous findings by Hallen *et al.*, (1996) but contradicted the findings of Dawson and colleagues who predicted that the transport protein had seven transmembrane regions. The amino acids not included in the predicated transmembrane regions corresponded to amino acids either on the outside of the brush border membrane (in the lumen of the small intestine) or on the inside of the brush border membrane (in the cytoplasm of the enterocyte).

13.5 2D modelling of the ileal Na^+/bile acid co-transport protein

To predict the 2D model of the transport protein, an amino acid sequence and the number of transmembrane regions were added into the 2D MEM MAP software (Antoniw and Mullins, 2000). This software produced a 2D model of the protein (figure 13.3) and could be used to display the distribution of hydrophobic, polar, basic and acidic amino acids. In figure 13.3, any amino acids that were not conserved between the 6 species were blocked.

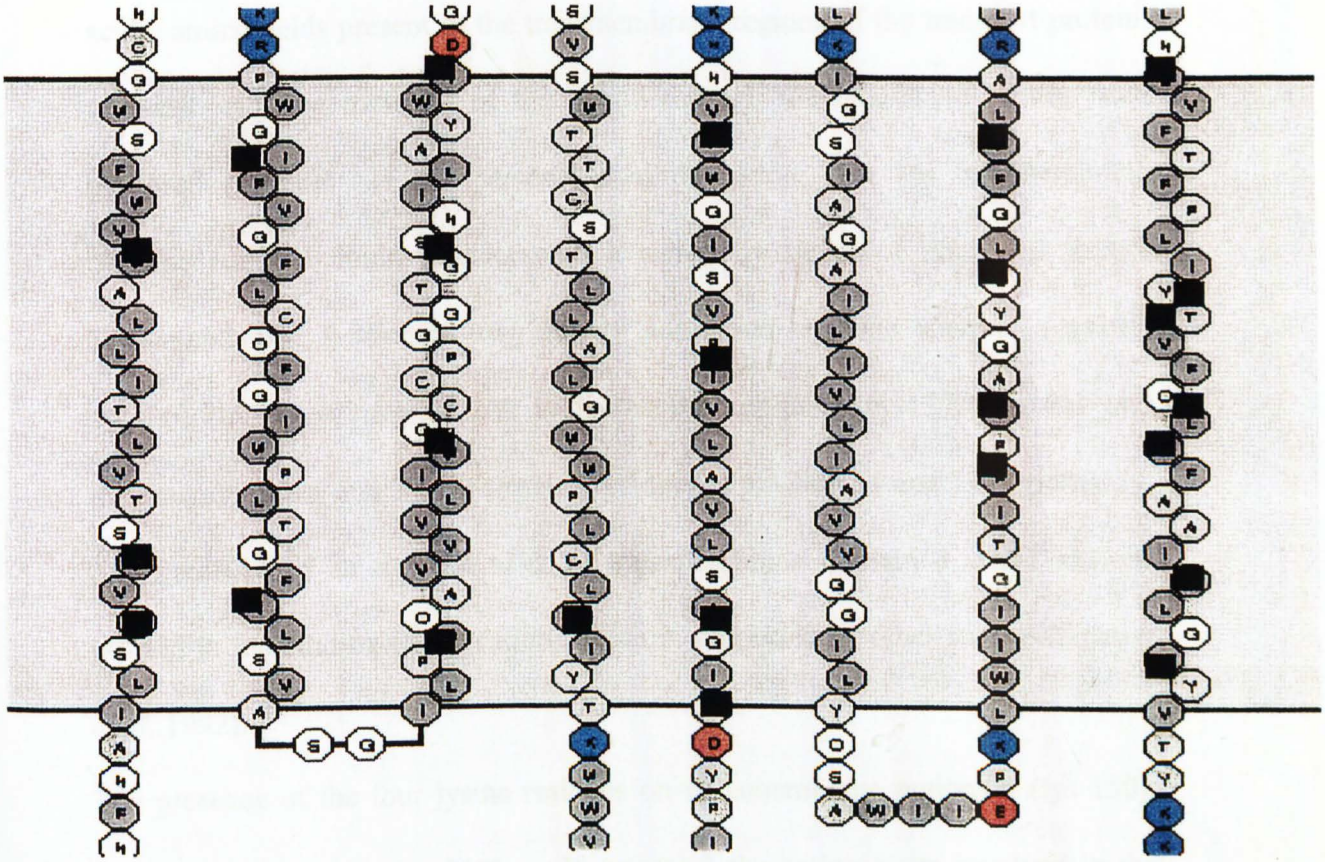
As can be seen in figure 13.3, the transmembrane regions largely consisted of hydrophobic amino acids (shown in grey), which is consistent with these amino acids residing in the hydrophobic brush border membrane.

Large bulky amino acids such as phenylalanine, tryptophone and tyrosine have long been proposed to be involved in interactions between with glycine residues on other transmembrane helices. The bulky aromatic side chain of the phenylalanine on one transmembrane helix has been proposed to fit into a gap created by a small glycine residue on another transmembrane domain, known as the ridge-groove arrangements (Chothia *et al.*, 1981). Transmembrane regions 2, 3 and 6 have 3-4 glycine residues, whilst transmembrane regions, 1,2 ,4 and 8 all have phenylalanine residues at a compatible depth. Therefore, it is possible that interactions between these putative transmembrane helices could occur within the brush border membrane.

The presence of the acidic amino acids, asparatic acid (D) and glutamatic acid (E) on the outside of the brush border membrane (in the lumen of the small intestine) appeared to be important in relation to the binding of the cation. The ileal Na^+/bile

Figure 13.3, 2D directional alignment of the ileal Na^+/bile acid co-transport protein

INSIDE OF ENTEROCYTE



OUTSIDE OF ENTEROCYTE

KEY: Amino acids blocked out are unconserved residues

To determine the 2D structure of the ileal Na^+/bile acid co-transport protein the software 2D MEM MAP was used. The amino sequence of the transport protein was entered and the settings for the position and number of transmembrane regions were altered to conform with this studies findings. The 2D model of the transport protein was produced by using the topology section of the 2D MEM MAP software.

acid co-transport protein has been shown to have an anionic binding site, which binds the positively charged cation (Hardison *et al.*, 1991: Kramer *et al.*, 1992). From the 2D model of the transport protein, the two negatively charged amino acids could be the proposed binding site for the cation. However, there are no acidic amino acids present in the transmembrane regions of the transport protein, to assist with the transport of the cation through the membrane. It has been proposed (Mullins *et al.*, personal communication) that the intramembrane cysteine residues could interact with a cation by virtue of their -SH group. Transmembrane 3 contains two highly conserved cysteine residues (cys105, cys106), transmembrane 4 has two cysteine residues (cys132, cys144) and transmembranes 1 and 2 have cysteine residues at position 51 and 74 respectively. The presence of an aspartic acid on transmembrane domain 3 could also be utilised in the binding of the cation, which is supported by other studies (Kramer *et al.*,1992).

The presence of the four lysine residues on transmembrane regions 4 (lys 150), 7(lys 223), 8(lys 312, lys 313) could represent the cationic site involved in the binding of the negatively charged bile acid to the protein in the lumen of the small intestine. The 2 consecutive lysine residues at the interfacial region of domain 8, have also been observed in hepatic canalicular ecto-ATPase, and could be a conserved feature of bile acid transport proteins (Mullins *et al.*,1998). On the cytoplasmic side of the transport protein, the basic amino acid arginine is present on transmembrane regions 2 (arg 91), and 7 (arg 246), whilst lysine and histidine are present on transmembrane regions 2 (lys 92), 5 (lys 185, his 184) and 6

(lys217). These 6 positively charged amino acids could also be involved in the transport of bile acids and could represent a possible binding site required for the bi-directional transport of bile acids out of the enterocyte.

13.6 Summary

The nucleotide sequence of the pig ileal PCR product, was converted to amino acids by using the software PC gene. To identify the protein for which the PCR product coded for, a BLAST search was carried out. The BLAST search revealed that the PCR product encoded an ileal Na^+/bile acid co-transport protein, which shared the greatest degree of homology with the hamster ileal Na^+/bile acid co-transport protein.

Computer modelling of the amino acid sequence, determined that the pig ileal Na^+/bile acid co-transport protein contained of 8 transmembrane regions. The proposed cation binding sites on the transport protein were the acidic amino acids, asparatic acid (D) and glutamatic acid (E) on the outside of the brush border membrane. The presence of the four lysine residues on transmembrane regions 4 (lys 150), 7(lys 223), 8(lys 312, lys 313) could be the proposed cationic site involved in the binding of the negatively charged bile acid. Possible cation and bile acid binding sites are also present on the cytoplasmic side of the transport protein, possibly facilitating in the bi-directional transport of bile acids.

CHAPTER FOURTEEN:

From this study it has been concluded that the pig ileal Na^+/bile acid co-transport protein does not have a strict dependency on Na^+, as previously proposed, and can use a range of cations for secondary active bile acid transport. This study has shown that ileal Na^+/bile acid co-transport protein is able to use K^+ for secondary active transport, which could have important physiological functions. In the terminal ileum there are low Na^+ concentrations, due to Na^+ being used for a wide range of transport processes in the duodenum and jejunum. Furthermore, within the lumen of the terminal ileum there is a high concentration of K^+, which can be used for the secondary active transport of bile acids.

Therefore, from the observations made in this study, it has been proposed that the ileal Na^+/bile acid co-transport does not have a strict dependency on Na^+, but rather a preference for this cation and can use other cations, such as K^+, for secondary active transport of bile acids. The following sections in this chapter explain how the final conclusion was reached.

14.1 Characterisation of the pig intestinal BBMV

Using enzyme enrichment assays pig intestinal BBMV were shown to have had an enrichment of 14-16 fold (table 8.1), which had previously been shown to be sufficiently pure to be used in uptake assays (Mullins *et al.*,1992; Lin *et al.*,1993; Kramer *et al.*,1993). Electron microscopy (figure 8.1) of the BBMV showed that the microvilli were on the outside of the BBMV and therefore would allow the transport of solutes into the vesicles.

The observation that BBMV isolated from all regions of the small intestine were able to actively transport D-glucose in the presence of an inwardly directed Na^+ gradient (figure 8.2) confirmed the functional viability of the BBMV. Furthermore, the distribution profile and cation dependency of D-glucose uptake into the BBMV corresponded to previous studies (Kessler *et al.*, 1978, Aoshima *et al.*,1987; Mullins *et al.*, 1992) and showed that the brush border membrane contained the SGLT1 transport protein.

Alterations in the osmotic potential of the BBMV (figure 9.1) showed that the BBMV were transporting taurocholate into the interior of the BBMV and that there was no binding of taurocholate to the surface of the BBMV.

Therefore, it was concluded that the BBMV isolated from pig small intestine could be used for functional characterisation of the Na^+/bile acid co-transport protein.

14.2 Functional characteristics of the Na^+/bile acid co-transport protein in BBMV

Using BBMV isolated from the duodenum, jejunum and ileum it was shown that Na^+/bile acid co-transport was localised to the ileum (figure 9.2), which corresponded to previous findings (Wilson, 1981; Kramer *et al.*,1983; Mullins *et al.*, 1992; Sorscher *et al.*, 1992; Wong *et al.*,1994).

However, the elevated uptake of taurocholate above passive diffusion levels in the presence of K^+ had not been observed in previous studies investigating the ileal Na^+/bile acid co-transport protein (figure 9.2).

To determine if taurocholate uptake in the presence of K^+ was a secondary active transport mechanism, the functional characteristics of the transport process were investigated further and compared to the functional characteristics of Na^+ dependent uptake of taurocholate.

Taurocholate uptake in the presence of either Na^+ or K^+ was found to be temperature sensitive (table 9.1). The temperature sensitivity of bile acid transport in the presence of Na^+ had been demonstrated in previous studies, with the inference that such sensitivity is indicative of an active transport process (Holt, 1964; Kramer *et al.*, 1994).

Previous studies have shown that the Na^+ /bile acid co-transport protein was able to transport bile acids against their concentration gradient (Lucke *et al.*,1978; Rouse and Lack, 1979). The removal of the inwardly directed taurocholate gradient (table 9.2) did not affect the transport of taurocholate into the BBMV in the presence of Na^+ or K^+. Furthermore, the uptake of taurocholate in the presence of both Na^+ and K^+ demonstrated an 'overshoot' (figure 9.3) which had previously been shown to only occur in the presence of active transport process (Mullins *et al.*, 1992; Kramer *et al.*,1992;1993). This observation again indicated that there was secondary active transport of taurocholate in the presence of Na^+ and K^+.

Secondary active bile acid transport has been shown to be dependent on the presence of an inwardly directed Na^+ gradient, with the removal of this gradient resulting in the reduction of taurocholate transport rates to that of passive diffusion (Lack and

Weiner, 1961; Holt, 1964; Mullins *et al.*, 1992; Kramer *et al.*, 1993*)*. In this study, the removal of both the inwardly Na^+ and K^+ gradients (figure 9.4) had the effect of reducing taurocholate transport to passive diffusion levels, which indicated that the inwardly directed Na^+ and K^+ gradients were essential for the secondary active transport of bile acids.

Therefore, the functional characterisation of taurocholate transport using ileal BBMV indicated that secondary active taurocholate transport could occur in the presence of Na^+ and K^+. Taurocholate transport in the presence of Na^+ was proposed to be occurring via the Na^+/bile acid co-transport protein. However, the observation of secondary active taurocholate transport occurring in the presence of K^+ had not been discussed in previous studies. The functional characterisation of taurocholate transport in the presence of Na^+ and K^+, did not, in itself, indicate whether the same transport protein was utilising both cations for secondary active transport or whether two different proteins were involved.

14.3 Determination of whether Na^+ and K^+ dependent taurocholate transport occurrs by the same transport protein

To determine if the same transport process utilised both Na^+ and K^+ for secondary active bile acid transport, inhibition profiles and substrate dependence, Michaelis Menten kinetics of the transport processes were investigated using ileal BBMV.

The inhibition of taurocholate uptake by the addition of another bile acid, taurodeoxycholate, occurred in the presence of both Na^+ and K^+. The competitive

inhibition of one bile acid by another has been observed in previous studies investigating Na^+ dependent bile acid transport by the Na^+/bile acid co-transport protein (Krag and Philips,1974; Schiff *et al.*, 1972; Wong *et al.*,1994). The inhibition of Na^+ and K^+ dependent taurocholate uptake, by the addition of taurodeoxycholate, both occurred to a similar percentage (table 9.3), and this was an initial indication that the uptake of taurocholate was probably occurring by the same transport process in the presence of either cation.

Secondary active bile acid transport in the presence of Na^+ and K^+ both displayed Michealis Menten kinetics. The affinity (Km) of taurocholate transport in the presence of Na^+ and K^+ was 20 and 60μM respectively (figure 10.2). This showed that the transport protein had a greater affinity for Na^+, but could use either Na^+ and K^+ to drive secondary active transport.

The maximal rate of taurocholate transport (Vmax) was 25 pmol/mg prot/s in the presence of Na^+ and 8.5 pmol/mg prot/s in the presence of K^+. The observation of increased velocity, generally 2-3 fold higher in the presence of Na^+ when compared to K^+, had been observed in all the BBMV experiments carried out in this study. The difference in transport rate in the presence of Na^+ and K^+ was not due to the affinity of taurocholate binding to the transport protein in the presence of the two different cations, as initially proposed, because the affinity (Km) of the transport proocess was 55mM for both cations (figure 10.3). This observation again indicated that

taurocholate was being transported by the same transport protein in the presence of either Na^+ or K^+.

Investigation of the stoichiometry of taurocholate uptake in the presence of Na^+ or K^+ showed that bile acid uptake was an electroneutral process with respect to both cations. A Hill plot (figure 10.5) of taurocholate uptake gave a ratio of 1:1 for both cations and taurocholate, which previous studies had shown to represent an electroneutral process (Wilson, 1981). Furthermore, the rate of taurocholate uptake was not affected by the alteration of the anion coupled to the cation (figure 10.7) or by the addition of valinomycin (figure 10.6), which again correlated with an electronetural transport process (Rouse and Lack, 1979; Wilson, 1981; Barnard and Ghishan, 1987). The observation that transport in the presence of Na^+ or K^+ was electroneutral again indicated that the same transport protein was able to use either Na^+ or K^+ for secondary active transport of bile acids.

Therefore, from these transport studies using BBMV isolated from pig ileum, it was concluded that rather than being a strictly Na^+-dependent process, the ileal Na^+/bile acid co-transport merely displayed a strong preference for Na^+, but could also utilise K^+ as a driving cation. This observation was proved by the similar accumulative concentration or "overshoot" profile, similar inhibition percentage of taurocholate uptake by taurodeoxycholate in the presence of either cation, the same stoichiometry and the close inverse relationship between the values of Vmax and Km observed in the presence of the two cations.

The ability of the Na^+/bile acid co-transport protein to utilise K^+ for secondary active taurocholate transport had not been clearly observed in previous studies.

From the data obtained in this study, it has been proposed that the Na^+/bile acid co-transport protein is able to also utilise K^+ for bile acid transport, due to the low concentration of Na^+ present within the terminal ileum. Throughout the small intestine Na^+ is utilised for a wide range of transport process, with the majority of these processes being present in the brush border membrane of duodenal and jejunal enterocytes. To ensure maintenance of an inwardly directed Na^+ gradient into the duodenal and jejunal enterocytes, the ATPase present on the basolateral membrane of enterocytes removes Na^+ from the cells and replaces it with K^+ (Clausen, 1998). Therefore, within intestinal enterocytes there is a low concentration of Na^+ and a high concentration of K^+. In this study, it has been proposed that the high concentration of K^+ within the enterocytes will pass through the brush border membrane and enter the lumen of the small intestine by the process of passive diffusion. Therefore, within the terminal ileum, the concentration of Na^+ is severely depleted whilst the concentration of K^+ is relatively high. From the data presented in this study, it is proposed that the protein can utilise another cation, K^+, to ensure sufficient absorption of bile acids to allow the maintenance of enterohepatic circulation.

14.4 Secondary active bile acid transport can be stimulated by other cations

The observation that secondary active taurocholate transport was observed in the presence of Na^+ and K^+ and that the same transport protein appeared to be utilising

both cations for bile acid transport, lead to the investigation into what other cations could induce secondary active transport of taurocholate. Using a range of cations it was determined that cations which had an ionic radius between 0.8 – 1.33Å (figure 10.4) stimulated taurocholate transport above the rates of passive diffusion.

Interestingly, the cations Ca^{2+} and Cd^{2+} were able to stimulate taurocholate transport to the same degree as Na^{+}. Whether Ca^{2+} was driving the uptake of taurocholate through an active transport protein, or merely enhancing observed rates of uptake through increasing the membrane potential of the vesicles, remains to be established. The high transport rates in the presence of Cd^{2+}, though interesting in terms of the consideration of the structure / function relationships of the transport process, are unlikely to be of any physiological importance. However, work into K^{+} channels have revealed that Cd^{2+} forms a bridge between amino acids residues cysteine and histidine which results in the opening of the K^{+} channel (Holmgren *et al.*, 1998). Work into the 3D structure of the intestinal bile acid transport protein has determined that the cation binding site of the transport protein could be composed of cysteine residues (Mullins *et al.*, unpublished data), as proposed in this study. Therefore, Cd^{2+} could be binding to cysteine residues, resulting in the opening/stimulation of the transport protein.

How the size of the cation affects active bile acid transport has yet to be proved, but the diameter of the pore of the transport protein (formed by the gap between transmembrane helices) could be involved in cation transport. Computer modelling of

the ileal Na^+/bile acid co-transport protein has revealed that the proposed pore of the transport protein, which the cations pass through, could have a diameter in the region of 2Å. Therefore, it could be speculated that for monovalent cations with ionic radii over the threshold of 0.8 Å, the velocity and affinity of transport is directly related to the size of the cation involved in driving the transport of bile acids, with the implication that Na^+ and K^+ use the same route. By this relationship, the relatively large ionic diameter of Rb^+ and Cs^+ prohibits the co-transport of bile acids, presumably by being too large to pass through the cation pore of the co-transport protein, whilst H^+ will be too small to stimulate the protein to transport bile acids.

The ability of Na^+ or K^+ to pass through the proposed cation pore in the Na^+/bile acid co-transport protein could be related to the difference in the rate of taurocholate transport in the presence of the two cations. Assuming spherical ionic dimensions the volume of the K^+ ion is $(1.33Å/0.95Å)^3 = 2.74$ times larger than the Na^+ ion, so the size of the cation appeared to be inversely related to the uptake rates observed. This observation indicated that the size of cation could control the rate of secondary active transport, with K^+ being nearly 3 times larger than Na^+ so leading to a 2-3 fold decrease in stimulation of taurocholate uptake.

14.5 Expression of the Na^+/bile acid co-transport protein in *Xenopus laevis* oocytes

The results obtained using ileal BBMV, gave an initial indication that the same transport protein was able to use both Na^+ and K^+ to transport bile acids. To ensure that the same transport protein was using both Na^+ and K^+ for secondary active bile

acid transport, the transport protein was expressed in *Xenopus laevis* oocytes in order to investigate the functional characteristics of the protein.

Expression of ileal mRNA in oocytes resulted in the elevated uptake of taurocholate in the presence of Na^+ and K^+ when compared to choline (figure,11.1). This showed that the protein expressed following injection of mRNA utilised K^+ as well as Na^+ for cation dependent transport.

To confirm if the transport process being expressed by ileal mRNA in oocytes was using both Na^+ and K^+, inhibition studies, Michaelis Menten kinetics and hybrid translation arrest experiments were carried out. The addition of the bile acid taurodeoxycholate inhibited taurocholate uptake into *Xenopus laevis* oocytes by 53% in the presence of Na^+ and 58% in the presence of K^+ (figure 11.2), which again indicated that the same ileal transport protein was using both cations for secondary active transport.

Michaelis Menten kinetics of Na^+ and K^+ dependent taurocholate transport (figure 11.3) revealed that the Km for taurocholate was 20μM in the presence of both cations, with the Vmax for Na^+ being 35 pmol/oocyte/hr compared to16.6 pmol/oocyte/hr for K^+. The identical Km values again indicated that the same transport protein was utilising both Na^+ and K^+ for secondary active transport. The difference in Vmax correlated to the previous findings in ileal BBMV that taurocholate uptake in the presence of Na^+ was 2-3 fold higher than for K^+. Therefore, the functional characterisation of the expression of ileal mRNA in *Xenopus laevis* oocytes supported

the BBMV data that the Na^+/bile acid co-transport protein appeared to be able to also use K^+ for secondary active transport of bile acids.

To gain molecular confirmation that the same transport protein was responsible for transport in the presence of both cations, hybrid translation arrest of the mRNA encoding the pig ileal Na^+/bile acid co-transport protein was carried out by the addition of a cDNA, which specifically encoded the hamster Na^+/bile acid co-transport protein, to pig ileal mRNA. The addition of the hamster cDNA had the affect of reducing Na^+ and K^+ dependent taurocholate to that of passive diffusion levels after expression in oocytes (figure 11.6). Therefore, the addition of the hamster cDNA had the affect of preventing the translation of the mRNA encoding the pig ileal Na^+/bile acid co-transport protein, which reduced taurocholate uptake in the presence of Na^+ and K^+ to passive diffusion. This finding indicated that the same protein was responsible for bile acid transport in the presence of Na^+ or K^+.

14.6 Identification of a possible carrier mediated bile acid transport process in pig jejunum

The injection of mRNA isolated from the duodenum, jejunum and ileum of the pig small intestine revealed that the mRNA encoding for the Na^+/bile acid co-transport protein was present in the ileum and duodenum (figure 11.5), which corresponded to previous work (Mullins *et al.*, 1992). However, the jejunal rate of Na^+ dependent taurocholate transport was reduced when compared to the rate of Na^+ dependent taurocholate transport in the duodenum and ileum. Furthermore, the rate of K^+

dependent taurocholate transport in the jejunum had similar levels to that observed in the ileum. The increased rate of jejunal taurocholate uptake in the presence of K^+, corresponded to the data seen in pig ileal BBMV (figure, 9.2). These findings indicated that a different transport mechanism could be present in the pig jejunum. This transport mechanism could be the carrier mediated transport mechanism which had been observed in the jejunum of other mammals (Amelsberg *et al.*, 1995; 1996).

14.7 Isolation and characterisation of the gene which encoded the pig ileal Na^+/bile acid co-transport protein

To enable further investigation of the gene which encoded the pig Na^+/bile acid co-transport protein, the region of DNA that coded for the protein was isolated from the rest of the genomic DNA.

Using the conserved regions between the nucleotide and amino acid sequences (figure 12.1) from the hamster, human and rat, 4 primers were designed (table 12.1 A –D) which corresponded to the N and C terminal regions of the Na^+/bile acid co-transport protein. The 2 sets of primers (both sets had a forward and reverse primer) both isolated a PCR product from the pig ileal DNA (figure, 12.2). The PCR products, produced by the two sets of primers were about 1-1.2 Kb in size which corresponded to PCR products isolated from hamster and human DNA, which encoded the Na^+/bile acid co-transport protein (Wong *et al.*, 1994). Furthermore, the PCR product isolated from the pig was the same size as the positive control, which was a cDNA that encoded the hamster Na^+/bile acid co-transport protein.

To functionally confirm that the isolated PCR product encoded the Na^+/bile acid co-transport protein, hybrid translation arrest of the mRNA which encoded Na^+/bile acid co-transport protein was carried out by the addition of the pig ileal PCR product to the mRNA. The mRNA/DNA complex was then expressed in *Xenopus laevis* oocytes, and analysis of Na^+ and K^+ dependent taurocholate (figure 12.3) revealed that the transport rates had been reduced to that of passive diffusion levels. This showed that the PCR product isolated from pig ileal DNA shared sufficient homology to bind to the mRNA that encoded the Na^+/bile acid co-transport protein. This observation indicated that the PCR product isolated from pig DNA coded for the transport protein.

The isolation of just one PCR transcript from the total pig genomic DNA supported the translation hybrid arrest data, in that only one gene encoded pig ileal Na^+/bile acid co-transport and that this protein was capable of utilising both Na^+ and K^+ for secondary active transport of taurocholate.

The pig ileal PCR product was cloned into a plasmid and the size of the DNA insert analysed by restriction digestion (figure 12.5). From the 50 clones analysed, only two clones contained an insert which corresponded to the size of the PCR product. These 1-1.2 Kb clones were proposed to be the PCR products isolated from the pig ileum and were sent away to MWG Biotech for nucleotide sequencing.

14.8 Molecular modelling of the pig intestinal Na^+/bile acid co-transport protein

The nucleotide sequence obtained from the sequencing company, MWG Biotech, was converted into an amino acid sequence (using the PC gene software (figure 13.1)) and

the protein was identified using the BLAST software. Sequence alignment (CLUSTALW) of the pig ileal Na^+/bile acid co-transport protein (figure 13.1) with sequences of the ileal Na^+/bile acid co-transport protein from other species revealed that the pig protein shared a 92% homology with the hamster Na^+/bile acid co-transport protein. A greater degree of sequence homology would possibly have been expected between the pig and human transport proteins, due to similar physiology. However, the human transport protein had the least number of conserved amino acids of the six species when compared with the pig sequence, though it still shared a 84% homology with the hamster (Dawson *et al.*, 1995).

Using amino acid sequences of the Na^+/bile acid co-transport protein from the 6 species for which it is known, it was determined that the protein had 8 transmembrane regions (tables 13.1-13.9), which agreed with previous findings (Hallen *et al.*, 1996). The proposed cation binding site on the protein consisted of asparatic acid (D) and glutamatic acid (E) present on transmembrane domains 5 and 7 respectively (Mullins *et al.*, personal communication). The presence of cysteine residues of transmembrane domains 1, 2, 3 and 4 have been proposed to be used in the transport of the cation through the membrane protein (Mullins *et al.*, unpublished data). Therefore, if these cysteine residues were important in the transport of the cation, then these residues would have to be in close alignment to allow the cation to pass through the membrane, by association with each of these amino acids in turn. From this observation, it could be proposed that these 4 transmembrane domains could form a

pore for the cation to pass through. These possible intramembrane binding sites could be used for the bi-directional transport of a cation into and out of the enterocyte, which would correspond to the bi-directional bile acid transport observed in this study (figure, 10.7).

The presence of the four lysine residues on the approaches to transmembrane regions 4 (lys 150), 7(lys 223) and 8(lys 312, lys 313) could be the proposed cationic site involved in the binding of the negatively charged bile acid To enable the bile acid to bind, these 3 transmembrane domains would have to be in an arrangement compatible with association with specific groups on the bile acid molecule. Furthermore, the presence of 6 basic amino acids on the cytoplasmic loops of the protein indicated that these regions could also be involved in bile acid binding. The presence of the 3 lysine residues near the ends of transmembrane domains 2 (lys 92), 5 (lys185) and 6 (lys217) represent the residues most likely to be involved in bile acid binding on the cytoplasmic side. The presence of a possible bile acid binding site on the inside of the membrane again supports the possibility of bi-directional transport observed in this study.

The presence of only one proposed cation pore, would indicate that the other cations, namely K^+ and Ca^{2+}, would also bind to these cysteine residues when being utilised to stimulate secondary active uptake of taurocholate. The affinity of taurocholate for the transport protein in the presence of Na^+ and K^+ has been shown to be the similar in BBMV and the same in oocytes (figure, 10.3, figure 11.4). Therefore, it could be proposed that the binding of the other cations (shown to stimulate secondary active

bile acid transport) to the proposed cation binding site should also stimulate the binding of taurocholate to the transport protein.

An important difference between the pig and hamster amino acid sequence could be the replacement for a glycine residue (present in all other species) for a serine residue (ser 71) in the pig. As mentioned previously (section 13.5), the presence of glycine on transmembrane domain 2 could be used in the formation of 'ridge-grooves' attachments between the transmembrane helices. Therefore, the absence of this glycine side chain could lead to some disruption in the arrangement of the helices involved in the cation pore. Furthermore, the replacement of the smallest amino acid glycine, with a larger amino acid (serine) could also have the effect of increasing the size of the proposed cation pore. This possible increase in the cation transport pore could explain why the pig ileal Na^+/bile acid co-transport protein can transport the larger cation K^+.

The findings of this study give further insight to functional properties of the ileal Na^+/bile acid co-transport protein. The presence of this ileal transport protein has been shown to be essential in the maintenance of efficient enterohepatic circulation of bile acids and can be involved in protecting the liver for cholestastic damage due to an accumulation of bile acids. The functional characterisation of the ileal transport has prompted recent investigations into using the transport protein to alter the circulating bile acid pool and also to deliver small peptides into the blood.

14.9 Future work:

14.9.1 Functional characterisation of the amino acids involved in the binding of the cation or bile acid to the Na^+/bile acid co-transport protein

Analysis of the amino acid sequence of the transport protein, in this study, has led to the identification of a number of amino acids proposed to be involved in cation or bile acid binding. To confirm which amino acids are involved in binding and transport, the amino acids that encode the proposed binding sites would need to be deleted and the ability of the Na^+/bile acid co-transport protein to transport either the cation or the bile acid would then be investigated.

To delete the amino acids proposed to encode the binding sites, the triplet codon of nucleotides, which encode the amino acids, would need to be altered to ensure that the nucleotides coded for a different amino acid. The alteration of the nucleotides could been done by site directed mutagenesis,.

The new PCR product could then be either be converted to protein by a transcription/translation technique, or be cloned into a Bluescript plasmid, which can be used to produce cRNA.

Functional characterisation of the new PCR product could be carried out by expression of cRNA in *Xenopus laevis* oocytes or expression of the protein in pancreatic microsomes. The transport capabilities of the new PCR products could be determined using the rapid stop filtration technique, as previously used in this study on BBMV and oocytes.

14.9.2 Further characterisation of the transport process identified in pig jejunum

Investigation into the distribution of the Na^+/bile acid co-transport protein, revealed that another bile acid transport process appeared to be present in the pig jejunum. From the data obtained in this study, it was proposed that this transport process was the carrier mediated transport process previously identified in other mammals, but not pigs. Therefore, further functional characterisation of the transport process is required to determine if it is a carrier mediated transport process and if it has any dependence on cations or anions. Furthermore, investigation into a gene that could code for this transport process, could allow comparison for any homology with the ileal Na^+/bile acid co-transport protein.

A

Abdullah.M., Whelan.W.J., Catley.B.J. (1977) The action pattern of human salivary alpha-amylase in the vicinity of the branch points of amylopectin. *Carbohydrate Research* **57**, 281-290

Acri.S.A., Ghishan.F.K. (1990) Active bile salt transport in the ileum: characteristic and ontogeny. *Journal of Paediatric Gastroenterology and Nutrition* **10**, 421-425

Adibi.S.A., Young.S.K. (1981) Peptide absorption and hydrolysis. *Physiology of the Gastrointestinal Tract* 1073-1095 . Edited by L.R.Johnson, Raven Press, New York

Ahrens.E.H., Borgstrom.B. (1955) Exchange of free fatty acids and glyceride fatty acids during fat digestion in the human intestine. *Second International Conference on Biochemical Problems of Lipids*, 665-675

Aiso.M, Takikawa.H, Yokote.M, Sano.N, Kitaura.K, Uegaki.S, Yamanaka.M (1996) Absorption of ursodeoxycholate and tauroursodeoxycholate in the rat intestine. *Bile acid meeting*, **38** Falk Symposium

Aldini.R., Roda.A., Lenzi. P., Ussia.G., Vaccari.M., Mazzell.D., Bazzoli.F., Galletti.G., Casanova.S., Montagnani.M., Roda.E.(1992) Bile acid active and passive ileal transport in the rabbit. *European Journal of Clinical Investigation* **22**, 744-750

Aldini.R., Roda.A., Montagnani.M., Roda.E. (1995) Bile acid structure and intestinal absorption in the animal model. *Italian Journal of Gastroenterology* **27**, 141-144

Aldini.R., Montagnani.M., Roda.A., Herlia.S., Biagi.P., Roda.E. (1996) Intestinal absorption of bile acids in the rabbit: Different transport rates in jejunum and ileum. *Gastroenterology* **110**, 459-468

Agellon.L., Torchia. E. (2000) Intracellular transport of bile acids. *Biochimica et Biophysica Acta (Netherlands)* **1486**, 198-209

http://www.alkami.com/primers/refprmr.htm Alkami Biosystems: Primer design Tips

Amelsberg. A., Schteingart. C.D., Hofmann. A.F. (1995) Carrier mediated absorption of conjugated bile acids in the jejunum of guinea pigs. *Bile Acids in Gastroenterology* **19,** 195-196

Amelsberg.A., Jochims.C., Richter.C.P., Folsch.U.R., Klinink.M. (1996) Jejunal absorption of conjugated bile acids. *Bile acid meeting*, **39** Falk Symposium

Ananthanarayanan.M., Von Dippe.P., Levy.D.(1988) Identification of the hepatocyte Na^+ dependent bile acid transport protein using monoclonal antibodies. *The Journal of Biological Chemistry* **263**, 8338-8343

Ananthanarayanan.M., Bucuvalas.J.C., Schneider.B.L., Sippel.C.J., Suchy.F.J. (1991) An ontogenetically regulated 48 kDa protein is a component of the Na^+ bile acid co-transporter of rat liver. *American Journal of Physiology* **261**, G810-17

Antoniw.J., Mullins.J . (unpublished data) 2d MEM MAP: A web enabled graphic user interface for viewing and analysing the 2D structure of membrane proteins.

Aoshima.H., Ishii.H., Anan.M. (1987) Expression of the functional D-glucose transport system in *Xenopus laevis* oocytes injected with mRNA of rat small intestine. *Brain Research* **388,** 263-267

Aoshima.H., Tomita.K., Sugio.S. (1988) Expression of amino acid transport systems in *Xenopus laevis* oocytes injected with mRNA of the rat small intestine and kidney. *Archives of Biochemistry and Biophysics* **265**, 73-81

www.amersham.com/life/lcat/nalan/tech_tip/tchtp_cw.htm Nucleic Acid labelling and Detection –Techtip-High sensitivity northerns, pp1-4

Armstrong.D., Rytina.E.R., Murphy.G.M., Dowling.R.H.(1994) Gastric mucosal toxicity of duodenal juice constituents in the rat. Acute studies using *ex vivo* gastric chamber model. *Gastroenterology* **39**, 327-339

Altschul. S.F., Gish. W., Miller. W., Myers. E.W., Lipman. D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410

B

Barnard, J.A., Ghishan, F.K., Wilson, F.A (1985) Ontogenesis of taurocholate transport by rat ileal brush border membrane vesicles. *Journal of Clinical Investigations* **75**, 869-73

Barnard, J.A., Ghishan, F.K. (1986) Methylprednisolone accelerates the ontogeny of sodium taurocholate cotransport in rat ileal brush border membranes. *Journal of Laboratory Clinical Medicine* **108**, 549-555

Barnard. J.A., Ghishan. F.K. (1987) Taurocholate transport by human ileal brush border membrane vesicles. *Gastroenterology* **93**, 925-933

Barnard. J.A., Thaxter.S., Kikuchi.K.,Ghishan. F.K. (1988) Taurine transport by rat intestine. *American Journal of Physiology* **254**, G334-338

Baringhaus. K., Matter. H., Stengelin. S., Kramer. W. (1999) Substrate specificity of the ileal and the hepatic Na^+/bile acid cotransporters of the rabbit. II. A reliable 3D QSAR pharmacophore model for the ileal Na^+/bile acid co-transporter. *Journal of Lipid Research (United States)* **40**, 2158-68

Bayle. D., Weeks. D., Hallen. S., Melchers. K., Bamberg. K., Sachs. G. (1997) In vitro translation analysis of integral membrane proteins. *Journal of Receptor Signal Transduction Research (United States)* **17**, 29-56

Benson.G.M., Haskins.N.J., Eckers.C., Moore.P.J., Ried.D.G., Mitchell.R.C., Waghmare.S., Suckling.K.E.(1993) Polydeoxycholate in human and hamster faeces: a major product of cholate metabolism. *Journal of Lipid Research* **34**, 2121-2135

Berant.M., Diamond.E., Alon.U., Mordichovitz.D.(1988) Effect of infusion of bile salts into the mesenteric artery in situ on jejununal mucosal transport functions in dogs. *Journal of Paediatric Gastroenterology and Nutrition* **7**, 588-593

Bessely. R.C., Faust. R.G. (1979) Sodium ion coupled uptake of taurocholate by intestinal brush border membrane vesicles. *Biochemical Journal* **178,** 299-303

http://www.biochem.ucl.ac.uk/~chen/protocls/oligo.html Oligosynthesis protocols

Blitzer.B.L., Terzakis.C., Scott.K.A.(1986) Hydroxyl/bile acid exchange. *The Journal of Biological Chemistry* **261**, 12010-12046

Bluett. M.K., Abumrad. N.N., Arab. N., Ghishan. F.K. (1986) Aboral changes in D-glucose transport by human intestinal brush-border membrane vesicles. *Biochemical Journal* **237**, 229-234

Bockman.D.E., Cooper.M.D.(1973) Pinocytosis by epithelium associated with lymphoid follicles in the buras of fabricius, appendix and Peyer's Patches. An electron microscope study. *American Journal of Anatomy* **136**, 455-478

Booth.C.C., Alldis.D., Read.A.E. (1961) Studies on the site of fat absorption. *Gut* **2**, 168-174

Brindley.D.N. (1978) Absorption and transport of lipids in the small intestine. *Intestinal Permeation* **4**, 350-362

Bry.L., Falk.P., Huttner.K., Ouellette.A., Midtvedt.T., Gordon.J. (1994) Paneth cell differentiation in the developing intestine of normal and transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 10335-10339

Buddington.R., Diamond.J.M, (1989) Ontogenetic development of intestinal nutrient transporter. *Annual Revue of Physiology* **51**, 601-609

Buddington.R., Randal.K., Puchal.A.A. Intestinal absorption of the bile acid taurocholate during postnatal development of pigs. Personal communication

Bundy.R., Mauskopf.J., Walker.J.T., Lack.L.(1977) Interaction of uncharged bile salt derivatives with the ileal bile salt transport system. *Journal of Lipid Research* **18**, 389-395

Burckhardt. G., Kramer. W., Kurz. G., Wilson. F.A. (1983a) Photoaffinity labelling studies of the rat renal Na^+/bile salt cotransport system. *The Journal of Biological Chemistry* **258**, 3618-3622

Burckhardt.G., Kramer.W., Kurz.G., Wilson.F.A.(1983b) Inhibition of bile salt transport in brush border membrane vesicles from rat small intestine by photoaffinity labelling. *The Journal of Biological Chemistry* **258**, 3618-3622

Burckhardt. G., Kramer. W., Kurz. G., Wilson. F.A. (1987) Photoaffinity labelling studies of the rat renal sodium bile salt cotransport system. *Biochemical et Biophysical Research Communications* **143**, 1018-1023

Busch.S., Burckhardt.B.C., Siffert.W. (1995) Expression of the human sodium/proton exchanger NHE-1 in *Xenopus laevis* oocytes enhances sodium/proton exchange activity and establishes sodium/lithium countertransport. *Pfluger Archives* **429**, 859-869

C

Cairnie.A.B. (1970) Renewal of goblet and paneth cells in the small intestine. *Cell Tissue Kinetics* **3**, 35-45

Campbell.B.J., Garner.A., Dockray.G.J., Hughes.J., Dimaline.R.(1994) The mechanism of action of gastrin releasing peptide (GRP) is stimulating avian gastric acid secretion. *Regulatory Peptides* **49**, 249-255

Castagna.M.,Shayakul.C.,Trotti.D.,Sacchi.V.F.,Harvey.W.R., Hediger.M.A.(1997) Molecular characteristics of mammalian and insect amino acid transporters: Implication for amino acid homeostasis. *The Journal of Experimental Biology* **200**, 269-286

Castagna.M.,Shayakul.C.,Trotti.D.,Sacchi.V.F.,Harvey.W.R., Hediger.M.A.(1998) Cloning and characterisation of a potassium coupled amino acid transporter. *Proceeding of National Academy of Sciences USA* **95**, 5395-5400

Chandan.R., Newell.S.M., Brown.D.R. (1988) Actions of gastrin releasing peptide and related mammalian and amphibian peptides in ion transport in the proximal jejunum. *Regulatory Peptides* **23**, 1-14

Chomczynski.P., Sacchi.N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156-159

Chothia.C., Levitt.M., Richardson.D (1981) Helix to helix packing in proteins. *Journal of Molecular Biology* **145**, 215-250

Clausen.T. (1998) Clinical and therapeutic significance of Na^+, K^+ pump. *Clinical Science* **95**, 3-17

Colman. C (1986) In: transcription and translation. A practical approach (Homes.B.D and Higgins.S. Journal editors) IRL, Oxford, England pp271-302

Cotton. F.A., Wilkinson. G. (1980) In : Advanced Inorganic Chemistry – A Comprehensive Text. John Wiley and Sons. pp 14

Craddock. A.L., Love. M.W., Daniel. R.W., Kirby. L.C., Walters. H.C., Wong. M.H., Dawson. P.A. (1998) Expression and transport properties of the human ileal and renal sodium-dependent bile acid transporter. *American Journal of Physiology* **274** G157-169

Crane. R.K. (1962) Hypothesis for mechanism of intestinal active transport of sugars. *Federation Proceedings* **21**, 891-895

D

Dahlqvist. A. (1964) Method for assay of intestinal disaccharidases. *Analytical Biochemistry* **7**, 18-25

Dakka.T., Dumoulin.V., Chayvialle.J., Cuber.J. (1995) Cholyltaurine absorption by isolated vasculary perfused rat small bowel. *Digestion* **56**, 165-170

Darnell.J., Lodish.H., Baltimore.D. (1990) In: Molecular Cell Biology, 2nd Edition. Scientific American Books, Inc, WH Freeman and Company, New York.

Davenport.A.(1975)A digest of digestion, the gastrointestinal system. Year Book Medical Publishers

Davidson.N.O., Hausman.A.M., Ifkovits.C.A., Buse.J.B., Gould.G.W., Burant.C.F., Bell.G.I (1992). Human intestinal glucose transporter : expression and localisation of GLUT 5. *American Journal of Physiology* **262**, C795-800

Dawson.P.A., Oelkers.P.(1994) Bile acid transporters. *Current Opinion in Lipidology* **6**, 109-114

Dawson P.A., Wong M.H. (1995) The apical membrane bile acid transporter of the ileal enterocyte. *Bile acids in Gastroenterology* **20**,197-204

Dawson.P.A and Oelkers.P (1996) Molecular genetics of ileal bile acid transport. *Bile acid meeting* **33**, Falk Symposium

Dawson.P., Rudel. L.L. (1999) Intestinal cholesterol absorption. *Current Opinions in Lipidology* **10**, 315-20

Dietschy.J.M. (1968) Mechanisms for the intestinal absorption of bile acids. *Journal of Lipid Research* **9**, 297-309

Dorando.F.C, Crane.R.K (1984) Studies of the kinetics of the Na^+ gradient coupled glucose transport as found in brush border membrane vesicles from rabbit jejunum. *Biochimica et Biophysica Acta* **772**, 273-287

Dowling.R.H., Murphy.G.M. (1995) Bile acid and acquired disease: old hypotheses, new concepts . Personal communction

Dyer.J., Ritzhaupt.A., Wood. I.S., De La Horra. C., Illundian. A.A., Shirazi-Beechey.S.P. (1997a) Expression of the Na^+/glucose co-transporter (SGLT1) along the length of the avian intestine. *Biochemical Society Transactions* **25**, 480S

Dyer.J., Garner.A., Wood.I.S., Sharma.A.K., Chandranath.I., Shirazi-Beechey.S.P. (1997b) Changes in the levels of intestinal Na^+/glucose co-transporter (SGLT1) in experimental diabetes. *Biochemical Society Transactions* **25**, 479S

E

Elsing.C., Fitscher.B.A., Boker.C., Kramer.W., Stengelin.S., Stremmel.W.(1999) Expression of a bile acid transporter in biliary epithelial cells from normal and cholestatic rat livers. *European Journal of Medical Research* **4**, 165-168

Eusufzai. S. (1995) Bile acid malabsorption: mechanisms and treatment. *Digestive Diseases* **13** , 312-21

http://www.embl-heidelberg.de/~toldo/JaMBW/5/2/index.html PcRimer results from EMBL

http://www.ebi.ac.uk/htbin/expasyfetch EMBL nucleotide sequences

http://www2.ebi.ac.uk/servicestmp/27170939221624.html EMBL Sequence alignment

F

Fasano.A., Verga.M., Raimondi.F., Guandalini.S. (1994) Effects of deconjugated bile acids on electrolyte and nutrient transport in the rabbit small intestine in vitro. *Journal of Paediatric Gastroenterology and Nutrition* **18**, 327-333

Feroci.G., Fini.A., Fazio.G., Zuman.P. (1996) Effect of divalent transition metal ions on the aggregation of trihydroxy bile salts. *Journal of Colloid and Interface Science* **178**, 339-347

Fondacaro.J.D ., Rodgers.J.B. (1978) Characterisation and effect of phospholipid on bile acid absorption by villi isolated from hamster small intestine. *Digestive Disease Science* **23**, 12-17

Frimmer.M., Ziegler.K.(1988) The transport of bile acids in liver cells. *Biochimica et Biophysica Acta* **947**, 75-99

Fujii.T., Yanagisawa.J., Nakayama.F. (1989) Absorption of bile acids in dog as determined by portal sampling evidence for colonic absorption of bile acids. *Digestion* **41**, 207-214

G

Gallagher. K., Mauskopf. J., Walker. J.T., Lack. L. (1975) Ionic requirements for the active ileal bile salt transport system. *Journal of Lipid Research* **17**, 572-577

Gerloff.T., Stieger.B., Hagenbuch.B., Madon.J., Landmann.L., Roth.J., Hofmann.A.F., Meier.P.J.(1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *The Journal of Biological Chemistry* **273**, 10046-10050

Gong. Y-Z., Zwarych Jr. P.P., Lin. M.C.,Wilson. F.A. (1991) Effect of antiserum to a 99 kDa polypeptide on the uptake of taurocholic acid by rat ileal brush border membrane vesicles. *Biochemical and Biophysical Research Communications* **179**, 204-209

Gong. Y.-Z.,Everett.E.T., Schwartz.D.A., Norris.J.S., Wilson. F.A. (1994) Molecular cloning, tissue distribution and expression of a 14kDa bile acid binding protein from rat ileal cytosol. *Proceeding of the National Academy of Science*, **91** 4741-4745

Gong.Y.G., Kato.T., Schwartz.D.A., Norris.J.S., Wilson.F.A. (1996) Ontogenetic and glucocorticoid-accelerated expression of rat 14 kDa bile acid binding protein. *Anatomical Record* **245**, 532-538

Gray.G.M.(1981) Carbohydrate absorption and malabsorption. *Physiology of the Gastrointestinal Tract.* Edited by L.R.Johnson. Raven Press, New York

Green.R.P., Cohn.S.M., Sacchettini.J.C., Jackson.K.E., Gordon.J.I. (1992) The mouse intestinal fatty acid binding protein gene: Nucleotide sequence, pattern of developmental and regional expression and proposed structure of its protein product. *DNA and Cell Biology* **11**, 31-41

Greenberger.N.J., Rodgers.J.B., Isselbacher.K.J (1966) Absorption of medium and long chain triglycerides. Factors influencing their hydrolysis and transport. *Journal of Clinical Investigations* **45**, 217-227

Gunther. R.D., Wright. E.M. (1983) Na^+, Li^+, and Cl^- transport by brush border membranes from rabbit jejunum. *Journal of Membrane Biology*, **74**(2), 85-94

Gunshin. H., Mackenzie. B., Berger. U., Gunshin. Y., Romero. M., Boron. W., Nussberger. S., Gollan.J., Hediger. A (1997) Cloning and characterisation of a mammalian proton-coupled metal-ion transporter. *Nature* (*England*) **388**, 482-8

H

Hagenbuch.B., Lubbert.H., Steiger.B,. Meier.P (1990) Expression on the hepatocyte Na^+/bile acid cotransporter in *Xenopus laevis* oocytes. *The Journal of Biological Chemistry* **265**, 5357-5360

Hagenbuch.B., Stieger.B., Foguet.M., Lubbert.H., Meier.P. (1991) Functional expression cloning and characterisation of the hepatocyte Na^+/bile acid cotransport system. *Proceeding of the National Academy of Science* ***88***, 10629-10633

Hallen.S., Bayle.D., Dawson.P.A., Sachs.G. (1996) Membrane topology of the human ileal Na^+ bile acid co-transporter by *in vitro* translation scanning. *Bile acid meeting* **40**, Falk Symposium

Hallen.S., Branden.M., Dawson.P.A., Sachs.G. (1999) Membrane inestion scanning of the human ileal sodium/bile acid co-transporter. *Biochemistry* **38**, 11379-11388

Hamosh.M. (1978) Rat lingual lipase:Factors affecting enzyme activity and secretion. *American Journal of Physiology* **235**, 416-421

Hardison.W.G., Heasly.V.L., Shellhamer.D.F.(1991) Specificity of the hepatocytes Na^+ dependent taurocholate transporter: influence of side chain length and charge. *Hepatology* **13**, 68-72

Harig.J.M., Barry.J.A., Rajendran.V.M., Soergel.K.H., Ramaswamy.K. (1989) D-glucose and L-leucine transport by human intestinal brush border membrane vesicles. *Gastrointestinal Liver Physiology* **19**, G618-G623

Harnett.K.M., Walsh.C.T., Zhang.L.(1989) Effects of Bay o2752, an hypocholesterolemic agent, on intestinal taurocholate transport and cholesterol esterification. *The Journal of Pharmacology and Experimental Therapeutics* **251**, 502-509

Hwang. S., Henning. S. (2000) Hormonal regulation of expression of ileal bile acid binding protein in suckling rats. *American Journal of Physiology (United States)* **278**, R1555-63

Hediger. M.A., Coady. M.J., Ikeda. T.S., Wright. E.M. (1987a) Expression cloning and cDNA sequencing of the Na^+/glucose co-transporter. *Nature* **330**, 379-381

Hediger. M.A., Ikeda. T.S., Coady. M.J., Gundersen.C.B., Wright. E.M (1987b) Expression of size selected mRNA encoding the intestinal Na^+/glucose cotransporter in *Xenopus laevis* oocytes. *Proceedings of the National Academy of Sciences of the USA* **84**, 2634-2637

Hediger. M.A.,Turk. E., Pajor. A.M., Wright. E.M. (1989) Molecular genetics of the human Na^+/glucose cotransporter. *Klin Wochenschr* **67**, 843-846

Hediger. M., Kanai. Y., You. G., Nussberger. S. (1995) Mammalian ion-coupled solute transporters. *Journal of Physiology (England)* **482**, 7S-17S

Herrington.C.S., McGee.J. (1992) In: Diagnostic Molecular Pathology: A practical Approach, volume 2. (Editors: Rickwood.D., Hamser.B.D) Oxford University Press, Oxford

Heubi.J.E., Gunn.T.D. (1985) The role of glucocorticoids in the postnatal development of ileal active bile salt transport. *Paediatric Research* **19**, 1147-1151

Heubi.J.E., Fellows.J.L.(1990) Absence of relationship between the postnatal development of ileal active taurocholate transport and microvillus membrane fluidity. *Journal of Developmental Physiology* **13**, 135-139

Higgins.J.V., Dumaswala.R., Heubi.J.E.(1993) Bile salt feeding up regulates ileal and hepatic taurocholate transport in the rat. *Hepatology* **18**, 966-972

Hingson.D.J., Diamond.J.M. (1972) Comparison of nonelectrolyte permeability patterns in several epithelia. *Journal of Membrane Biology* **10**, 93-135

Hirsch.J.R., Loo.D.F., Wright.E.M. (1996) Regulation of Na^+/glucose cotransporter expression by protein kinases in *Xenopus laevis* oocytes. *Journal of Biological Chemistry* **271**, 14740-147406

Hofmann.A.F. (1963) The function of bile salts in fat absorption. The Solvent properties of dilute micellar solutions of conjugated bile salts. *Biochemical Journal* **89**, 57-68

Hofmann. A.F., Roda. A. (1984) Physiochemical properties of bile acids and their relationship to biological properties: an overview of the problem. *Journal of Lipid Research* **25**, 1477-1489

Hofmann. A.F., Schteingart. C.D., Lillienau.J. (1991) Biological and medical aspects of active ileal transport of bile acids. *Annals of Medicine* **23,** 169-175

Hofmann.K., Stoffel. W. (1993) TMbase - A database of membrane spanning proteins segments *Biological Chemistry* **347**,166

Hold.K., De Boer.D., Zuidema.J., Maes.R. (1983) Saliva as an analytical tool in toxicology. *International Journal of Drug Testing* **23**, 1-33

Holmes.R., Lobley.R.W. (1989) Intestinal Brush Border revisited. *Gut* **30**,1667-1678

Holmgren.M., Shin.K.S., Yellen.G. (1998) The activation gate of a voltage-gated K^+ channel can be trapped on the open state by an intersubunit metal bridge. *Neuron* **21**, 617-621.

Holt. P.R. (1964) Intestinal absorption of bile salts in the rat. *American Journal of Physiology* **207**, 1-7

Hunter.C.K., Treanor.L.L., Gray.J.P., Halter.S.A., Hoyumpa.A., Wilson.F.A. (1983) Effects of ethanol in vitro on rat intestinal brush border membranes. *Biochimica et Biophysica Acta* **732**, 256-265

http://www.enzim.hu/hmmtop/server

I

Ikeda.T.S., Hwang.E.S., Coady.M.J., Hirayama.B.A., Hediger.M.A., Wright.E.M. (1989) Characterisation of a Na^+/glucose cotransporter cloned from rabbit small intestine. *Journal of Membrane Biology* **110**, 87-95

http://info.med.yale.edu/genetics/ward.tavi/p02.html

J

Johnson.L.R.(1991)In: Gastrointestinal Physiology, 4th Edition. (Editor: K. Kist) Mosby Year Book, Missouri, USA

K

Kato.T., Zwarych.P., Gong.Y.G., Wilson.F.A Evidence for the role of a 99 kDa protein in the ileal active transport of bile acid. Personal communication

Kaunitz. J.D., Gunther. R., Wright. E.M. (1992) Involvement of multiple sodium ions in intestinal D-glucose transport. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 2315-2318

Kessler.M., Tannenbaum.V., Tannenbaum.C. (1978) A simple apparatus for performing short times (1-2 seconds) uptake measurements in small volumes; its application to D-glucose transport studies in brush border vesicles from rabbit jejunum and ileum. *Biochimica et Biophysica Acta* **509**, 348-359

Kim.Y.S., Perdomo.J.M. (1974) Membrane glycoproteins of the rat small intestine chemical composition of membrane glycoproteins. *Biochimica et Biophysica Acta* **342**, 111-124

Kimmich.G.A. (1981) Intestinal absorption of sugar. *Physiology of the Gastrointestinal Tract*, pp1035-1061. Leonard R Johnson, Raven Press, New York.

Krag. E., Phillips. S.F. (1974) Active and passive bile acid absorption in man : Perfusion studies of the ileum and jejunum. *The Journal of Clinical Investigation* **53**, 1686-1694

Kramer.W., Bickel.U., Buscher.H.P., Gerok.W., Kurz.G.(1982) Bile salt binding polypeptides in plasma membranes of hepatoyctes revealed by photoaffinity labelling. *European Journal of Biochemistry* **129**, 13-24

Kramer. W., Burckhardt. G., Wilson. F.A., Kurz. G. (1983) Bile salt binding polypeptides in brush border membrane vesicles from rat small intestine revealed by photoaffinity labelling. *Journal of Biological Chemistry* **6**, 3623-3627

Kramer. W., Nicol. S-B., Girbig. F., Gutjahr.U., Kowalewski.S., Fasold. H. (1992) Characterisation and chemical modification of the Na^+-dependent bile acid transport system in brush border membrane vesicles from rabbit ileum. *Biochimica et Biophysica Acta* **1111**, 93-102

Kramer.W., Girbig.F., Gutjahr.U., Kowaleswki.S., Jouvenal.K., Muller.G., Tripier.D., Wess.G. (1993a) Intestinal bile acid absorption. *The Journal of Biological Chemistry* **268**, 18035-18046

Kramer. K., Wess.G., Baringhaus.K-H., Boger.G., Enshen.A., Falk.E., Freidrich.M.,Glombik.H.,Hoffmann.A.,Neckermann.C.,Pittius.C.,Urmann.M. (1993b) Intestinal absorption of peptides by coupling to bile acids. *Hepatology* **18**, Pt2, 971

Kramer.W., Girbig.F., Gutjahr.U., Kowaleswki.S., Wess.G., Baringhaus.K-H., Boger.G., Enshen.A., Falk.E., Freidrich.M., Glombik.H., Hoffmann.A., Neckermann.G., Pittius.C., Urmann.M.(1993c) Subunit composition of the Na^+/bile acid cotransport system form rabbit ileum. *Hepatology* **18**, Pt 2, 970

Kramer. K., Wess.G., Neckermann.G., Schubert.G., Fink.J., Girbig.F., Gutjahr.U., Kowalewsi.S., Baringhaus.K-H., Boger.G., Enshen.A., Falk.E., Freidrich.M., Glombik.H., Hoffmann.A., Pittius.C., Urmann.M. (1994) Intestinal absorption of peptides by coupling to bile acids. *The Journal of Biological Chemistry* **269** 10621-10627

Kramer. K., Wess.G., Baringhaus.K-H., Boger.G., Enshen.A., Falk.E., Freidrich.M., Glombik.H., Hofmann.A., Neckermann.G., Pittius.C., Schafer.H-L., Urmann.M. (1995a) Design and properties of ileal bile acid transport inhibitors. *Bile acids in Gastroenterology* **21**, 205-220

Kramer .W., Girbig.F., Gutjahr.U., Kowalewski.S. (1995b) Radiation inactivation analysis of the Na^+/bile acid co-transport system from rabbit ileum. *Biochemical Journal* **306**, 241-246

Kramer.K.(1996) Molecular structure and inhibition of the ileal bile acid transport system. *Bile acid meeting* **34,** Falk Symposium

Kramer. W., Stengelin. S., Baringhaus. K., Enhsen. A., Heuer H.,Becker. W., Corsiero. D., Girbig. F., Noll. R., Weyland. C. (1999) Substrate specificity of the ileal and the hepatic Na^+/bile acid cotransporters of the rabbit. I. Transport studies with membrane vesicles and cell lines expressing the cloned transporters. *Journal of Lipid Research (United States)* **40**, 1604-17

Kullak-Ulblick.G.A., Beuers.U., Paumgartner.G.(1996) Molecular and functional characterisation of bile acid transport in human hepatoblastoma HepG2 cells. *Hepatology* **23**, 1053-1060

L

Lack. L., Weiner.I. (1961) *In vitro* absorption of bile salts by small intestine of rats and guinea pigs. *American Journal of Physiology* **200**, 313-317

Lack. L., Walker. J.T., Hsu. C.-Y.H. (1977) Taurocholate uptake by membrane vesicles prepared from ileal brush borders. *Life Sciences* **20**, 1607-1612

Lammert.F., Paigen. B., Carey. M. (1998) Localization of the ileal sodium-bile salt co-transporter gene (Slc10a2) to mouse chromosome 8. *Mammalian Genome (United States)* **9**, 173-4

Lanzini.A., Northfield.T.C.(1988) Effect of ursodeoxycholic acid on biliary lipid coupling and on cholesterol absorption during fasting and eating in subjects with cholesterol gallstones. *Gastroenterology* **95**, 408-416

Levy.D., Von Dippe.P.(1989) Identification of bile acid transport protein in hepatocyte sinusoidal plasma membranes. *Methods in Enzymology* **174**, 165-179

Lillienau.J., Hagey.L.R., Borgstrom.B.(1991) Hepatic and ileal transport and effect on biliary secretion of norursocholic acid and its conjugates in rats. *American Journal of Physiology* **261**, G1057-1064

Lillienau.J., Crombie.D., Munoz.J., Lonmire-Cook.S., Hofmann.A.F. (1993) Negative feedback regulation of the ileal bile acid transport system in rodents. *Gastroenterology* **104**, 38-46

Lillienau. J. (1996) Zonal negative feedback regulation of active ileal bile acid transport in rodents. *Bile acid meeting* **42**, Falk Symposium

Lin.M.C, Weinberg.S.L, Kramer.W, Burckhardt.G, Wilson F.A (1988) Identification and comparison of bile acid binding polypeptides in ileal basolateral membrane. *Journal of membrane Biology* **106**, 1-11

Lin.M.C., Kramer.W., Wilson.F.A.(1990) Identification of cytosolic and microsomal bile acid bindings proteins in rat ileal enterocytes. *Journal of Biological Chemistry* **265**, 14986-14895

Lin.M.C., Gong.Y.Z., Geoghegan.K.F., Wilson.F.A .(1991) Characterisation of a novel 14 kDa bile acid binding protein from rat ileal cytosol. *Biochimica et Biophysica Acta* **1078**, 329-335

Lin. M.C., Mullady.E., Wilson F.A. (1993) Timed photoaffinity labelling and characterisation of the bile acid binding and transport proteins in rat ileum. *American Journal of Physiology* **265**, G56-G62

Lin.S.H.,Guidotti.G.(1989) Cloning and expression of a cDNA coding for a rat liver plasma membrane ecto-ATPase. *The Journal of Biological Chemistry* **264**, 14408-14414

Lin.S.H., Culic.O., Flanagan.D., Hixson.D.C.(1991) Immunochemical characterisation of two isoforms of rat liver ecto-ATPase that show immunological and structural identity with a glycoprotein cell-adhesion molecule with Mr 105 000. *Biochemical Journal* **278**, 155-161

Love.M.W., Kirby.L.C., Dawson.P.A .(1996) Identification of a mutant ileal bile acid transporter gene in a patient with familial hypertriglyceridemia. *Bile acid meeting*, **43** Falk Symposium

Louvard. D., Maroux.S., Baratti. J., Desnuelle. P., Mutaftschiev. S. (1973) On the preparation and some properties of closed membrane vesicles from hog duodenal and jejunal brush border. *Biochimica et Biophysica Acta* **291**, 747-763

Lowry. O.H., Rosebrough. N.J., Farr. A.L., Randall. R.J (1951) Protein measurement with the Folin Phenol reagent. *Journal of Biological Chemistry* **193**, 256-275

Lucke.H., Berner.W., Menge.H., Murer.H. (1978a) Sugar transport by brush border membrane isolated from human small intestine. *Pflugers Archives* **373**, 243-248

Lucke. H., Stange. G., Kinne. R., Murer. H. (1978b) Taurocholate - sodium co-transport by brush border membrane vesicles isolated from rat ileum. *Biochemical Journal* **174**, 951-958

Lutton.C. (1976) The role of the digestive tract in cholesterol metabolism. *Digestion* **14**, 342-356

M

Marcus. S.N., Schteinggart. C.D., Marquez. M.L., Hofmann. A.F., Xia.Y., Steinback. J.H., Ton-Nu. H-T., Lillienau.J., Angellotti. M.A., Schmassman.A. (1991) Active absorption of conjugated bile acids in vivo: kinetic parameters and molecular specificity of the ileal transport system in the rat. *Gastroenterology* **100**, 212-221

Miller.A., Ueng.T.H., Bronner.F.(1979) Isolation of a vitamin D-dependent, calcium-binding protein from brush borders of rat duodenal mucosa. *FEBS Letter* **103**, 319-322

Moe.A.J., Jackson. M.J. (1987) Isolation and characterisation of brush border membrane vesicles from pig small intestine. *Comparative Biochemistry and Physiology* **88**, 511-517

Montagnani.M, Aldini.R, Roda.A, Stagni.F, Fortugno.M, Roda.E (1996) Comparative inhibition on bile acid uptake into BBMV from rat and rabbit intestine in presence of polyclonal antibodies raised against the rat ileal bile acid transporter. *Bile acid meeting*, **44** Falk Symposium

Morsiani.E.,Pazzi.P., Moscioni.A.D., Rozga.J., Azzena.G., Demetriou.A.A.(1998) *In Vitro* morphological and functional characterisation of isolated porcine hepatocytes for extracorporeal liver support: bile acid uptake and conjugation. *Journal of Surgical Research* **79**, 54-60

Moyer. M.S., Heubi. J.E., Goodrich. A.L., Balistreri. W.F., Suchy. F.J. (1986) Ontogeny of bile acid transport in brush border membrane vesicles from rat ileum. *Gastroenterology* **90**, 1188-1196

Mullins. J.G., Beechey. B., Gould. G., Campbell. C., Shirazi-Beechey. S. (1992A) Characterisation of the ileal Na^+/bile salt co-transporter in brush border membrane vesicles and functional expression of *xenopus laevis* oocytes. *Biochemical Journal* **285,** 785-790

Mullins . J.G. (1993) Characterisation of the intestinal Na^+/bile salt cotransporter. Thesis submitted April 1993

Mullins.J.G., Punchard.N.A., Murphy.G.M. (1996) The identification of two different size mRNAs encoding the small intestinal Na^+/bile acid cotransporter: consequences for molecular regulation of the protein. *Bile acid meeting*, **45** Falk Symposium

Mullins.J.G., Antoniw.J., Galani.S., Murphy.G., Wilkinson.M. (1998) Molecular modelling of the mechanism of bile acid transport by the hepatoycte canalicular ecto-ATPase (CAM-105) *Biochemical Society Transactions* **26**, S123

Mullins.J.G., Knight.G.L., Murphy.G.M., Antoniw.J.F (unpublished data) Structure/function relationship of the intestinal active bile acid transport protein

Munck.B.G. (1981) Intestinal absorption of amino acids. *Physiology of the Gastrointestinal Tract* ,pp1097-1121. Edited by L.R.Johnson, Raven Press, New York

Murer.H., Hopfer.U. (1974) Demonstration of electrogenic Na^+ dependent D-glucose transport in intestinal brush border membranes. *Procedures of Natural Academic Science* **71**, 484-488

N

Neale.G. (1990) B_{12} Binding proteins. *Gut*, **31** 59-63

Novak.D.A., Sippel.C.J., Ananthanarayanan.M., Suchy.F.J.(1991) Postnatal expression of the canalicular bile acid transport system of rat liver. *American Journal of Physiology* **260**, G743-751

Nowicki.M.J., Shneider.B.L., Paul.J.M., Heubi.J.E.(1997) Glucocorticoids up regulate taurocholate transport by ileal brush border membrane. *American Journal of Physiology* **273**, G197-G203

O

Oelkers.P., Dawson.P.A.(1995) Cloning and chromosomal localisation of the human ileal lipid binding protein. *Biochimica et Biophysica Acta* **1257**, 199-202

Okada.Y. (1979) Solute transport in intestinal epithelial cells. *Membrane Biochemistry* **2**, 339-365

Oelkers.P., Kirby.L.C., Heubi.J.E., Dawson.P.A. (1997) Primary bile acid malabsorption caused by mutations in the ileal sodium dependent bile acid transporter gene (SLC10A2). *Journal of clinical Investigation* **99**, 1880-1887

Oelkers.P., Kirby.L.C., Heubi.J.E., Dawson.P.A. (1998) Primary bile acid malabsorption caused by mutations in the ileal sodium dependent bile acid transporter gene (SLC10A2). *Journal of Paediatric Gastroenterology and Nutrition* **26**, 236-237

P

Pennington. R.J. (1961) Biochemistry of dystrophic muscle. *Biochemical Journal* **80**, 649-654

Peters. T.J.(1976) Analytical subcellular fractionation of jejunual biopsy specimens: methodology and characterisation of the organelles in normal tissue. *Clinical Science and Molecular Medicine* **51**, 557-574

Pohl. P., Saparov. S.M., Antonenko. Y.N. (1998) The size of the unstirred layer as a function of the solute diffusion coefficient. *Biophysics Journal* **75**, 1403-9

Prieto.R.M., Stremmel.W., Sales.C., Tur.J.A.(1996) Oleic acid uptake by jejunal and ileal rat brush border membrane vesicles. *European Journal of Medical Research* **1**, 199-203

Promega (1996) Protocol and Application guide, 3rd edition. Printed in USA ISBN: 1-882274-57-1.

R

Ramasamy.M., Alpers.D.H., Tiruppathi.C., Seetharam.B. (1989) Cobalamin release from intrinsic factor and transfer to transcobalamin II within rat enterocyte. *American Journal of Physiology* **257**, G791-797

Reymann.A., Braun.W., Crobik.C., Woermann.C. (1989) Stimulation of bile acid active transport related to mucosal cyclic AMP content in rat ileum *in vitro*. *Biochimica et Biophysica Acta* **1011**, 158-164

Richieri.G.V., Ogata.R.T., Kleinfeld.A.M.(1992) A fluorescently labelled intestinal fatty acid binding protein. Interactions with fatty acids and its use in monitoring free fatty acids. *The Journal of Biological Chemistry* **267**, 23495-23501

Rose.R.C.(1981) Intestinal absorption of water soluble vitamins. *Physiology of the Gastrointestinal Tract* 1231-1241 . Edited by L.R.Johnson, Raven Press, New York

Ross.M.H., Romrell.L.J. (1989) In: Histology: A text and Atlas (2nd Edition). Williams and Wilkins.

Rouse. J.R., Lack. L. (1979) Ion requirements for taurocholate transport by ileal brush border membrane vesicles. *Life Sciences* **25**, 45-51

Rowell.A., Dyer.J., Hofmann.R.R., Shirazi-Beechey.S.P. (1997) Expression of Na^+/glucose cotransporter in the intestinal brush border membrane of ruminants with different feeding habits. *Biochemical Society Transactions* **25**, 482

Ruetz.S., Frickers.G., Hugentobler.G., Winterhalter.K.,Kurz.G., Meier.P.J.(1987) Isolation and characterisation of the putative canalicular bile salt transport system of rat liver, *The Journal of Biological Chemistry* **262**,11324-11330

S

Sacchi.F.A., Perego.C.(1994) Potassium activation of Na^+ dependent leucine transport in brush border membrane vesicles from rat jejunum. *Comparative Biochemistry and Physiology* **109**, 949-956

Sambrook.J., Fritsch.E.F., Maniatis.T.(1989)In: Molecular cloning: A laboratory manual, 2nd edition. Cold Spring Harbour Press. Cold Spring Harbour

Sauer.P, Fitscher.B.A, Kramer.W, Stengelin.S, Kloters-Plachky.P, Stremmel.W, Stiehl.A (1996) Effect of cholestasis on the intestinal absorption of bile acids. *Bile acid meeting* **37**, Falk Symposium

Sauer. P., Stiehl. A., Fitscher. B., Riedel. H., Benz. C., Kloters-Plachky. P., Stengelin. S., Stremmel. W., Kramer. W.(2000) Down regulation of ileal bile acid absorption in bile-duct-ligated rats. *Journal of Hepatology* **33**, 2-8

Schiff. E.R., Small. N.C., Dietschy. J.M. (1972) Characterisation of the kinetics of the passive and active transport mechanisms for bile acid absorption in the small intestine and colon of the rat. *The Journal of Clinical Investigations* **51**, 1351-1362

Schmitz. J., Preiser.H., Maestracci. D., Ghosh. B.K., Cerda. J.J., Crane. R.K. (1973) Purification of the human intestinal brush border membrane. *Biochimica et Biophysica Acta* **323**(1), 98-112

Schneider. B.L., Dawson. P.A., Christie. D.M., Hardikar. W., Wong. M.H., Suchy. F.J. (1995) Cloning and molecular characterisation of the ontogeny of a rat ileal Na^+ dependent bile acid transporter. *Journal of Clinical Investigation* **95**, 745-754

Schultz.S.G.(1981) Salt and water absorption by mammalian small intestine. *Physiology of the Gastrointestinal Tract* 983-989 . Edited by L.R.Johnson, Raven Press, New York

Schwarz.S.M., Bostwick.H.E., Danziger.M.D., Newmann.L.J., Medow.M.S. (1989) Ontogeny of basolateral membrane lipid composition and fluidity in small intestine. *American Journal of Physiology* **257**, G138-144

Schwarz.S.M., Watkins.J.B., Ling.S.C. (1990) Taurocholate transport by BBMV from the developing rabbit ileum: structure and function relationship. *Journal of Paediatric Gastroenterology and Nutrition* **10**, 482-489

Schwenk.M., Del Pino.V.L., Hegazy. E. (1982) Bile acid uptake by isolated intestinal mucosa cells of guinea pig. *Biochimica et Biophysica Acta* **721**, 247-52

Schwenk. M., Hegazy. E., Lopez Del Pino. V. (1983) Kinetics of taurocholate uptake by isolated ileal cells of guinea pig. *European Journal of Biochemistry* **131**, 387-391

Sharp.P.A., Debnam.E.S. (1994) The role of cyclic AMP in the control of brush border and basolateral membrane of rat jejunal enterocytes. *Experimental Physiology* **79**, 203-214

Shaw.S., Jayatilleke.E., Meyers.S., Colman.N., Herzlich.B., Herbert.V.(1989) The ileum is the major site of absorption for vitamin B_{12} analogues. *The American Journal of Gastroenterology* , **84** 22-27

Shirazi. S.P., Beechey. R.B., Butterworth. P.J. (1981) Potent inhibition of membrane-bound rat intestinal alkaline phosphatase by a new series of phosphate analogues. *Biochemical Journal* **194**, 797-802

Shirazi-Beechey. S.P., Gorverl. J.P., Beechey. R.B. (1988) In: Calcium and Phosphate Transport Across Biomembranes (Peterlik. M., Bronner. F. Eds New York, Liss pp 59-64)

Shirazi-Beechey.S.P., Kemp.R.B., Dyler.J., Beechey.R.B. (1989) Changes in the function of the intestinal brush border membrane during the development of the ruminant habit in lambs. *Comparative Biochemistry and Physiology* **94**, 801-806

Shirazi-Beechey.S.P., Davies.A.G., Tebbutt.K., Dyler.J., Ellis.A., Taylor.C.J., Fairclough.P., Beechey.R.B. (1990) Preparation and properties of brush border membrane vesicles from human small intestine. *Gastroenterology* **93**, 676-685

Siamak.R., Young.J. (1981) Peptide hydrolysis. *Physiology of the Gastrointestinal Tract* 1231-1241 . Edited by L.R.Johnson, Raven Press, New York

Simon.F.R., Sutherland.J., Sutherland.E.(1990) Identification of taurocholate binding sites in ileal plasma membrane. *American Journal of Physiology* **259**, G394-401

Singh.I. (1971) The distribution of paneth cells in the human small intestine. *Anatomical Annuals of Biology* **128**, 60-65

Singh.G., Parik.P.F., Anthone.G., Ortega.A., Simons.A.J., Beart.R.W. (1996) Effect of bile acids on colonic absorption of water and electrolytes. *Gastroenterology* **110**, A36

Sippel.C.J., Ananthanarayanan.M., Suchy.F.J. (1990) Isolation and characterisation of the cannicular membrane bile acid transport protein of rat liver. *American Journal of Physiology* **258**, G728-37

Sippel.J.C., Fallon.R.J., Perlmutter.D.H.(1991) Bile acid efflux mediated by the rat liver cannicular bile acid transport/ecto-ATPase protein requires serine 503 phosphorylation and is regulated by tyrosine 488 phosphorylation. *The Journal of Biological Chemistry* **269**, 19539-19545

Sippel.J.C., McCollum.M.J., Perlmutter.D.H.(1994) Bile acid transport by the rat liver canalicular bile acid transport/Ecto-ATPase protein is dependent on ATP but not on its own Ecto-ATPase activity. *The Journal of Biological Chemistry* **269**, 2800-2826

Sorscher.S., Lillienau.J., Meinkoth.J.L., Steinbach.J.H., Schteingart.C.D., Feramisco.J., Hofmann.A.F. (1992) Conjugated bile acid uptake by *Xenopus laevis* oocytes induced by microinjection with ileal poly A^+ mRNA. *Biochemical and Biophysical research communications* **186**, 1455-1462

Sriram.K., Gergans.G.A., Badger.H.(1989) Absorption of cabalamin (vitamin B12) administered via jejunostomy. *Journal of American College of Nutrition* **8**, 75-81

Stelzner. M., Hoagland. V., Somasundaram. S. (2000) Distribution of bile acid absorption and bile acid transporter gene message in the hamster ileum. *Pflugers Archive (Germany)* **440**, 157-62

Stengelin.S., Apel.S., Becker.W., Maier.M., Rosenbeger.J., Bewersdorf.U., Girbig.F., Weyland.C., Wess.G., Kramer.W.(1996). The rabbit ileal lipid binding protein. Gene cloning and functional expression of the recombinant protein. *European Journal of Biochemistry* **239**, 887-896

Stieger.B., Burckhardt.G., Murer.H. (1984) Demonstration of sodium dependent, electrogenic substrate transport in rat small intestinal brush border membrane vesicles by a cyanine dye. *Pflugers Archives* **400**, 178-182

Stieger.B, O'Neill.B. Meier.P (1992) ATP-dependent bile salt transport in canalicular rat liver plasma membrane vesicles. *Biochemical Journal* **284**, 67-74

Stiehl. A. (1995) Intestinal absorption of bile acids: Effect of ursodeoxycholic acid treatment. *Italian Journal of Gastroenterology* **27**, 193-195

St. Germain. D.L., Dittrich. W., Morganelli. C.M., Cryns. V. (1990) Molecular cloning by hybrid arrest of translation in *Xenopus laevis* oocytes. Identification of a cDNA encoding the type I iodothyronine 5'-deiodinase from rat liver. *Journal of Biological Chemistry* **265**, 20087-20090

Stolz.A., Takikawa.H., Ookhtens.M., Kaplowitz.N. (1989) The role of cytoplasmic proteins in hepatic bile acid transport. *Annuals Revue of Physiology* **51**, 161-176

Stratagene ProStar ^{TM} First Strand RT-PCR kit. Instruction Manual

Stravitz. R.T., Pandak.W.M., Vlahcevic.Z.R., Sanyal.A.J., Santos.M., Redford.K., Dawson.P.A. (1996) Induction of ileal bile acid transporter mRNA and protein by intestinal bile acids *Bile acid meeting* **41**, Falk Symposium

Stravitz. R.T., Sanyal.A.J., Pandak.W.M., Vlahcevic.Z.R., Beets.J.W., Dawson.P.A. (1997) Induction of sodium dependent bile acid transporter messenger RNA, protein, and activity in rat ileum by cholic acid. *Gastroenterology* **113**, 1599-1608

Stryer.L. (1988) In: Biochemistry, Internal student 3rd edition. W H Freeman and Company, New York

Sun.A.G., M Ananthanarayanan., Soroka.C.J., Thevananther.S., Shneider.B.J., Suchy.F.J.(1998) Sorting of rat liver and ileal sodium dependent bile acid transporters in polarized epithelial cells. *American Journal of Physiology* **275**, G1045-1055

Swaan.P.W., Szoka.F.C., Oie.S. (1997) Molecular modelling of the intestinal bile acid carrier: A comparative molecular field analysis study. *Journal of Computer-Aided Molecular Design* **11**, 581-588

T

Takikawa.H., Arai.S., Yamanaka.M.(1992) Binding of bile acids, organic acids, and fatty acids by bovine intestinal Z protein. *Archives of Biochemistry and Biophysics* **292**, 151-155

Thomson.A.B., Dietschy.J.M. (1981) Intestinal lipid absorption: major extracellular and intracellular events. *Physiology of the Gastrointestinal Tract* **46**, pp 1147-1187. Edited by Leonard R Johnson, Raven Press, New York.

Thomson. A.B., Keelan. M., Tavernini. M., Luethe. D., Lam.T. (1987) Development of active and passive transport of bile acids in rabbit intestine. *Mechanical Ageing and Development* **38**, 277-86

Thompson. J.D., Higgins. D.G., Gibson .T.J. (1994) CLUSTAL W: improving the sensitivity of progressiv multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680

Torchia.E.C., Cheema.S.K., Agellon.L.B. (1996) Co-ordinate regulation of bile acids biosynthetic and recovery pathways. *Biochemical and Biophysical Research communications* **225**, 128-133

Trier.J.S., Madara.J.L.(1981) Functional morphology of the mucosa of the small intestine. *Physiology of the Gastrointestinal Tract* **35** pp925-959. Edited by Leonard R Johnson, Raven Press, New York.

Tso.P., Balint.J.A (1986) Formation and transport of chylomicrons by enterocytes to the lymphatics. *American Journal of Physiology* **250**, G715-G726

Tuchweber.B., Yousef.I.M., Ferland.G., Perea.A.(1996) Nutrition and bile formation. *Nutrition Research* **16**, 1041-1080

Tulsiani. D.R.P., Opheim.D.J., Touster. O. (1977) Purification and characterisation of alpha-D-mannosidase from rat liver golgi membranes. *Journal of Biological Chemistry* **252**, 3227-3233

Tusnády.G.E., Simon.I. (1998) Principles Governing Amino Acid Composition of Integral Membrane Proteins: Applications to Topology Prediction. *Journal of Molecular Biology* **283** 489-506.

Turk.E., Zabel.B., Mundlos.S., Dyer.J., Wright.E.M. (1991) Glucose/galactose malabsorption caused by a defect in the Na^+/glucose cotransporter. *Nature* **350**(6316), 354-356

Turk.E., wright.E.M (1997) Membrane topology motifs in the SGLT cotransporter family. *Journal of Membrane Biology* **159**, 1-20

http://www.ch.embnet.org/TMPRED

U

Umbach.J.A., Coady. M.J., Wright. E.M. (1990) Intestinal Na^+/glucose cotransporter expressed in *Xenopus laevis* oocytes is electrogenic. *Biophysical Journal* **57**(6), 1217-24

V

Vander.A., Sherman.J, Luciano.D.(1990) Human Physiology International. Editors Nuners.I., SchanckD., Bradley.J, Von Hofmann Press, 4th Edition

Van Tilburg.A.J.P., De Rooij.F.W.M., Van Blankenstein. M.,Van Den Berg. J.W.O.,
Bosmann-Jacobs.E.P. (1990) Na^+ dependent bile acid transport in the ileum: The balance between diarrhoea and constipation. *Gastroenterology* **98**, 25-32

Voet.D., Voet.J.G.(1995) Biochemistry, 2nd Edition. John Wiley and Son Inc, New York.

Vondenlich.A.D., Gong.Y.G., Geoghegan.K.F., Lin.M.C., Lanzetti.A.J., Wilson.F.A. (1991) Identification of the 14kDa bile acid transport protein of rat ileal cytosol as gastrotrophin. *Biochemical and Biophysical Research Communications* **177**, 1147-1154

Von Dippe.P., Ananthanarayanan.M., Drain.P., Levy.D.(1986) Purification and reconstitution of the bile acid transport system from hepatocyte sinusoidal plasma membranes. *Biochimica et Biophysica Acta* **862**, 352-360

Von Heijne.G. (1992) Membrane Protein Structure Prediction, Hydrophobicity Analysis and the Positive-inside Rule. *Journal of Molecular Biology*, **225** 487-494

W

Walker.W.A., Isselbacher.K.J. (1977) Intestinal antibodies. *Physiology in Medicine* **297**, 767-773

Weinberg.S.L., Burckhardt.G., Wilson.F.A. (1986) Taurocholate transport by rat intestinal basolateral membrane vesicles. *Journal of Clinical Investigation* **78**, 44-50

Weinman. S.A., Carruth. M.W., Dawson. P.A. (1998) Bile acid uptake via the human apical sodium bile acid cotransporter is electrogenic. *The Journal of Biological Chemistry* **273**, 34691-34695

Wilson. F.A., Dietschy. J.M. (1974) The intestinal unstirred layer: its surface area and effect on active transport kinetics. *Biochimica et Biophysica Acta* **363**, 112-126

Wilson. F.A., Treanor. L.L. (1975) Characterisation of the passive and active transport mechanisms for bile acid uptake into rat isolated intestinal epithelial cells. *Biochimica et Biophysica Acta* **406**, 280-293.

Wilson. F.A., Treanor. L. (1979) Glycodeoxycholate transport in BBMV isolated from rat jejunum and ileum. *Biochimica et Biophysica Acta* **554**, 430-440

Wilson. F.A. (1981) Intestinal transport of bile acids. *American Journal of Physiology* **241**, G83-G92

Wolffram.S., Eggenberger.E., Scharrer.E.(1986) Kinetics of D-glucose and L-leucine transport into sheep and pig intestinal brush border membrane vesicles. *Comparative Biochemistry and Physiology* **84**, 589-593

Wolffram.S.,Wurmli.R., Scharrer.E.(1988) Distribution of Na^+ dependent taurocholate transport along the small intestine in sheep. *Journal Veterinary Medicine* **A35,** 236-239

Wong. M.H., Oelkers. P., Craddock. A.L., Dawson. P.A. (1994) Expression cloning and characterisation of the hamster ileal Na^+ dependent bile acid transporter. *The Journal of Biological Chemistry* **269,** 1340-1347

Wong.M.H., Oelkers.P., Dawson. P.A. (1995) Identification of a mutation in the ileal Na^+ dependent bile acid transporter gene that abolishes transport activity. *The Journal of Biological Chemistry* **270**, 27228-27234

Wong. M.H., Rao. P.N., Pettanat.M.J., Dawson.P.A (1996) Localisation of the ileal sodium bile acid co-transporter gene (SLC10A2) to human chromosome 13q33. *Genomics* **33**, 538-540

Wright.E.M ., Turk.E., Hager.K., Lescale-Matys.L., Hirayama.B., Supplisson.S., Loo.D.D. (1992) The Na^+/glucose cotransporter (SGLT1). *Acta Physiologica Scandinavica Supplementum* **607**, 201-207

Wright.E.M. (1993) The intestinal Na^+/glucose cotransporter. *Annual revue of Physiology* **55**, 575-589

Wright.E.M.,Loo.D.D.,Panayotova.M.,Lostao.M.P.,Hirayama.B.H.,Mackenzie.B., Boorer.K., Zampighi.G.(1994) Active sugar transport in Eukaryotes. *Journal of Experimental Biology* **196**, 197-212

Wright. EM., Loo. DD., Turk. E., Hirayama. B.A. (1996) Sodium co-transporters. *Current Opinions of Cell Biology* **8**, 468-73

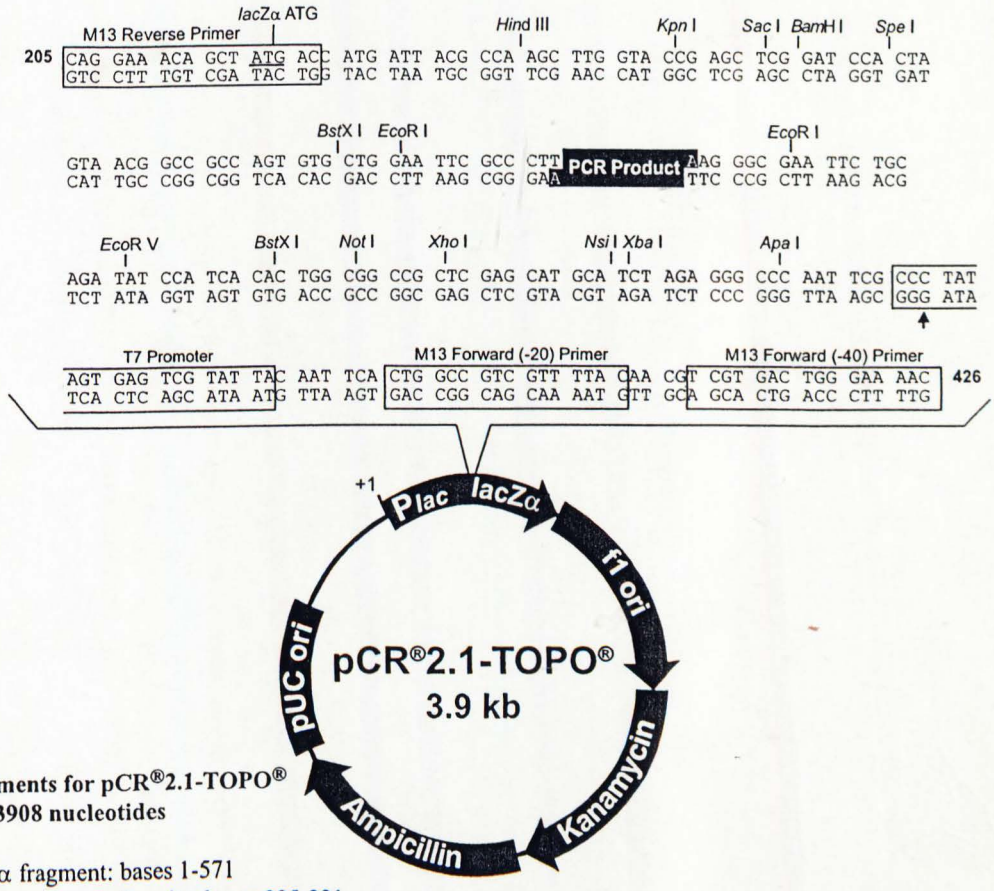
APPENDIX

Figure 1, Plasmid map used for cloning of the PCR product

Map of pCR®2.1-TOPO®

pCR®2.1-TOPO® Map

The map below shows the features of pCR®2.1-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrow indicates the start of transcription for T7 polymerase. For the full sequence of the vector, you may download it from our web site or call Technical Service (page 19).



Comments for pCR®2.1-TOPO®
3908 nucleotides

LacZα fragment: bases 1-571
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
M13 Forward (-40) priming site: bases 411-426
f1 origin: bases 548-962
Kanamycin resistance ORF: bases 1296-2090
Ampicillin resistance ORF: bases 2108-2968
pUC origin: bases 3113-3786