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Ollscoil Átha Cliath

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ASSESSMENT OF PERMEABILITY ENHANCERS FOR SALMON CALCITONIN (sCT) ACROSS INTESTINAL CELL CULTURE MODELS

being a thesis submitted for the degree of

DOCTOR IN PHILOSOPHY

in

PHARMACEUTICS

at

TRINITY COLLEGE DUBLIN

by

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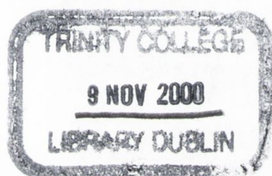
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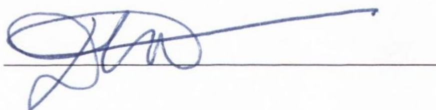
DECLARATION

This thesis is submitted by the undersigned to the University of Dublin, Trinity College, for examination for the degree of Doctor of Philosophy. It has not been submitted as an exercise for a degree at any other University. I myself carried out all the experimental work described, except where duly acknowledged. This manuscript was completely written by me with the aid of editorial advice from Dr. Caitríona M. O' Driscoll.



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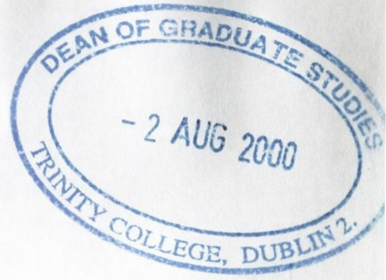

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Denis N. O'Driscoll

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"Every exist is an entry somewhere else"

Tom Stoppard

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The effect of micellar systems on the transepithelial transport of salmon calcitonin (sCT) using cell culture systems. Oral Presentation, TCD/QUB colloquium, 1998

Transepithelial transport of salmon calcitonin (sCT) in the presence of bile salts across CaCo-2 cell monolayers. Eur. J. Pharm. Sci., 1997, 5/52, P27

The effect of pH and excipients on the transport of salmon calcitonin (sCT) across CaCo-2 monolayer. Presented at 3rd European Intensive Course on New Forms and New Routes of Administration for Drugs, Marburg, Germany, March 1997

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AA	Amino acid
ABC	ATP Binding Cassette
ABL	Aqueous boundary layer
ACD	Heptakis (6-deoxy-6-aminopyridyl)-β-cyclodextrin sodium salt
ANOVA	Analysis of Variance
AP	Apical
Asn	Asparginine
Asp	Aspartamine
ATP	Adenosine triphosphate
AUC	Area under the curve
AVP	Arginine vasopressin
BA	Basolateral
BBMV	Brush Border Membrane Vesicles
BCD	β-cyclodextrin
BSA	Bovine serum albumin
C10	Sodium Caprate
Ca^{2+}	Calcium
CaCo-2	Human colon adenocarcinoma cell
CCDs	Commercially available cyclodextrins
CDs	Cyclodextrins
CFSLM	Confocal scanning laser microscopy
Cl^-	Chloride ion
CMC	Critical micellar concentration
CT	Calcitonin
CYP3A	Cytochrome P_{450} Enzyme system
Cys	Cysteine
DABCO	1,4-diazabicyclo[2.2.2]octane
DADLE	[D-Ala^2, D-Leu^2] enkephalin
Deoxy	Sodium deoxycholate
DMCD	2,3-dimethyl-β-cyclodextrin
DMEM	Dulbecco's modified Eagles medium

DHPE	{N-Texas-Red®sulphaonyl}-1,2-dihexadecanoyl-sn -glycero-3-phospoethanolamine triethylammonium salt
DMSO	Dimethyl sulphoxide
DPM	Disintegrations per minute
DSIP	Delta Sleep Inducing Peptide
EDTA	Disodium ethylenediamine tetracetate
EGTA	Ethylene glycol bis-(β-aminoethylether)-N,N,N,N' tetracetic acid
ELISA	Enzyme linked immunosorbent assay
ELLA	Enzyme lectin linked assay
EM	Electron microscopy
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocynate
GI	Gastrointestinal
Gln	Glutamine
Gly	Glycine
h	Hour
HBSS	Hanks balance salt solution
HCD	Heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin
hCT	Human calcitonin
HEPES	{N-(2 hydroxyethyl) piperazine-N-(2-ethanesulphonicacid)} sodium salt
hGH	Human Growth Hormone
His	Histidine
HIV	Human Immunodeficiency Virus
HPA	*Helix pomatia* peroxidase labelled lectin
HPCD	2-hydroxylpropyl-β-cyclodextrin
HPLC	High performance liquid chromatography
IFN-γ	Interferon Gamma
IGF I	Insulin-Like Growth Factor I
IGF II	Insulin-Like Growth Factor II
Ile	Iso-leucine
LA	Linoleic acid

Leu	Leucine
LM	Light microscopy
Met	Methionine
MA	Macula Adherens
MDR	Multi-drug resistance
μg	Microgram
mg	Milligram
min	Minutes
mM	Millimolar
msCT	Modified salmon calcitonin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Na^+	Sodium ion
N-AC	N-acetyl-L-cysteine
NaC	Sodium cholate
NaGC	Sodium glycocholate
NaTC	Sodium taurocholate
NCDs	Novel cyclodextrins
ng	nanogram
nM	Nanomolar
^0C	Degree Celsius
OPD	o- phenlyene- diamine
PAS	Periodic acid Schiff's stain
PBS	Phosphate buffered saline
PCD	Heptakis (6-O-sulphonatophenyl)- β-cyclodextrin sodium salt
PCC	Palmitoylcarnitine
PEG	Polyethylene glycol
P-GP	P-Glycoprotein
Phe	Phenylalinine
PI	Propidium iodide
pI	Isoelectric point
PPPM	Partially purified porcine mucin

Pro	Proline
psi	Pounds per square inch
rpm	Revolutions per minute
sCT	Salmon calcitonin
SDS	Sodium dodecyl sulphate
Ser	Serine
SEM	Scanning electron microscopy
TCA	Trichloroacetic acid
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
TNFα	Tumor Necrosis Factor
Thr	Threonine
TRH	Tyrotrophin Releasing Hormone
Tyr	Tyrosine
UV	Ultra-violet
V	Villi
Va	Vacuole
Val	Valine
ZA	Zona Adherens
ZO	Zona Occludens
ZOT	Zona Occludens Toxin

Symbols

A	Area of membrane (cm^2)
Å	Angstrom
C_0	Initial concentration (mol)
dQ/dt	Steady state flux (mol/sec)
F	Bioavailability
f_H	Fraction of the filter covered with Ht29GlucH cells
ka	Intestinal Perfusion rate Constant

Kd	Receptor Level expression (nM)
kp	True permeation (cm/sec)
η_{app}	Apparent viscosity
%	Percentage
Ω	Ohms
h_{aq}	Aqueous Boundary layer Thickness
Log P	Log Partition Co-efficient
MW	Molecular weight
Papp	Apparent permeability (cm/sec)
P_{cell}	Cellular permeability coefficient (cm/sec)
Pm	CaCo-2 cell monolayer permeability (cm/sec)
R_{theor}	Theoretical resistance
R_H	Resistance of the Ht29GlucH monolayer
R_C	Resistance of the CaCo-2 cell monolayer
t	Time (sec)
$T_{1/2}$	Half-life

CHAPTER 1

Origin and Scope

A large number of proteins are now being used or tested as therapeutic agents, enzyme inhibitors or antibodies to combat human disease, therefore they are receiving increased recognition for both their high activity at low concentrations and their fundamental role in human body systems. The advent of recombinant DNA technology has led to an explosion in quantities and varieties of proteins, which are highly purified and are non-contaminated with blood-bourne diseases. As a result, protein pharmaceuticals are becoming extremely important and indispensable tools that are enabling people to fight life-threatening diseases (Lloyd et al., 1998).

Apart from all this progress the vast majority of these peptide pharmaceuticals are being administered via the parenteral route. This route does not have high patient acceptability, especially with children, and allows for the increased risk of sharps injuries to both patients and the healthcare team. One of the major goals for pharmaceutical scientists has been the search for an alternative non-invasive route of administration for peptides. The oral route has proved to be the most desirable because of its acceptability and convenience. However, the development of suitable formulations for the oral delivery of peptides is a challenging task.

The barriers to peptide delivery may be summarised as follows: the susceptibility to enzymatic attack, poor intrinsic permeability across the intestinal epithelium, rapid post-absorptive clearance resulting in short half-life and chemical instability (Pettit and Gombotz, 1998). In general there are two routes of transport across the epithelium namely the paracellular and transcellular. The primary transport route is dependant on the physical and chemical properties of the compound. It is assumed that hydrophilic compounds go via the paracellular route and lipophilic compound via the transcellular route (Pade et al., 1997).

Various delivery strategies have been investigated as possible ways to improve oral protein drug absorption and systemic bioavailability. These strategies include: penetration enhancers, enzyme inhibitors, chemical modification and use of novel formulation approaches e.g. micro particles.

Enhancers, which have been used to overcome these barriers, include bile salts (Swenson et al., 1992) and cyclodextrins (Irie and Uekama, 1999). These excipients not only exhibit enhancement qualities but also have inherent enzyme inhibitory properties.

Bile salt micellar systems have been previously used in our laboratory to modify the absorption of lipophilic and hydrophilic compounds across the rat gastrointestinal tract (O' Reilly et al., 1994a and b; Lane, 1997) and the cell culture models CaCo-2 and CaCo-2: Ht29GlucH co-culture (Meaney, 1997; Meaney and O'Driscoll, 1999). A variety of effects have been suggested to explain the mechanism of absorption enhancement, including the micelles ability to enhance solubility (Hammad and Müller (1998a), an affect on the gastrointestinal membrane by either disruption of the lipid matrix of the cell (Kakemi et al., 1970; Feldman et al., 1973) or the integrity of the tight junction (O'Reilly et al., 1994a; Werner et al., 1996), and enzymatic inhibition (Hirai et al., 1981b). Understanding the mechanism is further complicated by reports that bile salts have reduced the absorption of compounds (Poelma et al., 1989, 1990). Therefore the precise mechanisms of absorption by bile salt micellar systems appear complex and as yet have not been fully elucidated.

Cyclodextrins (CDs) are cyclic oligo-saccharides consisting of 6-8 glucopyranose units. The α-1, 4-glycosidic linkage of the glucose units results in the formation of torus-like molecules with a hydrophilic outer surface and a hydrophobic interior cavity (Szejtli, 1988). As a consequence of this hydrophobic cavity, the CDs are able to form inclusion complexes with a wide variety of guest molecules (Corrigan and Stanley, 1982; Uekama et al., 1994). CDs offer several advantages in drug delivery including improved drug solubilisation (Duchêne et al., 1999), and protection against physical-chemical and enzymatic degradation (Shao et al., 1994). In addition the potential exists for enhanced absorption by direct interaction of the CDs with membrane components such as cholesterol, inducing changes in fluidity or a direct effect on the tight junctions, resulting in increases in permeability of the membrane (Shao et al., 1992; Irie et al., 1992).

In this project the barriers to the oral delivery of the peptide, salmon calcitonin (sCT) will be investigated at a cellular level using a range of cell culture models including the human adenocarcinoma CaCo-2 and a co-culture consisting of CaCo-2 and the mucus secreting clone Ht29GlucH, previously characterised in our laboratory (Meaney (1997; Meaney and O'Driscoll (1999)). These *in-vitro* models will be used to assess the effect that bile salt micellar systems and commercially available and novel cyclodextrins have on the permeability of the peptide, salmon calcitonin (MW 3414). Further to this two hydrophilic paracellular markers, mannitol (MW182) and PEG 4000 (MW 4000) will be used to assess the effect that these enhancers have on the paracellular route.

The mechanism of absorption of sCT will be assessed. Initial studies will attempt to decipher the route of transport to ascertain if it is passively transported across the CaCo-2 cell monolayer. The influence of the physical-chemical properties of sCT e.g. size and charge on transport will be investigated. Since sCT has an isolelectric point (pI) of approximately 9 this implies that it is positively charged at pH 7.4 (Duncan et al., 1995; Baudyš et al., 1996). Therefore the potential interaction of a cationic peptide with the paracellular route, which favours neutral or positively charged molecules, will be investigated. As size is known to influence permeability via the tight junction the dimensions of sCT will be measure using a molecular modelling package and compared to the size of the aqueous pore in the CaCo-2 cell monolayer in the presence and absence of enhancers.

Bile salt and cyclodextrins will be chosen as excipients as reports show they can not only enhance the apparent permeability (Papp) but also the stability in the presence of the enzyme systems of peptides.

The bile salt simple micelles of the dihydroxy sodium deoxycholate (Deoxy), and the trihydroxy unconjugated sodium cholate (NaC) and the taurine conjugated and glycine conjugated trihydroxy derivatives, sodium taurocholate (NaTC) and sodium glycocholate (NaGC) will be assessed. Mixed micelles of NaGC in combination with the long chain unsaturated fatty acid, LA will also investigated.

The cyclodextrins investigated will be the commercially available cyclodextrins: 2,3-dimethyl-β-cyclodextrin (DMCD), 2-hydroxylpropyl-β-cyclodextrin (HPCD) and Sulfobutyl-β-cyclodextrin (SBE_7CD). A range of novel cyclodextrins were synthesised to have amphiphilic characteristics and have the ability to form aggregates (Ramphul (1998); Holohan (1999)). These novel oligo-saccharide cyclodextrins: Heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin (HCD), Heptakis (6-O-sulphonatophenyl)-β-cyclodextrin sodium salt (PCD) and Heptakis (6-deoxy-6-aminopyridyl)-β-cyclodextrin sodium salt (ACD) will also be investigated. Further to this a preliminary study will be carried out to investigate if a combination of bile salt and CD caused any synergistic effect on sCT permeability.

The enhancers' ability to affect the transcellular and/or paracellular route will be monitored using a range of techniques including. transepithelial electrical resistance (TEER) measurements and the transmission electron microscopy (TEM). Toxicity of the excipients was examined by the intracellular dehydrogenase activity (MTT) and reversibility studies.

It has previously been shown that the mucus layer can act as a barrier to the drug permeability. Meaney and O'Driscoll (1999) developed a co-culture model capable of secreting mucus. Using this model they showed that the permeability of dextropropoxyphene, a lipophilic compound, which was ionised at pH 4.5, was decreased in the presence of mucus suggesting an electrostatic attraction between the mucus and the drug. Wikman-Larhed et al., (1997) reviewed the transport of differently charged molecules in mucus and suggested that the charge on the molecule influenced it passage through the extracted pig intestinal mucus. Therefore the possible interaction between positively charged sCT and mucus will be investigated.

Mucus may also be a barrier to excipients. Since mucus is a protective layer on the cell surface it may cause a decrease in diffusion of compounds or excipients to the membrane surface (Li et al., 1996). It has also been demonstrated that certain excipients can stimulate the release of mucus e.g. bile salt which may affect the excipients ability to

elicit an effect (Poelma et al., 1990; Shekels et al., 1995). Because mucus carries a net negative charge because of the sialic acid residues (MacAdam, 1993) there is a likelihood of electrostatic interactions. Therefore the effect that mucus has on the enhancement potential of bile salt simple and mixed micellar systems and novel and commercial CDs will be examined.

GENERAL INTRODUCTION

CHAPTER 2

Oral Peptide Delivery

2.1 Introduction

Recent advances in biotechnology have resulted in the significant increase in the number of therapeutic peptides and proteins that are reaching the market. This trend is expected to continue and even escalate in the future (Fletcher, 1998). Protein drugs have been recognised for both their high activity at low concentrations and their crucial roles in human body functions. However, due to the enzymatic instability and poor membrane permeability it appears that the major route for administration of these pharmaceuticals is via the parenteral route. This route has many disadvantages associated with it the most important being patient discomfort and the increased risk of sharps injuries with its associated consequences (Zhou and Po, 1991; Zhou, 1994). As a result of these problems there has been extensive research in attempting to delivery these protein drugs by a non-parenteral system. Various routes have been investigated including oral (Lee et al., 1992; TenHoor and Dressman, 1992), pulmonary (Patton et al., 1994; Deftos et al., 1997), nasal, buccal, vaginal, rectal (Sayani and Chien, 1996), and dermal (Matuszewska et al., 1994). However, the route that has proved to be most convenient and practical for administration is the oral route.

This review will discuss the following four interrelated aspects of oral peptide drug delivery a) the peptide drug stability b) the gastrointestinal (GI) barriers to protein absorption c) absorption mechanisms in the GI tract and d) approaches to improve protein drug absorption.

2.2 Peptide drug stability

The reasons why protein drugs are administered by injection rather than taken orally include enzymatic instability, poor membrane permeability due to large molecular weight and charge. This section will briefly review problems associated with instability.

2.2.1 Peptide stability

Proteins/peptides by their very nature are labile compounds. It is a major challenge to formulate these compounds and achieve maximal stability over their shelf life. Instability of peptides can be broadly classified as physical instability or chemical instability. Physical instability refers to changes that can occur in the peptides secondary, tertiary or quaternary structure and can lead to denaturation, aggregation, precipitation and adsorption to surfaces. Chemical instability is as a result of reactions such as racemization, hydrolysis, oxidation and deamidation. Since there may be more than one reaction site on a peptide they are susceptible to all of the above reactions.

2.2.1.1 Physical instability

Denaturation

This is usually when there is a disruption of the secondary or tertiary structure of the protein. Denaturation may be reversible or not and may be caused by thermal stress, extremes of pH or denaturing chemicals e.g. guanidine hydrochloride (Manning et al., 1989).

Protein aggregation behaviour

The most common physical instability of proteins is their ability to aggregate, which may be caused by a variety of factors such as pH or temperature changes (Charman et al., 1993) or by chemical transformation. In order to minimise thermodynamically unfavourable interaction between solvent and exposed hydrophobic protein residues aggregation occurs. This is considered to be one of the major driving forces for both protein aggregation and folding. A balance co-exists between folding and aggregation and represents the ratio of exposed to unexposed hydrophobic surface areas (Patro and Przybycien, 1996). The result of aggregation may lead to a partial or complete loss in activity (Runkel et al., 1998). It has been demonstrated that insulin undergoes fibrillation

which may cause aggregation and formation of viscous gels or insoluble precipitates (Brange et al., 1997). Protein aggregation may also lead to a reduction in solubility and altered immunogenicity.

Adsorption

As previously discussed, although the folded protein is hydrophilic it does have hydrophobic residues that lie within the interior of the molecule. If the protein comes into contact with a hydrophobic interface the protein may unfold to allow interaction between the interface and some of the hydrophobic residues leading to adsorption. The severity of adsorption is protein dependant and does not appear to depend on pI or the size of the protein (Burke et al., 1992). Evidence has shown that salmon calcitonin absorption to glass is primarily due to electrostatic interactions resulting in 209ng/cm^2 of sCT being adsorbed and therefore lost from the solution (Duncan et al., 1995). The adsorption process appears to be saturable with a plateau value of between 0.1-0.5μg/cm^2 (Horbett, 1992). Burke et al., (1992) amply demonstrated that the for five proteins alcohol dehydrogenase (ADH) α-chymotrypsinogen A, thyroglobulin, β-amylase and IgG the adsorption of these proteins was less than 0.5- 1μg/ml. Adsorption losses are significant for dilute solution but become less of a concern at concentrations greater than 200μg/ml as the percentage lost becomes negligible. In order to reduce the adsorption of proteins the inclusion of a surfactant has shown to be beneficial such as Tween 20 and Tween 80, both of which are non-ionic, (Duncan et al., 1995; Law and Shinh, 1999). Further to this competition between proteins can be used to prevent the adsorption of one over the other. This method of using one peptide to bind competitively to sites over another protein had been reported in case of insulin where albumin (0.1-1% w/v) was used to minimise its adsorption (Brange et al., 1993).

2.2.1.2 Chemical instability of peptides

Many chemical reactions are responsible for the inactivation of peptide/protein drugs. The reactions that most commonly occur are a) deamidation, b) oxidation, c) disulphide

bond, breakage or formation, d) hydrolysis, e) isomerization, and f) succinimidation, see Table 2.1. These reactions often occur simultaneously and as a result make it almost impossible to quantify degradation products e.g. basic fibroblast growth fibre (bFGF) undergoes multiple degradations in solution including succinimidation, hydrolysis and aggregation (Wang et al., 1996). These chemical reactions are also dependant on a number of factors such as pH and temperature. Lee et al., (1992) demonstrated that the degradation pathway for salmon calcitonin (sCT) was complex with the involvement of disulphide-exchange, racemization and oxidation. This study clearly showed that the detection of degradation products increased as the pH increased to more alkaline conditions and as the temperature was increased.

Therapeutic peptides and proteins can degrade by several pathways. Physical instability may occur by denaturation, aggregation or adsorption. Pathways of chemical instability include oxidation, racemization, deamidation and disulphide exchange. More often than not, more than one pathway of physical and/or chemical instability is responsible for the degradation of the peptide.

Table 2.1 Reactive peptide sequences and formulation stability (Banga, 1996)

Sequence [a]	**Primary Reaction**	**Major products**	**Est. Reactivity** [b]
-X-X-	Typical amide bond Hydrolysis	-X + X-	Slow
Asp-X- (X ≠ Pro)	Hydrolysis	-Asp + X- or -isoAsp + X-	Slow- moderate
Asp-Pro	Hydrolysis	-Asp + X- or isoAsp + X-	*May compromise shelf-life, fast*
-Asn-X (X ≠ Gly)	Deamidation	-Asp-X-, -isoAsp-X-Asp + X-isoAsp-Asp, iso-Asp L or D cyclic imide	May compromise shelf-life, moderate
-Asn-Gly	Deamidation	-Asp-Gly-, -Asp, -isoAsp-Gly-, isoAsp-Asp, iso-Asp L or D cyclic imide	*May compromise shelf-life, fast*
-X-Ser- or X- Thr-	Deamidation	-X + Ser- or –X + Thr-	Moderate
-Ser-, -Tyr-, -Phe, -X- (X ≠ Gly, Asp)	Racemization	-Ser-, -Tyr-, -Phe- X-	Slow-moderate
-Asp-	Racemization	-Asp-, (-isoAsp-)	*May compromise shelf-life, fast*
X-X-Gly-	Diketopiperazine formation	Cyclo-X-X + Gly-	*May compromise shelf-life, fast*
Gln-X-	Pyroglutamic acid formation	< Gln-X-	*May compromise shelf-life, fast*
-Met-	Oxidation	-Met(O)-	May compromise shelf-life, moderate
-Cys-	Oxidation,disulphide formation	-CysCys-	*May compromise shelf-life, fast*
-X- (X = many hydrophobic AA's)	Precipitation	Aggregates, liquid crystals	May compromise shelf-life, moderate

[a]X = any amino acid (AA) unless otherwise specified. AA drawn as –X or X- denotes the possibility of additional AA attached. [b]Reactions that are likely to compromise stability (t_{90} <2 yrs at 25^{0}C) at pH 5- 7 are shown in italics, where other limiting reactions are adjusted for their propensity to limit shelf life accordingly.

2.3 The gastrointestinal (GI) barriers to oral peptide drug delivery

The oral delivery of proteins is a worthy objective but extremely difficult (Davis, 1990). This is primarily due to the presence of a few difficult barriers in oral protein delivery. These include as previously outlined the chemical and physical instability of the peptides, the strong hostile acidic conditions of the stomach, the abundance of exogenous proteases and peptidases in the GI tract, the poor permeability across the GI membranes, first pass metabolism and rapid clearance from the body. The most difficult barriers in the GI tract, include the epithelial barrier and the enzymatic barrier.

2.3.1 The epithelial barrier

The GI epithelium is the interface between the external and the internal environment of the body. Its primary functions are protection from the external environment, terminal digestion and selective absorption and secretion of water and electrolytes.

2.3.1.1 The structure of the intestinal mucosae

The intestinal mucosa is composed of three distinct layers: the muscularis mucosae, the lamina propria and the epithelial cell layer. Underlying the epithelial cell layer is the lamina propria, which is a layer of connective tissue, lymphatics, capillaries and venules, this is supported by the deepest layer the muscularis mucosae which is composed of smooth muscle. The epithelium and the lamina propria projects out and forms finger-like villi, which increases the surface area. These villi are the site of electrolyte and nutrient absorption. The villus absorptive cells display uniform microvilli- 1μm in height and 0.1 μm in width, which further increases the absorptive surface area by 1-1.5 fold. This brush-border is where digestive enzymes are located. These anatomical features increase the surface area of the small intestine 50,000-fold relative to the area of a simple cylinder causing a surface area of approximately $200m^2$ in the human small intestine (Madara et al., 1994).

Between villi are well-like depressions, known as crypts. These crypts are sites of water and electrolyte secretion and IgA transport into the lumen (Madara et al., 1991). Crypts contain mainly undifferentiated cells as well as enterendocrine cells, Paneth cells, mucus producing goblet cells, tuft cells and cuplike cells. Except for the secretory Paneth cells all the other cell types migrate towards the villus surface and acquire functional and structural characteristics of mature cells before being sloughed off from the villus tip into the lumen. This process takes about 3-4 days with a complete turnover of the intestine in approximately one week.

2.3.1.2 Mucus

On top of the epithelium is a layer of mucus, which is secreted by the goblet cells and protects the underlying GI epithelium from physical, chemical and microbiological damage. Mucus is composed predominately of water (95%), the remainder being mucin glycoproteins, sloughed epithelial cells, proteins, electrolytes and bacteria. However, even though mucin glycoproteins only constitute 2- 3% of native mucus, they are considered to be responsible for the mucus gel properties (McAdam, 1993). Mucin molecules are composed of large molecular weight glycoproteins secreted from specialised glands and goblet cells. The glycoprotein molecule consists of a protein backbone with oligo-saccharides side-chains attached to specific amino acid residues. The oligo-saccharide side chains are always attached to the protein core via an O-glycosidic linkage between the α-1 position of the N-acetylgalactosamine and the oxygen of the hydroxyl group, found in the amino acid residues serine and threonine (Reid and Clamp, 1978). Depending on the source of the mucus the side-chains can contain varying amounts of sugar residues, up to 18 (Wilson et al., 1989). Usually only five sugars are present: galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine and N-acetylneuramic acid (also know as sialic acid) (Wesley et al., 1983). Since both sialic acid and the ester sulphates of galactose and N-acetylgalactosamine have pK_a values of less than 3 therefore these groups carry a negative charge at all but the lowest pH (MacAdam, 1993).

The thickness of the mucus layer is variable between species. A detailed study undertaken by Kerrs et al., (1982) demonstrated that the thickness of human, frog and rat gastric mucus layers varied from 5- 400μm. In-vitro mucus producing cell culture models such as Ht29GlucH have been successfully established with the mucus layer thickness being measured, 40- 60 μm (Wikman et al., 1993).

The significance of the mucus layer as a barrier to drug transport is still unclear. Nimmerfall et al., 1980 pointed out the importance of mucin as a decisive luminal barrier to the passage ergot alkaloids and β–blockers through the gut mucosae. In this study, the absorption of compounds of different chemicals structures was directly proportional to diffusion through isolated *in vitro* goblet cell mucin and inversely proportional to retention in mucin. It is important to realise that mucus gel layer is best described by a macromolecular network model, where the chains are held together by permanent entanglements of a physical and chemical nature. Molecular diffusion of the solute can be best described by a topological, free-volume-based model, which relates the drug diffusion coefficient to the molecular characteristics of the mucus gel network (Peppas et al., 1984). Not only is mucus a diffusional barrier but also, as previously mentioned, there is a negative charge associated with the sialic acid residues and therefore the possibility of electrostatic attraction or repulsion (MacAdam, 1993; Wikman-Larhed et al., 1998). The electrostatic attraction between mucus and a drug compound is best represented by tetracycline hydrochloride. Kearney et al., (1987) demonstrated that at pH 3 the tetracycline is positively charged and the sialic acid residues are negatively charged maximal binding occurs. The binding that occurs for pH values higher than 3 is associated with the hydrophobic association of the tetracycline with the globular protein region, or with the lipid associated with the glycoprotein molecule. A series of 13 β-lactam antibiotics and three aminoglycoside antibiotics (Niibuchi et al., 1986) and ergot alkaloids (Franz et al., 1980) all bound to rat intestinal mucus. Although it appears that mucus retards the transport of compounds it has been shown that the bioavailability remains unaffected (Brown et al., 1983). The formation of cell co-culture models consisting of the adenocarcinoma cell line, CaCo-2 and the mucin producing cell clone, Ht29GlucH have been used to predict the permeability of compounds across the GI

mucosae (Allen, 1992; Meaney, 1997). It has been shown that the permeabilities of compounds were affected by the mucus layer present in the co-culture model (Allen et al., 1991). Meaney and O' Driscoll, (1999) clearly showed that the high degree of ionisation of dextropropoxyphene at pH 4.5 and its subsequent binding to the negatively charged sialic acid residues, resulted in a decrease in its permeability across the co-culture model. It appears that drug-mucin interaction is complex and may involve a variety of interactions such as electrostatic, hydrophobic and hydrogen bonding to the carboxyl residue of the carbohydrate residue (Nimmerfall et al., 1980; Kearney et al., 1987; Wikman et al., 1993). The aggregation state of bile salt/phospholipid is affected by the presence of mucus, reducing the micellar size, which has implications for the transport of dietary lipids and solubilized drug through the mucus layer (Shiau et al., 1990; Li et al., 1996).

2.3.1.3 Apical cell membrane

After penetrating the mucus layer the next barrier is the mucosal membrane itself. This barrier is composed of an orientated lipid bi-layer matrix containing a distribution of integral and peripheral proteins. The proteins impart to the cell critical elements of structure, specific polarised transport of nutrients and metabolic activity. The lipid matrix, consisting of various classes of phospholipids and sterols, is thought to be a chief determinant of the fluidity of the cell membrane and help to maintain protein structure through specific protein-lipid interactions (Singer and Nicholson, 1972; Madara et al., 1994)). This membrane is therefore a barrier to compounds, which are hydrophilic, and in order for a compound to traverse this membrane it must be sufficiently lipophilic or be able to interact with a specific transporter (Burton et al., 1991). The passage of solutes across this barrier can be achieved via the transcellular or paracellular routes (Daugherty et al., 1998; Daugherty et al., 1999). Transcellular transport can be divided into (a) passive diffusion, (b) passive diffusion that is modified by an efflux pump, and (c) transport by an active transporter. Paracellular transport of peptides and proteins is limited by the presence of tight junctions (TJ).

2.3.1.4 Tight junctions

The transport of compounds via the tight junctional complex i.e. paracellular route is mainly confined to small hydrophilic drugs, nutrients, cofactors or vitamins. Peptides and proteins are poorly absorbed and usually require the application of enhancing agents. The surface area of this region represents a small fraction of the total area (0.01%) of the potential absorptive surface of the small intestine (Soergel et al., 1993; Lennernäs et al., 1998; Daugherty et al., 1999). This route may be viewed as a two-component system consisting of an apically located zone of junctional association between neighbouring cells and the underlying space between the lateral membranes of the opposing cells. It is the junctional complex that is the barrier for passive movement of hydrophilic solutes across the epithelium.

The intestinal epithelial cells adhere to each other by a series of junctional complexes: the zonula occludens (ZO) or tight junction near the apex of the cell, the zonula adherens (ZA) or the belt desmosome below and then macula adherens (MA) or spot desmosomes (Farquhar et al., 1963; Madara et al., 1994). The ZO, which is closest to the lumen, consists of a network of fusion sites ('kisses') made up of interconnecting P-face strands and complementary E-face grooves. Chains of 10nm particles form the strands, and the spaces between these particles represent the 'pores' that control the junctional resistance to permeation by small hydrophilic compounds. The resistance of the tight junction varies with its length (100 –600nm) and the number of stands that it contains. Many studies have shown that the paracellular route provides a higher resistance in the ileum than in the jejunum and between villus cells and crypt cells (Martinez-Palomo et al., 1975; Claude et al., 1978; Madara et al., 1987). The ZA located below the ZO and is another cell-cell contact system. These cell contacts appear along the lateral border of intestinal epithelial cells, holding adjacent cells in close not tight, proximity. The ZA is composed of the specific cell adhesion protein, E-cadherin. This glycoprotein is involved in forming associations between adjacent cells (Gumbiner et al., 1991). It appears that this zone is an important site for membrane insertion of actin filaments of the terminal web. The last structure of the junctional complex is the MA, which forms a

discontinuous band of focal rounded structures, unlike the previous structures, which form a continuous belt around the cell. These structures primarily act as fasteners between epithelial cells and as attachment sites for stabilising cytoskeletal cables. A further structure that is found in the junctional complex but normally not incorporated into its description are the gap junctions. These are communication channels between adjacent cells and allow the intercellular transfer of small molecules and ions.

The tight junction complex is a rate-limiting barrier to the permeability of small hydrophilic compounds. This complex completely circumnavigates each epithelial cell to form a continuous seal that segregates the apical from the basolateral component. This barrier appears to have size, charge and shape selective properties. The radii and distribution of the paracellular pores vary for each region of the GI tract. The apparent pore radii have been reported to be 7.5-8Å for the jejunum, 3-3.5Å in the ileum and 2-2.5Å in the colon with 10-fold more pores to be found in the ileum than in the jejunum (Ho et al., 1983). In the CaCo-2 cell monolayer the pore size radius has been estimated to be 12.0 ± 1.9 Å which, compares favourably to the predicted pore size range of the jejunum (Adson et al., 1994).

A further consideration is the predominance of negatively charged groups associated with the aqueous pores, neutral or cationic compounds should pass more easily through these paracellular aqueous pores relative to anionic compounds (Adson et al., 1994; Karlsson et al., 1999). In these studies it was shown that positively charged paracellular compound had greater permeability across the CaCo-2 cell monolayer compared to negatively and uncharged paracellular compounds. A further point to consider is that it is likely that not all pore sizes are the same in each region but a range of sizes co-exist. Regulation of the tight junction permeability is influenced by a number of interrelated factors including peptide hormones e.g. Tumour necrosis factor, the actin myosin ring, intracellular and extracellular Ca^{2+} levels and protein kinase activity (Hochman et al., 1994).

2.3.1.5 Apical efflux pump, p-glycoprotein (P-GP)

P-GP is a 170 kDa is a plasma membrane glycoprotein that belongs to the superfamily of the ATP-binding cassette (ABC) transporters (Gottesman et al., 1993). It functions as an ATP-dependant drug efflux pump originally discovered in multidrug resistance (MDR) in cancer cells upon treatment with anticancer drugs such as vinblastine, actinomycin D and dauromycin (Hunter et al., 1993). In addition, noncytostatic drugs have been reported to be affected by this efflux pump mechanism such as peptides (Cyclosporin A, valinomycin), verapamil and β-adrenoceptor e.g. celiprolol (Augustijns et al., 1993; Burton et al., 1993; Karlsson et al., 1993b; Sarkadi et al., 1994) and surfactant systems e.g. Cremophor EL and polysorbate 80 (Nerurkar et al., 1997). It appears to affect a wide range of chemically unrelated compounds and has been suggested that it is a secretory detoxifying system (Hunter et al., 1993). P-GP is expressed on the apical surfaces of many epithelial cells in a number of tissues including high levels in the columnar epithelial cells of the jejunum and colon, renal proximal tubules epithelium, and the luminal surface of the biliary hepatocytes (Zhang et al., 1998). In the GI tract the P-GP transporter may secrete drugs at low concentration out of the epithelium back into the lumen, however this process may be saturable once the concentration of drug is increased (Anderle et al., 1998). In the CaCo-2 cell monolayer P-GP is expressed but it can vary depending on the time in culture full function was observed on Day 17- 27 (Hosoya et al., 1996) and on the culturing conditions (Hoskins et al., 1993; Anderle et al., 1998).

2.3.2 The enzymatic barrier

The enzymatic barrier is considered to be one of the most important of the multiple barriers affecting the absorption of protein drugs in the GI tract because of the abundance and variety of peptidases. These enzymes are located in the gastric area, GI lumen brush borders and cytosol. Peptide/ protein drugs have a high susceptibility to the proteolytic enzymes and as a result are subject to huge degradation. Typically oral bioavailabilities of proteins are less than 1-2% (Pauletti et al., 1996). This is one of the major reasons why peptide drugs are unsuitable for oral delivery and may account for the relatively poor

success in this field concerning products being made available to the market (Horspol et al., 1998). Insulin, the most widely utilized protein drug, has been at the centre of oral peptide delivery research but a suitable oral formulation has remained an elusive goal (Carino et al., 1999). Multiple enzymes mediate the degradation of proteins simultaneously, which has made it difficult to study their exact mechanism of degradation. Lu et al., (1999) found that human calcitonin was metabolised by various enzymes including pancreatic enzymes and brush border membrane enzymes producing fragments that changed with time whilst in contact with these enzyme systems.

The proteases are classified according to their mechanism of action into four groups: a) serine, b) metallo-, c) cysteine and d) aspartic. They are commonly and abundantly present along the GI tract. Table 2.2 summarises the enzymes, their location and the degradation products that are produced.

As indicated maximal degradation begins in the stomach mainly by the pepsins whose maximal activity occurs at pH 2-3. Pepsins break the protein down into a mixture of polypeptides and these breakdown products move on into the duodenum. In the duodenum the pH increase to 6- 8 and this may cause denaturation or precipitation of the peptide/protein. The pancreatic proteases are active in the duodenum. These proteases may be endopeptidases such as trypsin, α-chymotrypsin and elastase or exopeptidases such as Carboxypeptidase A. Proteins may be further degraded by the brush border enzymes, which are mainly aminooligopeptidases, aminopeptidases and di- or tri-peptidases (Bai et al., 1992).

Table 2.2 Various kinds of proteases in the GI tract, their major sites of action and degradation products (Wang, 1996).

Categories	Enzymes	Major site of action/End product
Gastric proteases	Pepsins (aspartic proteases)	Broad activity, hydrolyse many peptide bonds
Intestinal pancreatic proteases	Trypsin (endopeptidase) α-chymotrypsin (endopeptidase) Elastase (endopeptidase) Carboxypeptidases	Peptide bonds of basic AA and peptides Peptide bonds of hydrophobic AA and peptides Peptide bonds of smaller and non-aromatic AA and peptides A: C-terminal amino acid B: C-terminal basic amino acid
Brush border proteases	Aminopeptidase A, N Aminooligopeptidase Dipeptidyllaminopeptidase Carboxypeptidase	Aminopeptidases are N-terminopeptidases degrading mostly 3-10 AA residues/ dipeptides and AA
Cystolic proteases	Di-tripeptidases	2-3 aminopeptidase/ amino acid

The distribution of these proteases has been shown to vary along the GI tract. In humans, the activities of aminopeptidase N and diaminopeptidase IV is higher in the ileum than the jejunum (TenHoor and Dressman, 1992). Stratford and Lee (1985 and 1986) had previously demonstrated that the activity of aminopeptidases in albino rabbit homogenates was greater in the ileum than in the jejunum than in the duodenum when L-leucine, L-alanine and L-arginine-4-methoxy-β–naphtylamide were used substrates. The presence of peptidases has been shown to exist on the apical side of CaCo-2 cells and Ht29GlucH cells (Howell et al., 1992). In the CaCo-2 cell monolayer the cell-surface peptidases, aminopeptidase N, dipeptidyl peptidase IV, peptidyl dipeptidase A,

aminopeptidase P, aminopeptidase W, endopeptidase 24.11, γ-glutamyl transpeptidase and membrane dipeptidase were all revealed by enzyme assays and immunochemically. The expression and level of activity appeared to be affected by passage number and days in culture. Thus, the CaCo-2 cell model is a plausible model for the *in-vitro* evaluation and characterisation of metabolism of drugs (Buur et al., 1992; Tamura et al., 1996). The use of the CaCo-2 cell monolayer in assessing metabolism of peptides was demonstrated for delta sleep-inducing peptide, a nonapeptide, which was rapidly metabolised in the apical chamber after 2-hours exposure (Augustijns et al., 1995). Also in this study it was clear that the aminopeptidase were mainly located on the apical side of the membrane and that the stability of this peptide was enhanced when peptidase inhibitors were used.

However, not only are the enzymes present in the lumen and the brush border there are also by cystolic enzymes contained within the intestinal cell (Langguth et al., 1997). It has been shown that some peptides are more adversely affected by these enzyme systems than luminal enzymes. The degradation of insulin has been shown to be greater in rat intestinal homogenates of duodenum and jejunum than for everted sacs of the same tissue, with almost 100% degraded compared to only 5-10% degraded after 30 minutes respectively (Schilling et al., 1990). This was confirmed by further work by Bai et al., (1995) who reported that 92% of insulin-degrading enzyme was found in the cytosol of the rat intestine. The CaCo-2 cell culture model has proved to be useful in assessing drug metabolism in the cytosol. Bai et al., (1995) found that 82% of insulin-degrading enzyme was found in the cytosol of CaCo-2 cell monolayer which had a pH optimum of 7.4 which was comparable to human and rat tissue.

Recently, species differences in peptidases activities have been reported. Intestinal tissue homogenates of the guinea pig had greater aminopeptidase activity than rabbit than dog than rat (Zhou et al., 1991). Irwin et al., (1994) reported inter-species differences for the intra-nasal delivery of the peptide, Leu-enkephalin. They demonstrated degradation and bioavailability differences for sheep nasal wash and homogenates compared to rat nasal cavity (Hussain et al., 1985) and human nasal washings (Hussain et al., 1990). The data contrasted with those found in other mammalian systems. In rats, the degradation of the

peptide occurred exclusively at the N-terminal end producing a tetrapeptide with the rate of hydrolysis of Leu-enkephalin being twice as fast as the tetrapeptide. In rabbits initial hydrolysis occurred at three sites producing three degradation products. In human nasal washing it was shown that the rate of degradation was lower than for rats with Leu-enkephalin and the tetrapeptide degraded at similar rates. In sheep it was shown that n-terminal hydrolysis was involved producing the tetrapeptide whose rate of hydrolysis was much faster than for Leu-enkephalin. This emphasises the care needed in choosing an animal model for investigating peptide delivery. Further to this is the effect that age has on the activity of peptidase in the GI tract. It has been shown that there is a 3.89-fold increase in the amount of intact Epidermal growth factor (ECF) absorbed across the intestinal wall in 14-day old suckling rats compared to 30-day old weanling rats. This difference has been ascribed to the higher activity of the ECF-degrading enzyme in the older animals (Thornburg et al., 1987). Proteases not only occur in large quantities in the GI tract but also occur in other tissue e.g. liver where their activities may be very different and in some cases even higher than in the GI tract. The enzyme, glutathione insulin transhydrogenase has been shown to exist in other tissue of the rat and it level of activity to be much greater in the liver and pancreas than in the intestine (Lee et al., 1988).

The types of enzymes encountered, the many locations of these enzymes in the body and the multiplicity of potential degrading sites on the molecule, suggests that there will be an upper limit to the percent of the applied dose of peptide that will reach the target site. Therefore it would appear that the enzymatic barrier is the most challenging barrier in oral protein delivery.

2.4 Absorption mechanisms

Drug molecules can move across the epithelia by two routes: transcellular or paracellular. The transcellular route involves intracellular transfer from the apical to the basolateral of an individual epithelial cell. This transport can take place either through specific uptake mechanisms of the cell or through sequential partitioning events from aqueous to lipid to

aqueous environments. The transcellular route incorporates simple passive diffusion, active transport, facilitated diffusion and endocytosis. The paracellular route involves passive transfer between adjacent cells through aqueous filled pores. This discussion will primarily deal with the transport processes that apply specifically to peptides and proteins.

Passive diffusion refers to the movement of a solute along its concentration and electrical gradient. As long as the diffusing molecule does not interact with the structural elements of the membrane the driving force behind the diffusion of a molecule through the lipid bilayer and/or aqueous pores of the membrane is the concentration difference of the compounds on the two sides of the membrane. Therefore the factors that most affect the passive diffusion of solutes are: lipophilicity, membrane composition and the state of ionisation of the compound. Passive diffusion of moderately and highly lipophilic compounds across the cellular barrier can occur via the transcellular or paracellular route or facilitated diffusion.

2.4.1 Paracellular transport

The paracellular route involves the passive movement of hydrophilic or charged/uncharged compounds between cells. The tight junctional complex is the barrier that prevents the movement of solute via this route (see section 2.3.1.4). The paracellular permeability is composed essentially of a diffusion component and a convective component, where the latter is the rate at which the compound is carried across the epithelium by the water flux (Artursson et al., 1991; Karlsson et al., 1999). The influence that water flux has on the intestinal absorption of small hydrophilic compounds and nutrients in the rat model has been reported by Pappenheimer and Reiss (1987). It was shown that the absorption of creatinine, PEG 4000 and inulin were linear functions of water absorption in the small intestine, which suggested that transport was by convective flow. Since glucose absorption was also observed to be a linear function of water absorption, they postulated that active sodium transport, stimulated by glucose, generated

the driving force for water flux via the paracellular pathway, accounting for 50% of total water absorption. It was proposed that glucose and the amino acids may be responsible for initiating contraction of the junctional actomyosin leading to expanded geometry of the occluding junctions and, consequently increased permeability.

In-vivo studies in man however have not as clearly demonstrated the solvent drag effect (Lennernäs et al., 1994; Lennernäs, 1995). In the presence of isotonic solutions containing high concentrations of the nutrients, D-glucose (20- 40mM) and L-leucine (30-60mM) no increase in water flux was observed across human jejunal segments. However, a hypoosmolar solution at the luminal side of the human jejunum stimulated water absorption, which may increase the uptake of compounds that are freely or partly transported by water (solvent-drag). When the permeabilities of small hydrophilic compounds, MW range 225-346 Da., traversing human jejunum in the presence of hypotonic solutions (170-180 mOsm/L) was investigated no increase was seen compared to when isotonic solutions were perfused (Nielsen et al., 1994; Lennernäs, 1995). Functional viability of the jejunal segments was demonstrated by the recovery of the marker molecule PEG 4000. Lennernäs (1995) outlined the shortfalls of preclinical permeability models such as cell culture, animal perfusion models and isolated tissue in ussing chambers compared to the human jejunal model. The observed differences between the human jejunal *in-vivo* model, these *in-situ* and *in-vitro* models could be attributable to species variation, available surface area and experimental conditions. The lack of blood supply and its consequences for ussing chambers may be a reason for the reduced permeabilities observed for this *in-vitro* model. It was also suggested that the flow rate in the *in-situ* intestinal models was an important factor because a high flow rate may result in deformation of the intestinal wall, flattening the intestine making the more permeable crypts accessible.

The charge, size and shape of molecules also have been shown to affect their permeability through the tight junctional complex. The charge selectivity of the paracellular pathway appears to favour cations (Nellans, 1991). In the cell culture model, CaCo-2 cell monolayer, this cation selectivity has been demonstrated (Knipp et al., 1997;

Karlsson et al., 1999). In the former study by Knipp et al., (1997), not only was charge selectivity demonstrated but also the importance of the radius of the compound. Formate, a negatively charged compound with a molecular radius of 2.51Å had a Papp of 4.39 ± 0.23 X 10^{-6} cm/sec and methylamine which is positively charged having a molecular radius of 2.65 Å had a Papp of 45.1 ± 4.62 X 10^{-6} (cm/sec). Thus demonstrating that for the cation of similar molecular radius to an anion the Papp is 10.3-fold greater. However, as the molecular radius increased to 4.80 Å for positively charged atenolol the Papp decreased 27-fold to 1.67 ± 0.16 X 10^{-6} (cm/sec) compared to methylamine, thereby demonstrating the importance of molecular radius as compared to the pore size.

In-vivo studies in humans have also demonstrated that the paracellular route is cation selective with a series of positively charged compounds transported to a greater extent than negatively charged compounds (Lennernäs et al., 1995). In general the cation selectivity is not considerable; in most cases a maximal of one order of magnitude difference was seen for a wide variety of inorganic compounds. As previously mentioned the tight junction behaves as a barrier not only because of the charge but also due to its radius. It has been estimated than the pore radius *in-vivo* human is approximately 6.7-8.8 Å in the jejunum and decreasing to 2.9-3.8Å in the ileum (Soergel, 1993). *In-situ* rat intestinal perfusion models the pore size has been predicted to be 9.2 ± 0.37Å (Lane et al., 1996). The *in-vitro* CaCo-2 cell-culture model the pore radius has been estimated to be 12 ± 1.9 Å (Adson et al., 1994), and 5.12 ± 1.2 Å (Knipp et al., 1997), the discrepancy may be attributable to the different culturing conditions implemented in these laboratories. In our laboratory the pore size of the CaCo-2 cell monolayer was estimated to be 8.43 ± 1.3 Å (Meaney, 1997). It has been shown by numerous authors that there appears to be relationship between MW of compounds and Papp, as MW increase a decrease in Papp is observed with some variability cited between animals (Ungell et al., 1997; Ho et al., 1995). However, MW of the compound is not the only consideration but also other factors such as molecular diameter (Lane et al., 1996), Log-D (Ungell et al., 1997) probe shape (Artursson et al., 1991; Adson et al., 1994) and dynamic surface properties (Palm et al., 1996) have been shown to influence paracellular transport.

Tight junctions are not static structures rather they exhibit a remarkable degree of plasticity and may be modulated by physical, pathological and physiological events (Daugherty et al., 1999). The transepithelial electrical resistance (TEER) measures the transjunctional flux of ions that are much smaller than the tight junction dimensions and is a useful indicator of junctional permeability (Adson et al., 1994). Tight junctions are where the plasma membranes of neighbouring cells are brought into close opposition forming contact areas, which have shown to have association with the actin cytoskeletal structure known as the perijunctional actin-myosin II ring (Hochman et al., 1994; Daugherty et al., 1999). It is contraction of this ring structure and protein-kinase, or phosphatase-mediated changes in tight junction protein phosphorylation, that have been proposed as mechanisms that may disrupt the tight junctional complex, thereby increasing the paracellular transfer of compounds. Agents, which are known to affect tight junctions and thereby alter paracellular permeability, are shown in table 2.3 and 2.4.

Intestinal cell lines have been extensively used to demonstrate that peptides can affect the tight junction by either increasing or decreasing paracellular permeability. However, these studies also have revealed that often the effect only occurs when the agent is placed in the basolateral chamber (McRoberts et al., 1992). It was shown that when the insulin growth factor or insulin was placed in the basolateral chamber of T84 cells that a 8-fold reduction in TEER was recorded over 3-4 days. The precise mechanism of action appeared to be associated with changes in protein synthesis and the structure of the cytoskeleton. As the GI tract is continuously exposed to a large spectrum of pathogenic agents its selective permeability properties provides a great deal of protection. However a number of pathogenic agents have acquired abilities to disrupt the structure or breach this barrier. ZOT (zona occludens toxin) that is released from Vibrio cholerae reversibly modifies actin polymerisation. It increases the permeability of TG to macromolecules by modification of protein kinase C pathway (Lutz et al., 1997; Daugherty, 1999). Previously Fasano et al., (1991) had shown that there was an increase in the permeability of rabbit ileum and by freeze-fracture techniques illustrating that the tight junctional complex had been simplified in the presence of ZOT. Some pathogens can directly disrupt the paracellular barrier function following interaction with epithelial cells similar

to what has been observed for *Mycoplasma pulmonis* which produced a two-fold increase in the paracellular permeability of tracheal epithelial cells (Dollery, 1999).

Table 2.3 Time course of action of some agents that are capable of tight junction disruption *in-vitro*

Agent	Approximate response time
Insulin	3- 4 days
Insulin-like growth factor-1 or –2 (IGF-1 or IGF-2)	4 days
Tumour necrosis factor (TNFα)	90 min
Interferon-gamma (IFN-γ)	2- 3days
Cytochalasin B or D	20 min
C. difficile toxin A	6- 8 hours
ATP depletion	30 min
Protein kinase A inhibition	1 hour
Protein kinase C activation	2 hours
Tyrosine phosphorylation	30 min
Oxidants	20- 30 min
Ca^{2+} chelators	10 min
Ca^{2+} ionophores	30 min
Na^{+}-linked nutrient uptake	20 min

The release of cytokines and lymphokines during the inflammatory and immune response following cell infection has been shown to affect paracellular integrity. Madara et al., (1989) demonstrated that IFN-γ caused a six-fold increase in the permeability of the paracellular marker, mannitol and inulin across T84 cells with a corresponding decrease in TEER. The time frame of hours-days associated with the response of this cytokine on

intestinal epithelial cells corresponds to the *in-vivo* response of intestinal infections. Other cytokines have also been shown to affect the paracellular pathway, such as TNFα, IL-1 and IL-2.

The cytoskeletal ring at the apical neck of the intestinal epithelial cells acts to coordinate and stabilize TG protein arrays at the membrane surface. *C. difficile* exotoxin affects the small GTP-binding protein whose function is required for the stabilisation of actin filaments in the perijunctional cytoskeletal ring (Daugherty et al., 1999). Cytochalasin B and D both are actin microfilament perturbing agents affecting the cytoskeletal structure causing an increase in paracellular permeability. Epithelial cell monolayers of MDCK and T84 have been used as models for investigating the effect of these perturbants (Meza et al., 1980; Madara et al., 1988). There was a decrease in TEER with a subsequent increase in the flux of the paracellular marker, mannitol and the microelectrode surface scanning studies showed a decrease in the number of TG strands which all indicated an increase in TG permeability.

Since TG structures are closely associated because of the formation of divalent Ca^{2+}-dependant links between the E-cadherin proteins in adjacent cells (Cereijido et al., 1993). This indicates the pivotal role that Ca^{2+} has in regulating the TG. The addition of the Ca^{2+} chelators EDTA to several cultured cell lines was seen to be polarised (Noach et al., 1993; Collares-Buzato et al., 1994). Epithelial permeability increase dramatically when EDTA was added to the basolateral side and even more so when added to the apical and basolateral sides, whereas no increase was observed with apical application only. This dependence may be explained by the presence of the calcium-dependant molecule, uvomorulin, in the ZA region, which is closest to the basolateral side. The use of the Ca^{2+} ionophore A23187, to increase the intracellular levels of Ca^{2+} has resulted in an increase in paracellular transport across, MDCK epithelial cells, CaCo-2 cells and rabbit tracheal epithelium (Rutten et al., 1991; Bhat et al., 1993). The precise mechanism of action of A23187 is incompletely understood but probably involves Ca^{2+}-dependant regulation of the cytoskeleton, phospholipases and proteases (Bhat et al., 1993).

Whilst the paracellular route is restricting to size there is evidence to show that peptides are transported via this route (Yen and Lee, 1995). In this study it was shown that pentapeptide, Pz-peptide was transported via the paracellular route in colonic and intestinal segments of albino rabbit. Pz-peptide when incubated in homogenates and sub-cellular fractions of various intestinal segments, underwent substantial degradation; however, it penetrated all intestinal segments with more than 80% of the peptide in the intact form. This suggests that the peptide permeated through the paracellular route and thus avoided intracellular proteolysis. Also the transport of thyrotropin releasing hormone has been shown to be transported via the paracellular route (Walter et al., 1994).

Opening of the intestinal paracellular barrier, through a disruption of TJ complexes, can enhance the uptake of orally delivered drugs. It is not clear as to what repercussions that may or may not arise from repeated or chronic disruption of this barrier. It has been shown that increased tight junctional permeability is associated with the development of colon cancer (Peralta Soler et al., 1999). With a continually improving knowledge of the structure-function relationships of TJ components, it may be possible to manipulate the TJ in a more dynamic and controlled fashion to enable the selective uptake of many poorly absorbed drugs, such as peptides and proteins at specific sites without the associated traumatic events.

2.4.2 Transcellular transport

Passive transcellular transport is the movement of molecules through cells. This route of transport is considered to be very important, as the surface area is several times greater than the paracellular route. The ability of the drug molecule to partition in to the cell membrane is of critical importance. Therefore, lipophilicity is traditionally considered to be an important molecular characteristic in determining passive diffusion through biological membranes because the membrane is considered to be lipophilic barrier. It has been demonstrated that for a variety of small inorganic compounds that the octanol/ water partition is a good predictor of their permeation of biological membranes. It has been

possible to establish a sigmodial relationship between the log partition coefficient and the effective permeability coefficient for the solute across the cell membrane (Ho et al., 1977). Interpreting this relationship the following points should be considered, a) Log octanol/water coefficients less than 0, transport across the membrane is slow due to poor membrane permeation properties b) the optimal log partition coefficients is about 1- 3 and c) values greater than 3 the permeability is independent of the partition coefficient and the aqueous boundary layer becomes the rate limiting step (Westergaard et al., 1974). In the CaCo-2 cell model the correlation of apparent permeability and log of the partition coefficient has not provided researchers with the same relationship as observed by Ho et al., (1977) had observed. Previously Artursson et al., (1991) had seen no correlation in the Log P value and the Papp for a series of 20 compounds with varying molecular weight and lipophilicities. In further studies where the number of compounds was increased to 51 the only trend observed was that it was possible to determine a Log P value above which all compounds were highly permeable (Yazdanian et al., 1998). However, Wils et al., (1994) using *in-vitro* cell models demonstrated that when the Log P values were lower than 3.5, the transepithelial permeability coefficient increased with lipophilicity for a range of compounds. However when the Log P values ranged from 3.5- 5.2, the transepithelial permeability decreased with lipophilicity. This suggested that there was a point where lipophilicity of a compound compromised its permeability characteristics. This reinforces the principle that single physical parameters such as partitioning values are but one factor involved in transcellular diffusion. Other factors that should be considered are solute size and shape, steric considerations e.g. chain branching and conformation (Burton et al., 1991). Other physical chemical parameters have shown simple linear relationships between CaCo-2 permeability coefficients including H-bonding capacity (Conradi et al., 1991; Kim et al., 1993) and molecular surface properties (Palm et al., 1996) for example. Evidence has shown that peptides/proteins are able to diffuse across the GI membranes, insulin appears to be transported partly by passive diffusion across rat intestine (Bendayan et al., 1994) and human calcitonin (hCT) transport across rat colon is predominated by the transcellular pathway (Hastewell et al., 1992).

2.4.3 Endocytosis

This route of transport is dedicated to a small number of macromolecules. Examples include ferritin and lectin in the ileal absorptive cells of suckling rats (Gonnella et al., 1984) and bradykinin and it analogues transport across CaCo-2 cell monolayer (Shimizu et al., 1997). The process of endocytosis is at least partly responsible for meeting nutritional requirements and host defence. Endocytosis mechanisms are either non-specific (fluid-phase endocytosis, pinocytosis and phagocytosis) or specific (absorptive endocytosis). Non-specific endocytosis is the engulfing of extracellular fluid containing dissolved protein. Specific endocytosis is a process of protein binding to the cell membrane followed by internalisation of vesicles. Non-specific endocytosis varies from specific endocytosis in the following features a) solute uptake is not concentration dependant, b) no competition between labelled and unlabelled solute and c) uptake rates are similar for different solutes. It appears that in epithelial cells that specific endocytosis is much greater than non-specific endocytosis (Steinman et al., 1983). Receptor mediated endocytosis is a special case of specific endocytosis where the substrate binds to specific receptors on the plasma membrane. Phagocytosis, engulfment of particles greater than 500nm is associated with specialised cells and areas of the GI tract e.g. Peyers patches. These sites have been suggested to be promising sites for the uptake of orally administered macromolecules (Neutra, 1998). However, because of the scarcity of these absorptive sites and the possibility that proteins entering via this route may induce antibody response it is considered not to be the most appropriate site for the delivery of pharmacological doses of peptides (Daugherty et al., 1999). The internalised proteins may have different fates within the cell. Proteins are endocytosed into endosomes, which have a pH of 5.0. In these vesicles proteins are concentrated and sorted out as to where they will be sent to: lysozymes where they are degraded, recycled back to the cell membrane or transcytosed i.e. diffuse to the basolateral chamber where they are exocytosed intact (Shen et al., 1992).

2.4.4 Active transport

Active transport is an energy dependant process and can occur against a concentration gradient. Energy is supplied in primary active transport by the hydrolysis of ATP. However, in secondary active transport, also known as coupled or co-transport, the energy is supplied by Na^+/ H^+ -exchanger, which generates and maintains the inward proton gradient on the luminal surface, while the Na^+/K^+ -ATPase present in the basolateral membrane maintains a low intracellular Na^+ concentration. As protons are co-transported this system is also known as H^+ -dependant cotransport system (Leibach et al., 1996). The transport of sugars, amino acids, bile salts and vitamins are by active transport process (Kararli et al., 1989). The active transport process is characterised by the following features: molecular oxygen requirements, inhibited by metabolic inhibitors e.g. 2,4-dinitophenol, which has shown to inhibit uptake of cefaclor, a peptidometic, by 81% across CaCo-2 cell monolayer (Dantzig et al., 1992), temperature dependence and competition from substrate analogues e.g. the cephalosporins, loracarbef uptake is inhibited by cefixime and visa-versa (Dantzig et al., 1994). The characterisation of a peptide transporter system in the intestine with its specificity has lead to new approaches in intestinal absorption. The strategy is to synthesis small, metabolically stable and recognisable by the transporter system. The intestinal peptide transport system accepts drug molecule with similar structural features to its physiological substrates as substrates and acts as a carrier for their absorption (Tsuji, 1987). β-lactam antibiotics were one of the first group of compounds to be identified as substrates for the peptide transporter system. The transporter system is dependant on an inwardly directed proton-pump gradient and on membrane potential. The carrier also mediates for ACE di and tri-peptides and renin inhibitors (Kramer et al., 1990). The transporter also takes up the β-lactam that exist as zwitterions such as the aminocephalosporins and anionic cephalosporins (Dantzig et al., 1992, 1994). Once concentrated in the enterocyte the compound is exited at the basolateral surface in to the blood stream. It has been shown that specific peptide transporters exist on the basolateral surface of CaCo-2 cell monolayers for cephalosporins (Inui et al., 1992). It appears that the rate-limiting step for

the peptide transporter is the release of the substrate into the blood stream (Dantzig, 1997).

2.5 Approaches to improve oral delivery of peptides

Even though some proteins can penetrate the GI tract the quantity absorbed is generally insufficient to produce significant pharmacological responses. It has been shown that even when pancreatic proteolytic enzymes *in-vivo* have been removed by total pancreatectomy, the total amount of insulin absorbed in human jejunum is approximately 0.5% (Crane et al., 1968). Therefore various oral protein delivery strategies have been investigated to improve peptide and protein absorption. The following is a review of the methods that have been employed: penetration enhancers, protease inhibitors, chemical modification and carrier systems. Wang (1996) provides an extensive table listing the methods that have been employed to enhance the transport of peptides with the results and effects of these methods. However, it has been suggested that a combination of all these methods may result in the delivery of peptides via the oral route (Aungst, 1993). The combination strategy has been one of the most successful to date for the delivery of insulin with microspheres having protease inhibitor and permeation enhancing characteristics (Carino et al., 1999).

2.5.1 Absorption enhancers

Penetration enhancers can be used to enhance the transport of compounds by the paracellular and/or transcellular route. Ideally these systems should have the following desirable attributes (Junginger et al., 1998):

- The enhancing action should be immediate and unidirectional, and the duration of the effect should be specific, durable and predictable.

- Following removal of the material to the applied membrane the tissue should fully recover to its normal barrier functions.
- No systemic or toxic side effects should be seen
- No irritation or damage of the affected membrane
- Wide range of compatibility with other pharmaceuticals, excipients and drugs

As yet no single enhancer fulfils the above criteria, table 2.4 summarises the major classes of enhancers, which have been investigated for oral macromolecular absorption (Muranishi, 1990; Junginger et al., 1998).

Penetration enhancers improve protein absorption by one or more of the following mechanisms 1) altering rheological properties of mucus, 2) fluidising the membrane lipid bilayer, 3) altering the intercellular tight junctions (TJ), 4) inhibiting enzymatic activity and 5) increasing the thermodynamic activity of proteins (Lee et al., 1991). In the CaCo-2 cell monolayer the use of SDS (sodium dodecyl sulphate) a surfactant enhancer that extensively interacts with the membrane has been shown to affect the permeability of compounds and the morphology of the cells (Anderberg et al., 1993a). In this study the absorption of mannitol, vasopressin and PEG 4000 increased via the paracellular route. This was demonstrated by a reduction in TEER, the disbanded actin filaments and the morphological changes in the monolayer. A dose dependant effect of SDS on the epithelium was observed, with a recoverable response only seen for cell monolayers exposed to the surfactant for 20min and no recovery when exposure time was increased to 2 hrs.

Table 2.4 Classes of absorption enhancers and their proposed mechanism of action.

Class	Example	Mechanism	Transport route
Surfactants	Na-laurylsulphate Polyoxyethylene-9-laurylether Bile salts: Sodium glycocholate (NaGC) Sodium deoxycholate (Deoxy) Sodium taurocholate (NaTC)	Phospholipid acyl chain perturbation Peptidase inhibition Mucus viscosity reduction	Transcellular ↑ Paracellular ↑
Fatty Acids	Oleic acid Short fatty acids	Phospholipid acyl chain perturbation	Transcellular ↑ Paracellular ↑
Cyclodextrins	α-, β- and γ-cyclodextrins Methylated β-cyclodextrins	Inclusion membrane components	Transcellular ↑ Paracellular ↑
Chelators	EDTA Polyacrylates	Complexation of Ca^{2+} TG opening	Transcellular ↑ Paracellular ↑ Paracellular ↑
Positively charged polymers	Chitosan Trimethyl chitosan	Ionic interaction with negatively charged glycocalix groups	Paracellular ↑
Amino acid conjugates	E352 E414 Derivatized α-amino acids	Affect specific receptors or structural features of the drug	Unknown

The bile salts have extensively been used as absorption enhancers. These systems have been used to promote the absorption of peptides and proteins across epithelia. Insulin absorption was increased to 2.3% in the presence of sodium glycocholate (NaGC) (30mM) in the jejunum/proximal ileum segment of the rat intestine (Shao et al., 1993). The formation of mixed micelles (NaGC: LA) has shown to improve the absorption of insulin across rat nasal epithelium as compared to simple bile salt micellar systems of

NaGC. The nasal bioavailability was about 15-17% for the mixed micellar system (Tengamnuay et al., 1990). Mixed micelles have also enhanced the transport of human calcitonin (hCT) across rat colonic segments (Hastewell et al., 1994). In this study by Hastewell et al., (1994) for both man and rat model the bioavailability of intra-colonically administered hCT is less than 1%, however with the use of sodium taurocholate and monoolein (40:40mM) the absorption of hCT increased 9-fold. The type of fatty acid used in mixed micelles has been shown to influence results. O' Reilly et al., (1994a; 1994b) observed that the greatest absorption of clofazamine, a lipophilic drug, was increased in the presence of NaTC and unsaturated fatty acid, linoleic acid. Mesiha et al., (1994) also observed that the hypoglycaemic effect of orally administered insulin in rabbits was increased in the presence of NaGC and saturated fatty acids as compared to unsaturated fatty acids or non fatty acid systems with the same chain length.

Cyclodextrins have also been used to increase the oral bioavailability of peptide compounds. The methylated CDs, DMCD 10% w/v appeared to enhance the bioavailability of insulin from a negligible value (approx. 0.06%) to 5.63% when administered enterally in the lower jejunal/upper ileal segment of rat intestine (Shao et al., 1994). The use of CDs as nasal absorption enhancers has been adequately demonstrated for peptides and hormones: insulin (Merkus et al., 1991; Shao et al., 1992; Verhoef et al., 1994), the adrenocorticotropic hormone analogue (ACTH) (Schipper et al., 1993) and salmon calcitonin (Schipper et al., 1995).

The use of chitosan as bioadhesive drug delivery systems for peptide drugs has shown potential (Dodane et al., 1998. 1999). Using an *in-vitro* cell culture model it has been reported that chitosan derivatives were able to significantly increase the permeabilities of a series of peptide compounds including vasopressin, buserelin, 9-desglycinamide and insulin but without preventing these compounds from being degraded by proteolytic enzymes (Kotzé et al., 1997).

2.5.2 Protease inhibitors

Protease inhibitors may also promote the oral absorption of therapeutic peptides and proteins by reducing their proteolytic breakdown in the GI tract. The effect of five different protease inhibitors, sodium glycocholate, aprotinin, bacitracin, soya bean trypsin inhibitor and camostat mesilate were all compared when coadministered with insulin directly into isolated loops of the small and large intestine of rats (Yamamoto et al., 1994). This study concluded that these inhibitors particularly, NaGC bacitracin and camostat mesilate were much more effective at improving the amount of available insulin to the large intestine compared to the small intestine. These inhibitors proved useless in the small intestine and this may be attributable to the overwhelming amounts and varieties of enzymes in that locale. *In-vitro* transport of vasopressin across alveolar epithelial cell monolayers have shown that the stability of the peptide was enhanced in the presence of camostat mesilate (aminopeptidase inhibitor) but not in the presence of leupeptin (a serine protease inhibitor), which indicates the importance of knowing the susceptibility of the peptide to which enzyme systems (Yamahara et al., 1994). The stability of the DSIP (delta sleep-inducing peptide) across the CaCo-2 cell monolayer was investigated and found to be enhanced in the presence of a 'cocktail' of peptidase inhibitors, bestatin (aminopeptidase inhibitor), diprotin (dipeptidylpeptidase IV inhibitor) and captopril (peptidyl dipeptidase A inhibitor). This combination increased the stability of this peptide remaining in the apical chamber after 2h incubation to 95.1 ± 1.6% from 8.2 ± 1.1% (Augustijns et al., 1995). As previously mentioned bile salts are capable of not only increasing the permeation of compound but also the stability since they are inherent enzyme inhibitors (Hirai et al., 1981a, b). In these studies the *in-vitro* stability of insulin in the presence of 0.27% of sodium glycocholate in rat intestinal homogenates increased more than 10-fold from 9% for control to 92% after 60 min incubation. The stability of [D-Ala^2, D-Leu^2] enkephalin (DADLE), an analgesic pentapeptide, was enhanced in the presence of puromycin, amastatin and NaGC in rat intestinal homogenates. It was also shown that NaGC (10mM) in combination with bestatin increased the permeability and stability of DADLE traversing rat intestine mounted on modified Ussing chambers (Uchiyama et al., 1997).

CDs have also been reported to provide some enzymatic protection. Irwin et al., (1994) reported the stability of leucine enkephalin in sheep nasal homogenate was increased substantially with the $T_{1/2}$ increasing from 44 min to 75 min in the presence of β-cyclodextrin. The stability of insulin was also improved in the presence of CDs in rat nasal homogenates with DMCD providing the most protection to the peptide (Arima et al., 1990)

2.5.3 Chemical modification

This approach is usually more applicable to small peptides rather than to larger proteins because of the structural complexity associated with proteins (Samanen et al., 1996; Pauletti et al., 1997). A peptide can be chemically modified to increase its enzymatic stability and/or membrane permeation (Conradi et al., 1992; Bai et al., 1992). Langguth et al., (1997) demonstrated that increasing met-enkephalin enzymatic stability by chemical modification did not affect the permeability across everted intestinal rings. Another strategy is to increase the lipophilicity of a hydrophilic compound. The synthesis of new lipophilic derivatives of tetragastrin, TRH, insulin and lysozymes increased their permeability whilst retaining their biological activity (Muranishi et al., 1991). It was also suggested that this modification resulted in an increase in enzymatic stability of these compounds. Cyclization of molecule results in an increase in lipophilicity due to the removal of the N and C-termini. Also, more lipophilic synthetic amino acids can be used to make peptide analogues providing that biological activity is not reduced or lost. The formation of these lipidic amino acids and their homooligomers represents a class of compounds, which have the structural features of lipids with those of amino acids (Toth et al., 1994a, c). Because of this bi-functionality these may be conjugated to a wide variety of functional groups, creating new compounds or prodrugs. The resultant compound possess a high degree of affinity for the membrane thereby facilitating its transfer across the epithelium and cause steric hindrance with the alkyl side chains in preventing enzymatic degradation (Toth et al., 1994a). The conjugation of the enzymatically labile peptides, LHRH and TRH to various lipidic peptides increased the

half-life in CaCo-2 cell homogenates. LHRH $T_{1/2}$ was increased 9-fold on conjugation to one lipidic peptide and when this was increased to two lipidic peptide conjugations the half-life increased 72-fold as compared to the unconjugated LHRH (Toth et al., 1994b). However, chemical modification does not always cause an increase in the permeability of the compound in question. The diacyl derivatives of insulin were more prone to enzymatic degradation compared to the parent moiety in the rat intestine. This was due to the compound not being able to associate because of the diacylation allowing more monomers to be made available for degradation (Asada et al., 1994). The targeting of specific transporters by chemical modification is a promising mechanism for the delivery of small-peptide derived drugs. For example bile acid transporters can be utilised for site-specific delivery, cephalexin was bound to bile acids, which resulted in an increase in its absorption across the intestinal epithelium of rabbits and also demonstrated liver specificity (Kramer et al., 1997).

2.5.4 Carrier systems

The use of various carrier systems such as nanoparticles, microspheres, liposomes or erythrocytes can be used to enhance peptide and protein absorption (Swenson et al., 1992; Wang, 1996). It has been shown that the primary route of transport of these systems is via Peyers patches and endocytosis. Particle absorption is size dependant with little to no absorption seen for particulates greater than 2- 5μm (Jani et al., 1990). However, several reports have suggested that the bioavailability of peptide drugs have increased with these systems. Damge et al., (1988) have shown that the hypoglycaemic effect of insulin was enhanced in nanoparticles (220nm) in diabetic rats. The improvement was attributed to the increase in the enzymatic protection offered by these systems. The bioavailability of peptides via this route appears to be prolonged. Polyalkylcyanacrylate nanoparticles administered to rats caused a 50-60% reduction in glycaemia. However, this effect appeared two days after administration and was maintained for a period of up to 20 days (Damge, 1988). This prolonged or sustained activity of these systems was reported for isobutylcyanoacrylate nanoparticles

encapsulating insulin and calcitonin in rats (Lowe et al., 1994). It was suggested that the peptide was slowly released into the lumen with small amounts being absorbed because of the variable absorption profile.

The delivery of starch microspheres across the CaCo-2 cell monolayer has been investigated by a number of authors (Björk et al., 1995; Illum et al., 1990). These systems induced pulsatile delivery of insulin across the monolayer for 1-2hr by causing a reversible dilation of the tight junctional complex. It would appear however that the even with the lower sized particles the bioavailability is poor, it may be useful for vaccine delivery (Augnst, 1993).

CHAPTER 3

Models for Assessing Gastrointestinal Peptide Absorption

3.1 Introduction

Models to assess intestinal drug absorption can be classified as, *in-vivo*, *in-situ* or *in-vitro*. A brief introduction will be given to include some examples of these different methods. Particular emphasis will be placed on the *in-vitro* cell culture model, as this was the model utilised in this project. The merits and criticism of the cell culture model, in particular CaCo-2 model, will be discussed together with the *in-vitro: in-vivo* correlations, which have been published.

3.2 *In-vivo* models

These studies involved direct sampling of biological fluids e.g. blood or urine from intact human or conscious animal models after administration of a formulation. There are many techniques employed for *in-vivo* systems including: intubation technique using a multichannel tube e.g. Loc-I-Gut which allows by inflating two balloons at either end of a tube to study isolated segments of the intestine (Lennernäs et al., 1995), high frequency capsule (Lee et al., 1999), direct administration to the site of administration by surgically implanted chronic catheters (Sinko et al., 1999), capsule pharmacoscintigraphy (Rouge et al., 1996). *In-vivo* studies give information regarding the absolute or relative bioavailability of the compound. However, limitations do exist with this model especially when information is required regarding the specific transport pathway at cellular level. Another factor is the considerable intersubject variability because of the individual differences that exits (Sinko et al., 1999; Lee et al., 1999). However, this model is the ultimate test in ensuring that a product formulation is worthy of further investigations.

3.3 *In-situ* intestinal models

Usually the *in-situ* methods involve anaesthetising experimental animals (rat, dog, monkey or rabbit) and isolating the chosen intestinal segment for perfusion of the test formulation. The methods employed include both open loop technique such as single-

pass perfusion, recirculating perfusion and oscillating perfusion and closed loop technique (Rouge et al., 1996). The relative merits of the above procedures have been extensively reviewed by Schurgers et al., (1986) and Doluisio et al., (1969). In drug perfusion studies the drug or metabolites is sampled from the blood stream (portal vein) or directly from the loop lumen (Blanchard et al., 1990). To assess water flux across the intestinal wall the inclusion of poorly absorbable marker molecule e.g. PEG 4000 or inulin is added to the perfusion solution. *In-situ* studies provide information on the rate-limiting and rate-determining factors of drug absorption in the intestine (Ho et al., 1983). These methods have advantages over the *in-vitro* methods including the presence of an intact lymphatic and blood vessel supply and extended tissue viability. Advantages over *in-vivo* methods include drug dissolution and by-passing sites of drug degradation and allowing control over drug input and choice of intestinal segment to be perfused (Banga, 1996). However, these methods do have disadvantages such as the trauma of surgery to the animal and in addition the anaesthetic may affect the absorption kinetics. In order to overcome these disadvantages Poelma et al., (1989) developed the chronic isolated intestinal loop procedure that allows absorption to be studied without the interference of anaesthesia and surgical trauma. This model also permits cross over experimental designs. In general direct *in-vivo* correlation to human data may be difficult because of the anatomical and physiological differences between animals and man i.e. a species difference.

3.4 *In-vitro* models

The successful application of *in-vitro* models to the study of drug absorption across the intestinal mucosae depends on how closely the *in-vitro* model mimics the characteristics of the *in-vivo* biological barrier. These models allow for the study of absorption at a cellular level and tend to be less complicated than *in-vivo* models. Although it is very difficult to reproduce *in-vitro* all of the characteristics of the intestinal mucosae, various systems have been developed which, to varying degrees, mimic the characteristics of the intestinal mucosae.

3.4.1 Excised tissue

The tissue sample that is required is surgically removed from the animal. Once removed the tissue may be used to prepare: intestinal segments, everted sacs, intestinal rings or stripped or unstripped mucosae for mounting in Ussing chambers. These methods have disadvantages including the lack of vasculature, the unphysiological thickness of the membrane due to the muscle layer and the reduced viability of the tissue. Ussing chambers has tissue mounted in a chamber which allows the researcher to determine the flux of compounds from the apical to the basolateral side in the presence and absence of excipients and monitor TEER which gives this technique a distinct advantage over the other excised tissue techniques (Jezyk et al., 1992).

3.4.2 Isolated cells

Isolation of intestinal enterocytes can be accomplished using proteolytic enzymes (Hartman et al., 1982), chelating agents (Weiser et al., 1973) or mechanical disassociation (Harrison et al., 1969). These cells once isolated have complex media requirements to be sustained, have a short life span and are used in suspension. Therefore, experiments must be carried out as quickly and efficiently as possible. These systems allow for uptake experiments to be carried out but do not distinguish between apical or basolateral polarity of this uptake. The major advantage of this system is that they are useful for studying the uptake and metabolism of compounds without interference from submucosae and underlying musculature. The experimental conditions can be tightly controlled and multiple compounds tested on enterocytes isolated from a single animal.

3.4.3 Membrane vesicles

Membrane vesicles allow further simplification of the system. These brush border membrane vesicles (BBMV) are prepared through a series of homogenisation and centrifugation steps and the resulting spheres can be best described as intestinal

liposomes (Hillgren et al., 1995). These vesicles are devoid of any basolateral membrane and cellular components. In the isolated BBMVs, the transport properties of solutes can be investigated without the interference of cellular metabolism and energy. Moreover, the diffusion characteristics of the brush border can be studied independently of the basolateral membrane. Using these vesicles, the transport characteristics of choline, glucose, several amino acids and small peptides have been studied (Yuasa et al., 1993; Kramer et al., 1990).

3.4.4 Cell culture models

Monolayers of intestinal cells grown in culture have been widely used as a method to predict intestinal absorption (Hidalgo et al., 1989; Wilson, 1990; Artursson, 1990). Unlike isolated tissues, cell culture can have extended viability when supplied with nutrients and oxygen. Immortalised human cell lines differentiate in culture and have been used to study drug transport and metabolism. This section will describe the three of the immortalised cell lines that are frequently used: CaCo-2, CaCo-2: Ht29GlucH and T84 and will briefly review the future developments for cell culture models.

3.4.4.1 T84

T84 cells display the characteristics of colonic crypt cells and have been widely used to study electrogenic Cl^- secretion, the transport event responsible for mucosal hydration (Dharmsathaphorn et al., 1989). They grow as very tightly packed colonies and when confluent display abundant microvilli on the apical surface facing the culture medium. They differ from CaCo-2 cells in that the brush border is not as well developed and they do not express microvillous membrane hydrolases (Zweibaum et al., 1991)

3.4.4.2 Ht29

The Ht29 cell line was established in culture from a colon adenocarcinoma in 1964 by Fogh (Zweibaum et al., 1991). Under standard cell culturing conditions, Ht29 cells

remain undifferentiated, growing as a multilayer of polarised cells with irregular microvilli and desmosomes. The activity of the brush border associated enzymes is low and sucrose isomaltase (SI) is absent. The two principle features that have brought this cell type to the forefront of intestinal research is their 1) ability to differentiate under certain culturing conditions and 2) mucus producing clones have been isolated (Pinto et al., 1982; Huet et al., 1987). Pinto et al., (1982) induced the cell line to differentiate by replacing glucose with galactose in the culturing media therefore they were termed Ht29Gluc. This differentiated phenotype was characterised by polarisation of the cell layer, with apical brush borders and tight junctions, and by low levels of SI (Zweibaum et al., 1985). Louvard et al., (1984) isolated the Ht29-18 clone from Ht29 cells grown in glucose enriched media. When these cells were grown in galactose approximately 10% exhibited signs of differentiation to form mucus secreting cell types whereas the rest were differentiated absorptive cells. Many other Ht29 cell clones have been isolated that are mucus producing e.g. Ht29-18-C_1 and Ht29-18-N_2, the former has been used as a cell culture model for studying drug transport (Wils et al., 1993).

Treatment of the Ht29 cell with 5mM sodium butyrate produced cell clones exhibiting morphological signs of either enterocytic (junctional complex, dome formation) or goblet-cell (mucus secretion) differentiation (Hillgren et al., 1995). The selection of these goblet-cells is possible by using glucose-deficient media, and with different concentrations of cytotoxic drugs e.g. methotrexate or 5-fluorouracil (Lesuffleur et al., 1991a and b), yielding derivative populations or clones that are able to maintain a differentiated phenotype in the absence of the selective agent(s).

The development of a monolayer of mature goblet cells that had the ability to secrete mucin was demonstrated for Ht29GlucH cell clone under standard culturing conditions (Allen (1992); Wikman et al., 1993; Meaney (1997)). This monolayer was grown on permeable supports and demonstrated the existence of a mucin layer after 2 weeks in culture with the thickness and surface coverage increasing exponentially after 14 days to give a thickness of 40-60μm and surface coverage of >90% after 4 weeks (Wikman et al., 1993). In this study the apparent permeability of testosterone, a lipophilic compound was

shown to be delayed in the presence of mucus with the Papp increasing 2-fold following a washing of the mucus layer. This cell type permits examination of the effect that the mucus layer has on the permeability of compounds under controlled cell culturing conditions. Karlsson et al., (1993b) demonstrated that the transport of the highly lipophilic compound testosterone was 4.6-fold higher in the CaCo-2 cell monolayer as compared to the Ht29GlucH monolayer. In this study it was determined that for the Ht29GlucH that the mucus layer was the rate determining step in the absorption of the compound.

3.4.4.3 CaCo-2

Fogh (1974) established the CaCo-2 cell line from human colon adenocarcinoma. The characterisation of the CaCo-2 cell line has been extensive leading to a wealth of knowledge on the structural and functional aspects of this cell type. It has been shown the confluent cells differentiate to form columnar shape cells representing villus enterocytes, the presence of a well developed brush border facing the culture media and the formation of tight junctions (Pinto et al., 1983; Rousset et al., 1985). Domes can be observed in confluent cultures reflecting the presence of tight junctions. When the cells were grown as monolayers on semi-permeable supports the measurement of the transepithelial electrical resistance (TEER) confirm the formation of tight junctions, with the transepithelial electrical resistance (TEER) of about 150- 300Ω.cm ^{2} (Hidalgo et al., 1989). The most distinctive feature of the CaCo-2 cell monolayer, making them the first good model for differentiated enterocytes *in-vitro*, is their ability to produce several brush border digestive enzymes. Both immunohistochemical and biochemical studies have revealed the presence in post confluent cells of sucrase-isomaltase (SI), γ-glutamyl transpeptidase (GT), alkaline phosphatase (AP), dipeptidylpeptidase IV (DPPIV), aminopeptidase N (APN), aminopeptidase W (APW), aminopeptidase P (APN) and angiotensin I-converting enzyme (Howell et al., 1992; Jalal et al., 1992; Chantret et al., 1994). Recently the activities of the cystolic enzymes were compared to the behaviour of the rat colonic and rectal mucosae, which demonstrated that the cytosolic enzymes from the CaCo-2 cells had greater activity (Bai, 1995). While no direct comparison to the

activity of the small intestinal cytosolic enzymes was carried out, the difference in activity implies that the CaCo-2 cell may resemble the small intestinal in terms of enzymatic activity. The expression of many other enzyme systems have been verified in the CaCo-2 cell model, see table 4.1 for a brief summary.

Table 3.1 Enzyme systems that are expressed in the CaCo-2 cell monolayer and their site of activity.

Enzyme	Site of activity	Reference
Insulin degrading enzyme	Cytosol, degrading insulin	Bai et al., 1994
Glutathione-S-transferase isoenzyme (GST)	Detoxifying enzymes, expressed to similar levels as found in the small intestine	Peters et al., 1992
Phenol sulfotransferase (PST)	Levels increased as the cell matured	Baranczyk-Kuzma et al., 1991
Cytochrome P450 1A1 isoenzyme (CYP1A1)	Responsible for the activation of precarcinogens	Boulenec et al., 1992
7-ethoxyresourufin o-deethylation (EROD)	Enzyme system associated with CYP1A1, levels increase to maximum 25 days post-seeding	Rosenberg et al., 1993
Antioxidant enzymes		Baker and Baker 1992

In summary CaCo-2 cells have been shown to express the main enzyme systems involved in drug metabolism. However, the polyclonal population fails to express the predominant enzyme of the cytochrome P450 family, CYP3A. This may possibly explain differences observed in permeabilities of compounds that were substrates for CYP3A between the CaCo-2 cell model and intestinal tissue. Nevertheless, there is a clone of the CaCo-2 cell lineage that does express this enzyme system, the TC7 clone (Caro et al., 1995). The use of this clone as a model for the intestinal wall has yet to be demonstrated although from initial studies it appears to be a useful tool to study transport processes and to evaluate the role of the intestine in the biotransformation of drugs (Caro et al., 1995; Hu et al., 1999).

It recently has been demonstrated that the altering the culturing conditions affected the expression of CYP3A (Schmiedlin-Ren et al., 1997). In that study it was shown that incorporating 1α, 25-dihydroxyvitamin D_3 into the medium resulted in a dose- and duration-dependant increase in the metabolically active CYP3A by confluent CaCo-2 cells.

The CaCo-2 cells express many of the carrier systems that are found *in-vivo* including peptide and amino acid carriers, bile acid transporters, receptor and carrier-mediated transport and efflux pumps.

Thwaites et al., (1993, 1994), confirmed the presence and role of an apically localised Na^+, H^+ exchanger in H^+-coupled dipeptide absorption in CaCo-2 cells using glycylsarcosine (Gly-Sar) as the substrate. This transporter was dose and pH-dependant and higher without Na^+. Further to these studies the existence of a dipeptide transporter in the basolateral membrane of the CaCo-2 cells was demonstrated (Saito et al., 1993). It has been shown that the ACE inhibitors, captopril and enalaprilat were able to stimulate the H^+-flux, suggesting that transport was via a di/tripeptide transport system (Thwaites et al., 1995).

Many orally active compounds such as cephalosporins, TRH, β-lactam antibiotics are substrates for the intestinal peptide transporter system (Yang et al., 1999). It appears that the transporter system level of expression varies depending on the passage number of the cells as shown for TRH (Walter et al., 1994). In this study the transport of TRH was shown to be active when the CaCo-2 cells were at the higher passage of 98-99 and that TRH was transported across the monolayer at lower passages (30- 34) via the paracellular route. Dantzig (1997) has shown that the dipeptide transporter for the β-lactam antibiotics can be reduced in the presence of dipeptides suggesting a competitive binding to the transporter.

The amino acid transporter has been shown to exist in the CaCo-2 cells with a non-specific Na^+-dependant mechanism (Hidalgo and Borchardt, 1990a). It was shown that

phenylalinine transport was decreased in the presence of glucose, oubaine and sodium azide, that the carrier was specific for the L-isomer and cationic amino acids inhibited its transport. This suggested that the cationic amino acid carrier transports cationic and neutral amino acids. The polarity of the carrier was seen, as the apical to basolateral transport of phenylalinine was 10 times faster than in the basolateral to apical direction, this is consistent with the *in-vivo* situation. The transport of L-α-methyldopa in the CaCo-2 cell monolayer was decreased 50% in the presence of 25mM phenylalinine, which suggests that this compound is transported via the amino acid carrier (Hu and Borchardt, 1990).

The expression of the bile acid transporter has shown to vary depending with time in culture, plateauing at Day 28 (Hidalgo et al., 1990b), and with the growth supports for the cells. It has been verified by Nicklin et al., (1992) that the flux of bile salt, NaTC across aluminium oxide filters was only 50% of what was seen for nitrocellulose or polycarbonate filters. The expression of the bile transported has also been shown to vary in 43 different stable clones, which showed that some cells transport NaTC whilst others transported significantly higher amounts of NaTC than the parent population (Woodcock et al., 1991). Exploitation of this carrier system for the enhanced transport of peptide drugs has been investigated with limited success (Kim et al., 1993; Kramer et al., 1997). However, the bile salt carrier expressed in the CaCo-2 cells is approximately 250-fold lower than that of the human ileal (Hidalgo et al., 1990b).

Many nutritional components, particularly essential vitamins, have an intrinsic low permeability caused by their hydrophilic properties. Therefore specific uptake systems, such as receptors are required. Furthermore, growth factors exert their action upon binding to the receptor that causes a cascade of events within the cell. Also a variety of pathogens translocate upon binding to the cell surface receptors. CaCo-2 cells reveal the presence of several receptors that serve the above purposes.

The absorption of vitamin B_{12} is receptor mediated (Levine et al., 1984). In the stomach vitamin B_{12} forms a complex with intrinsic factor, which binds to specific receptors on

the apical membrane of absorptive cells in the distal ileum. Dix et al., 1990 demonstrated that CaCo-2 cells express the protein capable of binding the complex. The proteins level of expression depended on the differentiation of the cells with levels peaking 10-15 days post-seeding. After uptake the complex (Vitamin B_{12} and intrinsic factor) was transported to the basolateral side of the cells upon binding to a protein having similar immunological properties to transcobalamin. These studies were confirmed by other investigators who further demonstrated the specificity of the endocytic process (Dan et al., 1994). The use of the vitamin B_{12} pathway has been exploited for the delivery of large peptides e.g. erythropoietin or granulocyte-colony-stimulating factor by forming conjugates with the vitamin. Results indicated an increase in the transport of these compounds across the CaCo-2 cell monolayer having a similar pathway of absorption to the vitamin itself (Russell-Jones et al., 1995).

The presence of epidermal growth factor (EGF) receptors has been shown to exist on the apical and basolateral sides with a 2.5: 1 ratio of distribution in favour of the basolateral chamber (Hidalgo et al., 1989b). The level of expression for these receptors peaks at Day 10 in culture but the binding levels are comparable to rat foetal and adult intestinal microvillous membranes (K_d= 0.67, 1.03 and 2.31nM respectively). Biotin, which is essential for normal cellular function and growth, has been shown to be absorbed by a Na^+-dependant saturable process (Ng and Borchardt, 1993), which is what was seen for human intestinal BBMV (Said et al., 1987). However Cogburn et al., (1991) had shown that biotin was transported across the CaCo-2 cell monolayer by a passive mechanism. This apparent difference may be associated with the different culturing techniques of the laboratories in question and the fact that the passage numbers used by Cogburn et al., (1991) were very high and therefore the level of expression of the transporter was lower.

The pathogenesis of most infections involves cell adhesion of the pathogen prior to infection. Cruz et al., (1994) has demonstrated that the adhesion of *E. coli* is via a mannose- specific receptor, which was located on the apical side of CaCo-2 cell monolayer post-confluency. It has been demonstrated that the adhesion of the HIV virus

occurs in the CaCo-2 cells because of the similarity in the glycolipid character of the CaCo-2 cells and human intestine (Fantini et al., 1993).

An ATP-dependant drug efflux pump, which has been identified as p-glycoprotein (P-GP), is present on the apical surface of CaCo-2 cells (Burton et al., 1993). Hunter et al., (1993) demonstrated the presence of P-GP in CaCo-2 cell monolayers of passage 85-100 using a monoclonal antibody. They also showed that vinblastine flux was greater via the basolateral to apical route 14-15 days post-seeding. This flux could be decreased in the presence of several P-GP modulators such as verapamil, nifedipine, taxotere and 9-dideoxiforskolin. This was confirmed by later studies at lower passage number (<30) (Mah et al., 1996). It has been shown that the functionality and expression levels of P-GP change during the time course of the cell culture. Hosoya et al., (1996) reported continuous expression of the P-GP during the 28-day period of culturing of CaCo-2 cells on filters. Altering the constituents in the culture media can modify the regulation of the P-GP efflux system e.g. desacetylvinblastine sulphate which up-regulates the P-GP (Hoskins et al., 1993). This suggests the presence of an efflux system in the CaCo-2 cells and that this cell model would be a suitable screen for potential P-GP substrates.

The development of new intestinal cell lines, which mimic the in-vivo situation, better than CaCo-2 cells is an area of research that is rapidly expanding (Paul et al., 1993; Tavelin et al., 1999). Tavelin et al., (1999) have established 2/4/A1, a cell line originating from foetal rat intestine, that fully differentiate to form tight junctions, with an increased expression of brush border enzymes and a paracellular permeability that is comparable to the human small intestine. This cell line is an example of a transformed intestinal epithelial cell line that at least recalls the well-coordinated processes, which control cell proliferation and differentiation. This makes it interesting alternative to the high resistant CaCo-2 cell monolayer. The electrical resistance of this cell line is comparable to excised human intestine (Paul et al., 1993). The cultivation period is only 4 days and therefore much shorter than the CaCo-2 cell model which is 21 ± 2 days. The transport of hydrophilic marker molecules was reported to be comparable to the *in-vivo*

situation e.g. mannitol had a P_e of 15.5 ± 2.09 X 10^{-6} cm/sec in 2/4/A1 and for human jejunal studies it was 38 ± 17 X 10^{-6} cm/sec.

3.4.4.4 Co-culture models

The establishment of a co-culture model between CaCo-2 cells and Ht29GlucH cells has been reported (Allen, 1992)). From preliminary work it was shown that the mucus layer affected testosterone and warfarin permeabilities. Wikman-Larhed et al., (1995) attempted to set up the same model but without success as the model failed to produce mucus although the barrier properties of the model were similar to the human intestine. Meaney (1997) established and characterised the CaCo-2: Ht29GlucH co-culture model in our laboratory and demonstrated the effect that mucus had on the enhancing properties of micellar systems of NaTC and the transport of the lipophilic compound, dextropropoxyphene. Meaney and O'Driscoll, (1999) demonstrated that the mucus layer was a barrier to the enhancement potential of the micellar systems of NaTC. In addition mucus was shown to be a barrier to ionised drugs e.g. dextropropoxyphene which was ionised at pH 4.5 and there appeared to be a electrostatic interaction with the mucus, which decreased its' transport by 4-fold in the presence of mucus (Meaney and O' Driscoll, 1999).

A co-culture model that produces the main characteristics of lymphoid follicle-associated epithelium (FAE) and M-cells was established by cultivation of Peyers patches lymphocytes with the differentiated human intestinal cell line CaCo-2 (Kernéis et al., 1997). This model was characterised by analysing the expression of two proteins, which are associated with brush border organisation, villin and sucrase-isomaltose. The levels of expression of these proteins were indicators of the formation of a co-culture between these cell types. The sucrase isomaltase (SI) down regulated and the villin was redistributed into the cytoplasm, which is similar to FAE *in-vivo* as the digestive functions of this cell type are reduced. TEM demonstrated the formation of the co-culture with the lymphocytes inducing reorganisation of the bush border. The transport of FITC-labelled particles was shown to be transcytosed only in the presence of the co-

culture and not for CaCo-2 cell monolayers. It was concluded that major features of M cells i.e. vectorial translocation of inert particles and bacteria had been reproduced *in-vitro* by the co-culture of PP lymphocytes and CaCo-2 cells. Using this model it should be possible to detect and quantify the signals produced by lymphocytes responsible for M-cell formation, to characterise the mechanisms mediating cytoskeletal reorganisation and transcytosis and to analyse the cellular mechanism of bacterial translocation through M-cells. This should facilitate the design of oral vaccines and more efficient mucosal delivery systems.

3.5 Applications of the CaCo-2 cell culture model

The CaCo-2 cell monolayer when grown on microporous membrane inserts become polarised and provide a good *in-vitro* model for conducting intestinal drug transport studies (Wilson, 1990). Separation of the apical from the basolateral fluids allows for the study of solute movement from apical to basolateral or basolateral to apical. It is important that experiments are carried out under sink conditions to prevent the diffusion of drug molecules back from the basolateral to apical chamber. The type of filter used to grow the cell monolayer is important. As previous studies have shown aluminium oxide filters reduce the absorption of compounds and increase the electrical resistance of the monolayer compared to nitrocellulose (Nicklin et al., 1992). The membrane of choice is polycarbonate as there is less adsorption to the membrane by peptides and drugs compared to nitrocellulose (Artursson 1990b; Sergent –Engelen et al., 1990). The problem with this filter is that they are translucent and thus makes it difficult to ensure cells are on the surface. Sergent-Engelen et al., (1990) have also suggested the use of PTFE filter since they are transparent and have shown the successful formation of differentiated monolayer on the apical surface. Another consideration is the pore size of the filter. Tucker et al., (1992) have shown that CaCo-2 cells are able to traverse the membrane and successfully grow on the basolateral surface when the pore diameter of the filter was 3μm. The growth rate of the CaCo-2 cell monolayer has been shown to decrease with increasing pore diameter of the filter (0.3- 4.1μm). For transport studies the normal pore diameter for the filter is 0.4μm.

The CaCo-2 cell culture model has been used 1) to elucidate transport pathways 2) to screen absorption enhancers 3) for metabolism studies 4) to determine structure transport relationships 5) to assess the influence of the aqueous boundary layer and 6) to assess solvent drag (Delie and Rubas, 1997).

Elucidation of transport pathways:

Passive transport of drugs in the CaCo-2 cell culture model has been investigated with β-blockers (Artursson et al., 1990b), arginine vasopressin (AVP) and its analogue dDAVP (Lundin et al., 1990), thyrotropin releasing hormone (TRH) and prodrug (Lundin et al., 1991; Walter et al., 1994) and Pz-peptide (Yen and Lee, 1994). The transport of these compounds has shown to be independent and polarised. The permeability of the β-blockers was shown to be comparable to published data for the rat ileum, but atenolol, which was the most hydrophilic compound, was transported at a slower rat in the CaCo-2 cell monolayer. This was attributed to the tighter junctional complexes formed in the CaCo-2 model as compared to rat ileal enterocytes. TRH has been shown to be passively transported across the CaCo-2 cell monolayer by the paracellular route (Thwaites et al., 1993) but the route of transport has been shown to change with passage number with the predominate pathway being an active transport mechanism (Walter et al., 1994).
Walter et al., (1995) assessed the transport of a series of modified peptidometic renin inhibitors across the CaCo-2 cell monolayer. In this study striking differences in the transport of the renin inhibitors was observed, with the difference between the highest and the lowest apparent permeability being 370-fold. The molecular structure, lipophilicity, and molecular masses were similar for all eight compounds investigated. It was suggested that these inhibitors were transported via the paracellular pathway because of the relationship between their Papp and the inverse of TEER. This relationship was also shown for the paracellular marker molecule, fluorescein.

The effect of P-GP on drug transport has been shown by studies carried out by Burton et al., (1996). It was revealed that $AcPhe(NmePhe)_2NH_2$ was released from the apical surface of CaCo-2 cells by a saturable, verapamil-sensitive apical efflux system. In

contract, $AcPheNH_2$ was not a substrate for this apically polarised pump. Further studies with this peptidometic series demonstrated that Cremophor EL and polysorbate 80 increased the transport of $AcPhe(NmePhe)_2NH_2$ (Nerurkar et al., 1996). These surfactant systems did not affect the apical to basolateral flux of $AcPheNH_2$. This suggested that these surfactant systems blocked the apically polarised efflux pump to allow the P-GP substrate, $AcPhe(NmePhe)_2NH_2$ to be transported.

In recent studies with human growth hormone (hGH) it has been shown that conjugation of the hormone to amino acid derivatives have made it become a substrate for P-GP (Wu and Robinson, 1999). It was demonstrated that hGH itself was poorly transported across the CaCo-2 cell monolayer but once conjugated that the basolateral to apical flux was 1.5-fold greater than the apical to basolateral flux. This flux was reduced in the presence of P-GP inhibitors with the apical to basolateral permeability of the conjugates increasing. This suggests that on conjugation with these amino acid derivatives hGH can be effluxed in a P-GP mediated fashion thus implying that P-GP has become more lipophilic in the presence of these delivery agents.

The CaCo-2 cell monolayer expresses carrier systems as previously outlined in section 3.4.4.3. Several studies characterising these transporters have been carried out in the CaCo-2 cell monolayer (Dantzig et al., 1990; Zheng et al., 1994; Dantzig et al., 1997). These studies confirmed the localisation of the transporters on the apical and/or basolateral sides and that the level of expression depended on a number of factors including, days in culture, passage number and culturing media. However, the existence of peptide transporters on the CaCo-2 cell systems need to be further confirmed and verified. Further characterisation of the carrier should provide useful information concerning the structural requirements necessary for transport by the peptide carrier, which has been found to carry a variety of compounds (Hidalgo et al., 1995). In attempts to predict the mechanism of transport across the intestinal epithelium the development of a pharmacophore map of the intestinal peptide carrier has been undertaken (Swaan and Tucker, 1997). The information provided by this study would within limits allow researchers to predict the transport behaviour of untested compounds; that is if they

possess any affinity for the transporter and if they possess the correct geometry for translocation.

Screening absorption enhancers and drug delivery systems

The screening of absorption enhancers and drug delivery systems has been extensively studied using the CaCo-2 cell culture model. However the CaCo-2 cell model appears to be mores sensitive especially to surfactant systems than whole animal tissue models (Anderberg, 1993b). The first enhancer system that was studied using the cell culture model was EGTA, a Ca^{2+}-chelating agent (Artursson, 1990). In this study it was shown that EGTA acted on the tight junctional complex because it caused a reversible reduction in TEER and the TEM demonstrated that there was dilation of the tight junction. This enhancer increased the permeability of the more hydrophilic β-blockers by 2-9 fold with no difference observed on the Papp for the more lipophilic compounds. In a further study (Anderberg et al., 1992) excipients including the synthetic anionic surfactants: sodium dodecyl sulphate and sodium dioctylsulfosuccinate, the non-ionic surfactant: polysorbate 80 and polyoxyl 40 hydrogenated castor oil and the bile salts: sodium taurocholate, sodium taurodihydrofusidate and taurodeoxycholate were investigated. A dose dependant effect was observed in the presence of all surfactant systems investigated producing a decrease in the intracellular dehydrogenase activity, an increase in the permeability of marker compounds, a decrease in transepithelial electrical resistance and changes in morphology with increasing concentrations of surface active agents. The results correlated with published animal data. The study also demonstrated that the toxicity and effectiveness of enhancer systems could be evaluated using three fundamental methods: a) MTT assay for determining the intracellular dehydrogenase activity, b) permeability of marker compounds to assess the increase in paracellular permeability and c) recovery experiments to ensure that the response elicited by the enhancer was reversible. The TEER data was shown to be a less sensitive measure than the flux of the hydrophilic paracellular markers, mannitol and PEG 4000 or the MTT. The usefulness of the CaCo-2 cell monolayer as a screening for the cytotoxicity of enhancers has been demonstrated (Schasteen et al., 1992; Sakai et al., 1998). In the latter

study the cytoxtoxicities of sodium deoxycholate, sodium caprate and dipotassium glycyrrhizinate were evaluated using trypan-blue exclusion test, protein-release assay, neutral-red assay and propidium iodide staining. The reversibility of the enhancement was monitored using TEER.

The pharmaceutical surfactant, sodium dodecyl sulphate (SDS) has been extensively studied as an enhancer (Anderberg 1993a; Boulenec et al., 1995). The investigations carried out by Anderberg et al., (1993a) suggested that SDS affected the paracellular route as well as the apical membrane. These assumptions were supported by assays including: marker permeabilities, TEER, intracellular Ca^{2+} levels changes, propidium iodide staining, rhodamine-labelled phalloidin staining of the actin fibres, and TEM. Boulenec et al., (1995) demonstrated that above levels of 100mg/L ($\approx$ 0.35mM) SDS caused irreversible damage to the CaCo-2 cell monolayer, which was similar to the finding in the previous study by Anderberg et al., (1993a).

Anderberg et al., (1993a) demonstrated the enhancer effects of sodium caprate on CaCo-2 cell monolayer. It was suggested that the enhancer affected tight junctional permeability and also it was shown that the presence of Ca^{2+} decreased its activity probably attributable to the formation of soaps. The effect that Ca^{2+} had on the permeability of PEG 4000 in the presence of sodium caprate was investigated further (Tomita et al., 1994). In this study it was suggested that sodium caprate was unaffected by the presence or absence of mucosal calcium. This indicated that the mechanism of action attributed to C10 was not only due to chelation with calcium but also some other mechanism was involved. The mechanism of action of sodium caprate (C10) has subsequently been extensively studied and it appears that the primary mechanism of action is by increasing the intracellular calcium levels through interaction with phospholipases C in the membrane and enhances the permeability by opening the tight junctions through activation of the calmodulin-dependant contraction of the actin microfilament by the released calcium (Tomita et al., 1995, 1996; Lindmark 1995, 1998; Hayashi et al., 1999). Further studies on the mechanism of absorption enhancement by medium chain fatty acids were carried out (Lindmark et al., 1995). In the latter study the enhancing effects of

medium chain fatty acids, C6 (caproate), C8 (caprylate), C10 (caprate) and C12 (laurate) were investigated using the CaCo-2 cell model. Although these fatty acids are structurally similar they demonstrated different mechanism of actions e.g. C8 only enhanced absorption under hypotonic conditions whereas C10 demonstrated enhancement potential under both hypotonic and hypertonic solutions in the apical chamber.

Palmitoylcarnitine (PCC) and other long chain acyl -carnitines are zwitterionic surfactant, which have unique features, which make them attractive as absorption enhancers. *In- vivo* studies with these surfactants have shown that the effect is spontaneously reversible and they are able to promote intestinal drug absorption under conditions, which do not produce any apparent damage to the intestinal epithelium (Fix et al., 1986). Similar to its effects *in-vivo*, PCC, promoted drug absorption and diminished TEER in the C aCo-2 cell monolayer without significant signs of cell lysis (Hochman et al., 1994). Hochman et al., (1994) failed to observe significant changes in the morphology of cell monolayer and with TEM and freeze-fracture EM suggests that PCC acts by disrupting the tight junction. The co-administration of PCC with fluorescent-labelled dextrans and electron dense lanthanum resulted in an accumulation of these compounds in the tight junctional complex with essentially no cytoplasmic accumulation of these compounds. Its precise mechanism of action is unclear as its effect appears to be Ca^{2+} independent and it does not affect the actin filament distribution in the CaCo-2 cell monolayer. The enhancing mechanism of acetyl-carnitines involves intracellular acidosis and/or depletion of ATP, which results in an increase in the calcium levels. The increase in the calcium levels activates the actomyosin contraction by activation of cytoskeletal destabilisation or through other processes leading to an opening of the tight junction. The mechanism of action differs from sodium caprate (C10) in that this mechanism is calmodulin and myosin light chain kinase independent (Hayashi et al., 1999).

The CaCo-2 cells have been used to study the toxicity and absorption processes of a range of other excipients including the bile salts sodium glycocholate (Jørgensen et al., 1993), sodium taurocholate simple and mixed micelles (Werner et al., 1996; Meaney, 1997), chitosan derivatives (Kotzé et al., 1997, 1998, 1999), lysophosphatidylcholine

(Høvgaard et al., 1995a), cyclodextrins (Høvgaard et al., 1995b; Haeberlin et al., 1996; Tötterman et al., 1997) and non-ionic surfactants e.g. polysorbate 80, polyoxyethylene (24)-cholesterol-ether (Drewe et al., 1993; Nerurkar et al., 1996).

For effective and safe use of absorption enhancers, the following physiological examinations are a prerequisite: clarification of the exact enhancing mechanism, prediction of the epithelial barrier changes induced by the enhancer, examination of the reversibility of enhancement and assessment of the potential for the practical use of the enhancer.

Drug metabolism studies

Since CaCo-2 cell monolayers are able to expresses a vast array of enzyme systems as previously outline in section 3.4.4.3, table 1, this cell culture system serves as a good model for the metabolism of peptides. Delta-sleep inducing peptide (DISP) degradation was studied in the apical and basolateral chambers of the CaCo-2 cell monolayer (Augustijns et al., 1995). DISP was metabolised to produce Trp-Ala and Trp with the degradation being more prevalent in the apical side of the monolayer. Metabolism of DISP could be reduced by the addition of the peptidase inhibitors, bestatin an aminopeptidase inhibitor, diprotin A, a dipeptidylpeptidase IV inhibitor and captopril, an ACE inhibitor. Although the addition of the various enzyme inhibitors led to the stability of DISP, they failed to increase the permeability of DISP. Taumra et al., (1996) using intact CaCo-2 cell monolayer and homogenates of the monolayer showed that the L-isomer of the tripeptide Val-Val-Val was degraded in 1h and 2 h respectively. However when the isomers were altered to give L-Val-L-Val-D-Val the degradation rate decreased with $\approx$ 37% intact and $\approx$ 69% intact in the homogenates and apical chamber respectively. The other six isomers synthesised demonstrated complete stability in the homogenate.

Determine structure transport relationships

CaCo-2 cell monolayer has been used to determine the effect that altering the structure of peptides has on their transport (Conradi et al., 1991,1992). This study demonstrated that there was an inverse relationship between the permeability of a series of D-phenylalinine containing peptides and the hydrogen bonding potential. There was no apparent correlation between the flux of the compound and their lipophilicity. The hydrogen bond potential was reduced due to the alkylation of the amide bonds, which caused an increase in permeability. This relationship between H-bond potential and permeability had been observed for *in-vivo* intraduodenal dosing of rats (Karls et al., 1991).

The relationship between apparent permeability (Papp) and a series of charged and uncharged hydrophilic compound with varying molecular radii was investigated to determine the pore radius in the CaCo-2 cell monolayer (Adson et al., 1994, Knipp et al., 1997). The values obtained were 12.0 ± 1.9Å and 5.12 ± 1.2 Å respectively, which compares favourably with the *in-vivo* human jejunal epithelial pore radius of 6-8Å. In our laboratory the pore radius of the CaCo-2 cell model was determined to be 8.43 ± 1.3 Å (Meaney, 1997). Adson et al., (1994) developed a model, which provided information on the relationship between molecular size, Papp and changes in junctional pore size with the addition of perturbants to the system. It further demonstrated that a relationship exists between TEER and flux but this relationship was influenced by solute size and charge.

Assess the influence of the aqueous boundary layer

Transport studies are normally carried out using Transwells ®, which results in the formation of a non-physiological unstirred water layer or aqueous boundary layer at the surface of the cell monolayers. This unstirred water layer will be the rate-limiting step for the transport of lipophilic compounds across the monolayer. Several methods have been used to determine the thickness of this aqueous boundary layer in the CaCo-2 cell monolayer (Karlsson et al., 1991). In this study utilised an ELISA plate shaker to agitate

the monolayer in their inserts in a controlled manner. A linear relationship was observed between Papp and the agitation rate, which allowed for the cellular permeability coefficient (P_{cell}) and the aqueous boundary layer thickness (h_{aq}) to be calculated. The h_{aq} decreased from 1554 ± 142μm to 128 ± 10μm as the agitation rate went from 0→ 1090rpm, which corresponded, to a 2.8-fold increase in the Papp of the lipophilic compound, testosterone. The contribution of the resistance of the aqueous boundary layer in the absence of agitation was 70% of the total diffusional resistance, this reducing to 16% when agitation was increased to 1090 rpm. The development of a system where agitation was produced by a 5% CO_2/O_2 gas lift in novel diffusional cells was introduced (Hidalgo et al., 1991). This system of agitation and the results obtained correlated with the previously published data (Karlsson et al., 1991). Meaney et al., (1999) and Meaney (1997) investigated the effect that the h_{aq} had on the permeability of the lipophilic dextropropoxyphene using the Costar Snapwell® and diffusion chamber system. The results showed that the Papp linearly increased with an increase in the agitation of the system, which corresponded to a decrease in the thickness of the h_{aq}.

Assessment of solvent drag

Water flux across the intestinal epithelium occurs via the transcellular and paracellular routes. Although the relative magnitudes of the flux for each route are not known it has been suggested that a significant fraction is paracellular. Karlsson et al., (1999) investigated the effect that a hypotonic solution had on the permeabilities of three similarly sized but differently charged compounds, creatinine (cationic), erythritol (neutral) and foscarnet (anionic). It clearly confirmed that in the presence or absence of a hypotonic solution that the paracellular route is charge selective with the Papp for creatinine> erythritol> foscarnet. The permeabilities of all three compounds were increased in the presence of the hypotonic solution in both the apical to basolateral direction and visa-versa. From fluorescence and transmission electron microscopy it would appear that the enhancement in transport is due to dilation of the tight junctions rather than a solvent flux. In isolated rat ileum segments the hypotonic solution only had marginal effects on the TEER and on the enhancement of the markers permeabilities.

This suggested that a more pronounced disruption of the tight junctions than that obtained through stimulation of epithelial absorption of water flux is required for more efficient enhancement of paracellular intestinal drug absorption. Jørgensen et al., (1993) demonstrated the effects that a hypertonic solution (700mOs) had on the CaCo-2 cell monolayer morphology. From TEM studies the cells showed disordered microvilli, a disorganised terminal web and many intracellular vacuoles but no effects on the apical cell membrane and tight junctions was observed.

3.6 Advantages and disadvantages of cell culture models

Cell culture models offer many advantages over other drug absorption models (Borchardt, 1990; Hillgren et al., 1995)

- Rapid evaluation of the potential permeability and metabolism of drugs, thereby providing an invaluable screening tool for drug development.
- Minimise the amount of animals required for further studies
- Require less compound for analysis
- Easy to work with once established
- The environment is easily controlled and therefore reduces variation.
- Relatively clean samples are prepared for analysis and usually do not require any cleanup, purification or extraction processes involved with most biological fluids
- Transport process can be characterised because of access to the apical and basolateral surfaces
- Isolation of certain cell types is possible and therefore it is possible to delineate the relative importance of different cell populations in the absorption and metabolism of compounds
- The system is very simple and does not have any of the complications associated with tissues and animal systems
- It is possible to utilise human cells

There are disadvantages in cell culture models:

- Labour intensive procedures for maintaining adequate cell stock
- Expensive to set-up and run
- Cell culture have to be continuously monitored for infections e.g. bacterial, mycoplasma
- They are of cancer origin
- Cell lines are not necessarily phenotypically stable. The properties of the cell line are equal to the sum of the properties of the sub-population (Artusson, 1990b).
- Because of their polyclonal nature it is important that the passage number is defined and limited
- Cell lines have to be characterised each time they are established in a laboratory
- The reported inter-laboratory variation in results.
- Colonic origins of the CaCo-2 cells
- The topography of the cell culture monolayer compared to *in-vivo* intestine is different (Artusson et al., 1996)

3.7 *In-vitro- in-vivo* correlations

The *in-vivo-in-vitro* correlation between drug permeability in the CaCo-2 cell monolayer and percent drug absorption in humans after oral administration was investigated by Artusson and Karlsson, (1991). A positive S-shaped correlation between transport rate and lipophilicity was shown. Highly absorbed drugs were found to have a high permeability coefficient $>1 \times 10^{-6}$ cm/sec, drugs which had absorption >1% and < 100% had Papp of 0.1 to 1×10^{-6} cm/sec and incompletely absorbed drugs (< 1%) had a Papp $<1 \times 10^{-7}$ cm/sec in the CaCo-2 cell monolayers. Rubas et al., (1993) reported a similar correlation between the permabilities of a series of hydrophilic and lipophilic compounds across the CaCo-2 cell monolayer and absorption in human, however the absolute values were increased by an order of magnitude. This difference was attributed to inter-laboratory variation in passage number, culturing and experimental conditions.

Similarly good correlations have been reported between CaCo-2 cell model and *in-situ* isolated animal tissue models (Conradi et al., 1993; Kim et al., 1993; Rubas et al., 1993, 1995). Rubas et al., (1995, 1996b) compared the permeability of the CaCo-2 cell monolayer to the permeability of various animal colonic segments. The results demonstrated that there was good correlation found between the cell model and the rabbit (R^2= 0.969), moderate for monkey (R^2= 0.84) and none for dog (R^2= 0.684). However, the data further suggested that the permeability in the CaCo-2 cell monolayer was 2-fold greater than for the isolated rabbit colonic tissue. The flux of these compounds was assessed in *in-vitro* human colonic tissue and calculated permeabilities compared well with the rabbit data, suggesting that the CaCo-2 cell monolayer is a suitable *in-vitro* model of human colon. Kim et al., (1993) obtained a good correlation (R^2= 0.94) between the permeabilities of a series of model peptides for the *in-situ* perfused rat ileum model and the *in-vitro* CaCo-2 cell monolayer. The permeability of this series of peptides was lower in the rat ileum compared to the cell culture model. These results suggest that the permeability values determined in the CaCo-2 cell monolayer may be a good predictor of the intestinal permeability of peptides. However, Conradi et al., (1993) demonstrated that the correlation of a series of renin inhibitory peptides with rat intestinal absorption and CaCo-2 cell monolayer was not as good with a R^2= 0.85. This reduction in the correlation coefficient was attributed to the proposed route of transport of these peptides. It has been shown that a good correlation exists between rat perfusion studies and the CaCo-2 cell monolayer whereas none was observed for CaCo-2 cell monolayer and the *in-vitro* everted rings model (Stewart et al., 1995). The use of *in-vitro* CaCo-2 cell model and the *in-situ* perfused rat intestine to predict intestinal absorption *in-vivo* was further illustrated by Ribadeneira et al., (1996). In that study a series of structurally modified angiotensin II receptor antagonists permeabilities were assessed in each of the absorption models, the CaCo-2 permeation coefficient (on a log scale) and *in-situ* rat intestinal perfusion absorption rate constant (k_a) correlated well (R^2= 0.97 and 0.92 respectively) with bioavailability (F) in rats.

The permeability of passively transported compounds across the CaCo-2 cell monolayer has shown good correlation with *in-vivo* rat jejunal studies, R^2=0.99 for a series of

lipophilic compounds (Yamashita et al., 1997). Therefore it was suggested that because of the simple monolayer structure of a cultured cell system, it provides a distinct advantage in predicting *in-vivo* drug absorption compared to more complex animal gut perfusion studies.

Artusson et al., (1993) demonstrated that the permeability of a series of polyethylene glycols (MW 194- 502) decreased in a comparable fashion in the CaCo-2 cell monolayer and the human intestine *in-vivo*, although the permeabilities of these compounds was 100 times lower in the cell model. It was suggested that there were fewer pores in the CaCo-2 cell monolayer but that the pore radii were comparable for both systems. The correlation of the permeabilities of drugs, which are transported primarily via the paracellular route, is a qualitative relationship between CaCo-2 cell monolayer and human jejunal perfusion studies (Lennernäs et al., 1996). The permeability of six structurally different compounds was investigated which resulted in the lipophilic compounds, naproxen, antipyrine and metoprolol having comparable Papp in either system but the more hydrophilic compounds, terbutaline and atenolol Papp decreased 79 and 29-fold respectively in the cell culture model compared to the jejunum.

Active and passive transport of drugs have been compared in CaCo-2 cells and in the human jejunum *in-vivo* (Lennernäs et al., 1996). The carrier mediated transport rates of L-dopa, L-leucine and L-glucose were shown to be much slower in the cell culture model compared to the human jejunum. The lower permeability was ascribed to the colonic origins of the CaCo-2 cell monolayer as previously it had been reported that CaCo-2 cells displays a variable and generally lower expression of carrier-mediated transport than seen *in-vivo* (Hu and Borchardt, 1990). Therefore it would appear that prediction of human active transport in CaCo-2 cell monolayer will only be possible after rigorous characterisation of the transport system and subsequent introduction of a scaling factor to compensate for the different expression of the carrier in the CaCo-2 cell monolayer from that seen *in-vivo*.

Polli and Ginski, (1998) have progressed further in attempting to directly interpret absorption kinetics from oral dosage forms in humans and CaCo-2 cell monolayer permeability values. It would appear from their preliminary studies using three compounds, metoprolol, ranitidine and piroxicam, that a rank order relationship was observed for both apparent and true permeation rate constants *in-vivo* with CaCo-2 cell monolayer permeability. Because of the potential utility of measuring drug permeability in CaCo-2 cell monolayers to predict human absorption kinetics of oral dosage candidates, dosage forms of more drugs need to be evaluated in mapping the relationship between true permeation (k_p) and CaCo-2 cell monolayer permeability (P_m).

It appears that correlations exist with *in-vivo* systems, and this instils confidence that the cell culture model can be used to predict *in-vivo* absorption. The CaCo-2 cell culture model is the best characterised among intestinal cell culture models with regards to the transport properties of drugs. However, it is important to be aware of the limitations associated with cell culture models. This may be attributable to the polyclonal nature and the diversity of the cell line. Further to this it is necessary that culture conditions, age of cells, passage number and cell source are all controlled to avoid discrepancies in results.

CHAPTER 4

General Properties of Bile salts

4.1 Introduction

Bile salts are synthesised in the liver from cholesterol and enter into the duodenal lumen where they form of mixed micelles with lecithin and cholesterol. In the intestinal lumen, bile salts serve as detergents, which aid the solubilisation of dietary fats (Atwood and Florence, 1983). The detergent activity results from the amphiphilic nature of these compounds, which possess a polar and a non-polar head.

4.2 Chemical structure

Bile acids are C_{22} to C_{26} carboxylic acids with a cyclopentenophenanthrene nucleus containing branched side chain of three to nine carbon atoms ending in a hydroxyl group. Most naturally occurring bile acids are C_{24} saturated acids. Bile salts are generally classified according to the number of hydroxyl groups attached to the nucleus: mono-, di- and tri-hydroxy (Small, 1971). The trihydroxy bile salt, cholic acid has three hydroxyl groups attached to the nonpolar steroid nucleus at positions, 3, 7 and 12; the dihydroxy bile salt deoxycholate and chenodeoxycholate has hydroxyl groups at position 3 and 12 and 3 and 7 respectively and the monohydroxy bile salt lithocholic has one hydroxyl group at position 3. Conjugating groups may be present on the nucleus or side chain e.g. taurine or glycine. The structure of the trihydroxy bile salt cholic acid and its taurine and glycine conjugates and the dihydroxy bile salt, deoxycholate are shown in Figure 4.1.

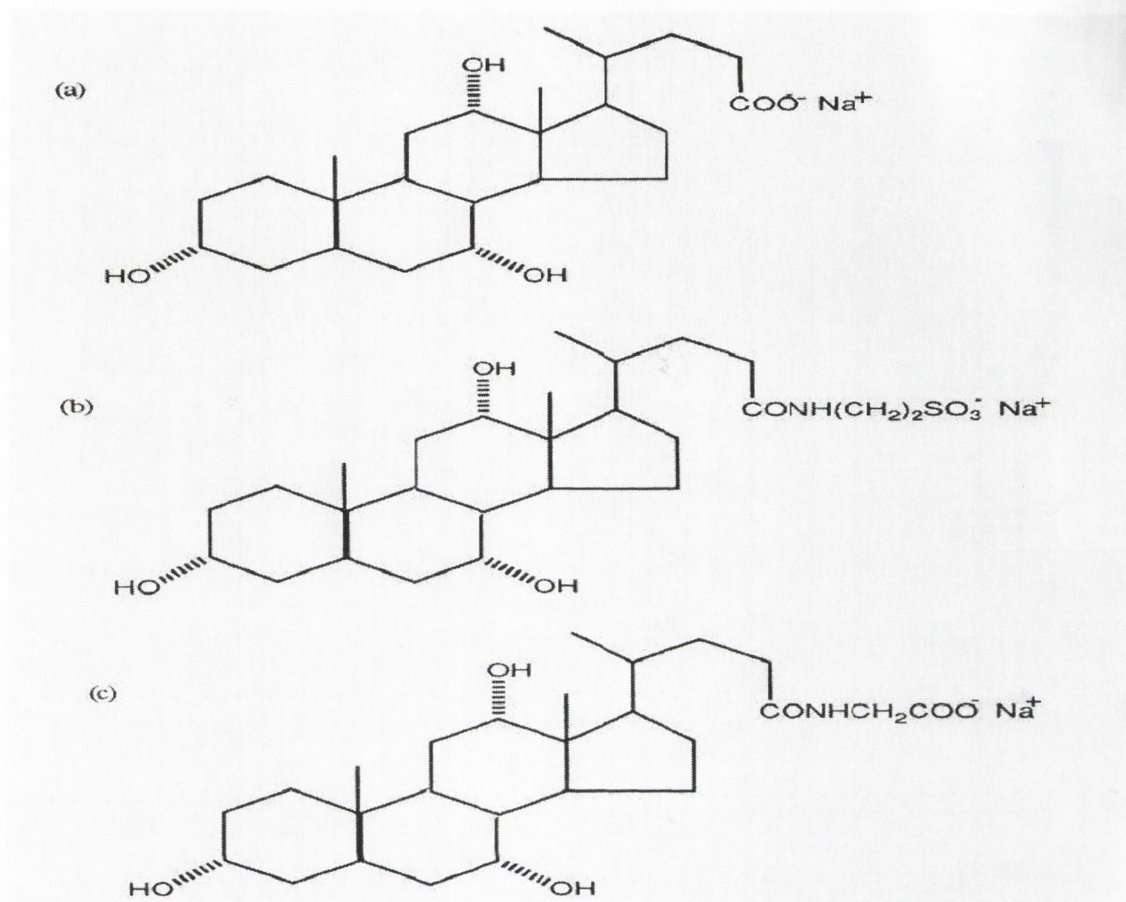


Figure 4.1 The trihydroxy bile salt (a) sodium cholate, (b) sodium taurocholate and (c) sodium glycocholate (Lane, 1997)

4.3 Physicochemical properties

Bile salts carry extensive hydrophobic portions in each molecule that attempt to reduce their contact with water (Carey and Small, 1972). This is reflected in rapid dynamic association-dissociation equilibrium to form 'self-aggregates' or micelles as the total concentration of bile acid solute is increased above the CMC (Critical micellar concentration). A number of interrelated factors influence the CMC and the size of micelles including 1) bile salt structure, 2) the presence and concentration of lipids, 3) temperature, 4) the presence and concentration of counterions and 5) pH. It would appear that the generally the CMCs are higher for trihydroxy bile salts (3-8mM) than for the less hydrophilic dihydroxy bile salts which have CMC in the range (2-4mM) (Atwood et al.,

1983). The CMC for conjugated species increases further because of the influence that conjugation has on the pK_a of the acid. Also the CMC is generally higher for the taurine conjugate because it is more hydrophilic than the glycine conjugate. The CMC for bile acids has been measured using various methods and solvent systems and because of this there are differences in values quoted (Small, 1971). However, the CMC for the trihydroxy bile salt sodium cholate (NaC) was 3 ± 1mM and for the glycine (NaGC) and taurine (NaTC) residues 6 ± 2 mM and 5± 3 mM respectively (Carey, 1985). Generally the CMC decreases with increasing concentration of counterion e.g. Na^+ present in the medium (Small, 1971). It has been shown that the CMC of NaTC decreases rapidly when the NaCl is >0.7mM. Further studies have shown that the size of the trihydroxy micelle increases from dimers in water to 6-9 molecules per micelle in 1m NaCl. On the other hand the counterion concentration has a marked effect on the dihydroxy bile salt size e.g. sodium glycodeoxycholate which forms dimers in water and forms large micelles with an aggregation number of about 63 in 0.5 N NaCl. The effect that the counterion has on bile salt depends on the number of hydroxyl groups rather than on the position of these groups. It appears that the dihydroxy bile salts 'salt-out' at much lower concentrations that the trihydroxy bile salts. Temperature appears to have an effect on the micellar size with the dihydroxy bile salts decreasing in size as the temperature is increased. The pH has also shown to have an effect on the bile salt systems most particularly for the dihydroxy systems. It has been shown that as the pH is decreased very large micelles are formed e.g. at pH >8 the aggregation number for Deoxy is 15 which increases to 18 when pH is lowered to 7.8-8 and increases to 500 when the pH is 7.3 (Small, 1971). In the presence of organic solvent systems the dihydroxy bile salts are know not to form micelles.

The sodium and potassium salts of the free di- and tri-hydroxy bile acids are very water soluble unlike the protonated free bile acids. As the concentration of bile acid increases above its CMC it becomes solubilized in the micelle thus enhancing its own solubility. The taurine conjugates of dihydroxy and trihydroxy bile acid are strong acids, which are soluble down to pH 1. Generally precipitation of the bile salt occurs as the pH is decreased but depends on the nature of the bile salt. As the pH decreases towards the pK_a

of the bile acid the sparingly soluble protonated species begins to form, solubilisation occurs until saturation levels are reached and then precipitation occurs. Free bile acids precipitate at near neutral pH 6.3, a level frequently found in jejunum, whereas the glycine conjugate doesn't precipitate until the pH has decreased to ≈ 4 and the taurine conjugate is soluble at all physiological pHs. The pK_a for the bile acids and their conjugates is shown in table 4.1 (Small, 1971)

Table 4.1 The pK_a of the dihydroxy and trihydroxy bile salts

Bile salt	pK_a
Deoxycholate (Deoxy)	5.02
Sodium cholate (NaC)	4.98
Sodium glycocholate (NaGC)	3.95
Sodium taurocholate (NaTC)	1.85

4.4 Absorption of bile acids

When a meal is ingested, contraction of the gall bladder and relaxation of the sphincter of Oddi occurs, bile enters the duodenum and promotes lipid digestion (Physiology of the Gastrointestinal Tract, 1994). The bile acids are deconjugated by bacterial hydrolysases resulting in the formation of the unconjugated bile acid and trihydroxy acids are converted to dihydroxy acids. The efficient intestinal conservation of bile acids is a result of passive and active absorption of conjugated bile acids from the small intestine and passive absorption of unconjugated acids formed by the bacterial hydrolysases of conjugated acids in the distal region of the small intestine and the large intestine. These are returned to the liver via the portal vein. The mean bile salt composition found in human bile has been reported to be 30 mol% glycocholate, 30 mol% glychenodeoxycholate, 15 mol% glycodeoxycholate, 10 mol% taurocholate, 10mol% taurochenodeoxycholate and 5 mol% taurodeoxycholate (Swenson et al., 1992). The total bile pool in humans is estimated to be between 3-5g and it is estimated that the average bile acid molecule makes 10-20 cycles in the enterohepatic circulation before it

is lost from the exchangeable bile acid pool. The average half- life of bile in humans is 2-3 days and the mass of bile acids in the enterohepatic circulation is maintained by bile acid biosynthesis, which is a negative feedback mechanism (Hofmann, 1994).

4.5 Formation of mixed micelles

Under normal physiological conditions bile salts are not found as singular chemical entities but in mixed micelle formation with other biliary lipids and with normal digestion lipids. Mixed micelles have been defined as any micelle, which is made up of more than one lipid-like chemical species with at least one of the chemical species able to form micelles alone in solution (Small, 1971). It has been shown that mixed micelles of the trihydroxy bile salts are more damaging and more effective enhancers than bile salt alone (Swenson et al., 1992).

4.6 Effect of bile salts on the bioavailability of drugs

The aqueous solubility and dissolution rate of many compounds with varying chemical structures increase in the micellar solution of bile salts, because the interaction of the solute molecule with the hydrophobic domains of the micelles leads to enhanced aqueous solubility (Hammad et al., 1998a, b). They may also play a role in enhancing drug transport across the intestine by influencing the resistance of the aqueous boundary layer and the membrane epithelium, to the passage of drugs. The absorption enhancer activity of bile salts has been shown to depend on the hydrophobicity and conjugation. *In-vitro* studies have shown that the absorption promoting activity of bile salts correlated with hydrophobicity and lysis of sheep erythrocytes (Murakami et al., 1984). It was shown that the more hydrophobic the bile salt was the greater the enhancement seen in the permeability of ampicillin across *in- situ* rat intestine. Fricker et al., (1996) suggested that ursodeoxycholate and chenodeoxycholate caused an increase in the absorption of the orally active peptide, octreotide in the *in-vitro* CaCo- 2 cell monolayer, the rat model and in the *in-vivo* human model. The mechanisms of action were attributed to an effect on the paracellular route because of the increase in permeability of PEG 4000 and on the

transcellular route due to the increase in LDH levels. Bile salts have shown that they enhance the stability of peptides and proteins in rat nasal homogenates indicating their duality as enhancers and enzyme inhibitors (Hirai et al., 1981a,b). Bile salts have been extensively used to promote the absorption and stability of peptides including thrombin inhibitor (Werner et al., 1996), insulin (Jørgensen et al., 1993; Shao et al., 1993) and calcitonin (Hastewell et al., 1994).

Meaney (1997) observed that the NaC and NaTC bile salt simple and mixed micelles increased the solubility and absorption of the lipophilic compound, dextropropoxyphene across the CaCo-2 cell monolayer. It was shown that the more hydrophobic bile salt NaC caused more changes to the morphology of the monolayer, and, that the mixed micelles of either bile salt significantly enhanced the transport of dextropropoxyphene and the paracellular marker compounds compared to the bile salt alone. This had previously been observed *in-situ* rat perfusion studies using a lipophilic compound, clofazamine (O'Reilly et al., 1994a, b). Greater enhancement potential was seen for mixed micelles formed with long chain unsaturated fatty acids e.g. linoleic acid, oleic. Lane (1997) observed that the stability of insulin in rat perfusate was enhanced in the presence of the conjugated bile salts, NaTC and NaGC compared to NaC. In this study it was established that mixed micellar systems increased the absorption of insulin in the *in-situ* rat perfusion model to a greater extent than the corresponding simple micellar system. This increase in enhancement was accompanied by a concomitant increase in the degradation of insulin in the intestinal effluent suggesting that cytosolic enzymes were being released from the cells due to a membrane effect of the mixed micellar systems.

The mechanism of absorption enhancing action of bile salt simple and mixed micelles has yet to be completely elucidated. The mucus layer has been suggested as a potential barrier to their absorption potential (Martin et al., 1978; Poelma et al., 1990; Shekels et al., 1995). It has also been suggested that a transcellular mechanism is involved because of their ability to disrupt the phospholipids in the membrane (Hoogdalem et al., 1992). In addition it has been suggested that bile salts act on the paracellular route by affecting the tight junctional complex (Werner et al., 1996; Meaney and O' Driscoll, 1999).

CHAPTER 5

General Properties of Cyclodextrins

5.1 Introduction

Cyclodextrins (CDs) are a series of cyclic oligo-saccharides, which are torus shaped and consist of six, seven or eight α-1, 4 –linked D- (+)-glucopyranose units. CDs, which consist of smaller or larger number of glucopyranose units, exist but are rare (Szeijtli, 1982). The number of glucose units present in the cyclodextrin may be denoted by the use of the Greek letters: α-cyclodextrin which has six units, β-cyclodextrin which has seven units and γ-cyclodextrin which has eight units. The structures for these parent cyclodextrin molecules are shown in figure 5.1.

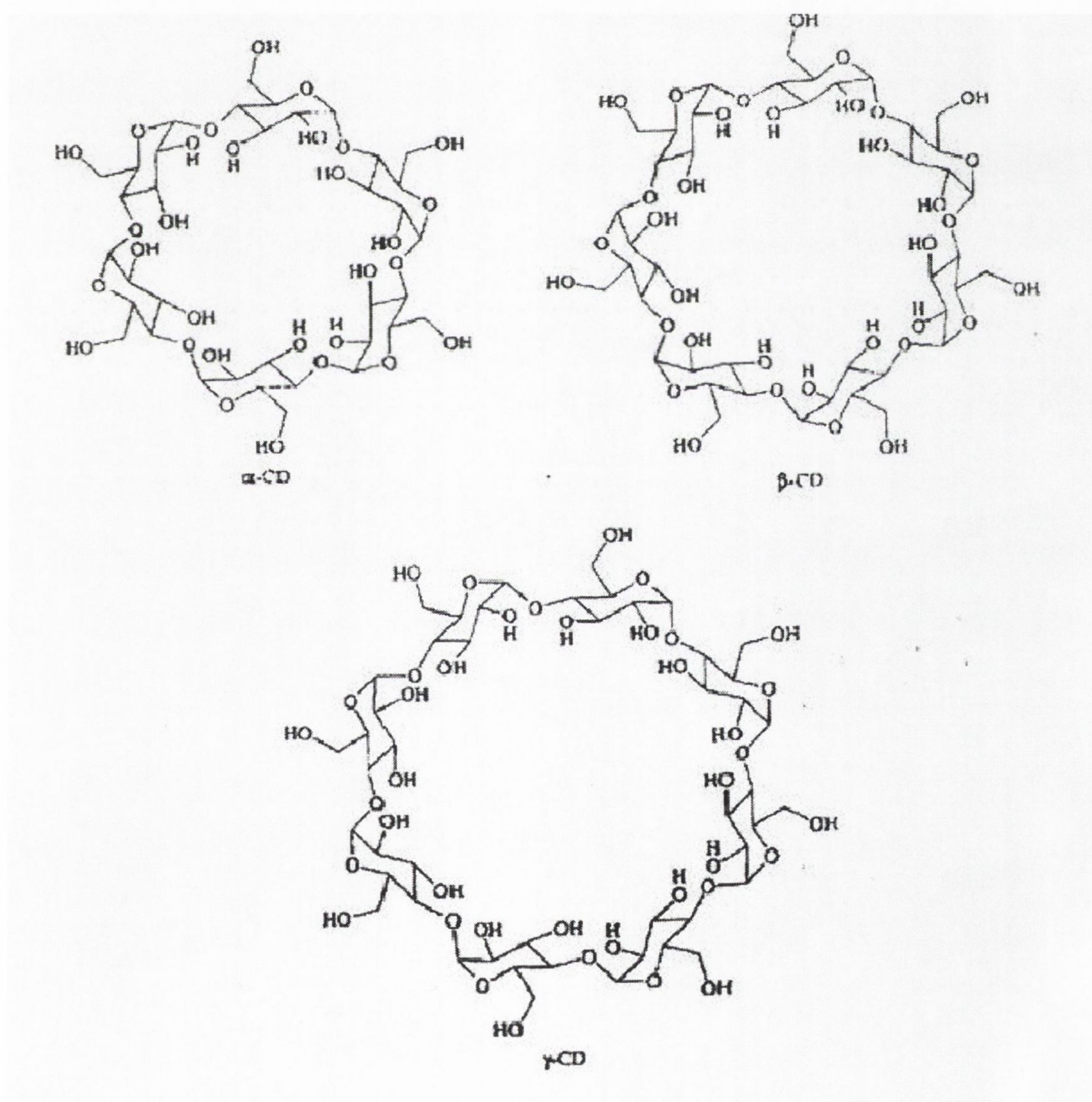


Figure 5.1 Structures of α-, β- and γ-cyclodextrins

5.2 Structural features of cyclodextrins

The three major cyclodextrins are crystalline, homogenous, nonhygroscopic substances, which are torus-like macrocycles built up from glucopyranose units (Smith et al., 1984). The cylindrical shape results from the lack of rotation around the $^4C_1$ glycosidic bond. The primary glucose hydroxyl groups, which are attached to the C-6 carbons, are oriented at the narrower side of the torus and the secondary glucose hydroxyl groups are attached to the C2 and C3 carbons at the wider face of the torus. The cavity of the CDs is lined with hydrophobic groups and the outside surface is hydrophilic in nature. The characteristics of α-, β- and γ-cyclodextrins are shown in table 5.1. Interestingly the aqueous solubility of β-cyclodextrin is less than the other two this is due to H-bond interactions between the C2 and C3 hydroxyl groups which results in the formation of a rigid structure (Szeijtli, 1982).

Table 5.1 Dimensions and physicochemical properties of α-, β- and γ-cyclodextrins (Strattan, 1991).

Feature	α	β	γ
No of glucose units	6	7	8
Mol weight	972	1135	1297
Aq. solubility (%)	14.5	1.85	23.2
Cavity diameter Å	4.7- 5.3	6.0-6.5	7.5- 8.3
Height of torus Å	7.9	7.9	7.9
Diameter of outer periphery Å	14.6	14.6	14.6
Approx. Vol. of cavity $Å^3$	174	262	472

5.3 Chemically modified cyclodextrins

Recently, various kinds of CDs derivatives have been prepared to extend the physiochemical properties and inclusion capacity of natural CDs as novel carriers

(Uekama et al., 1994). Generally the chemically modified CDs can be divided into three groups i.e. hydrophilic, hydrophobic and ionizable derivatives. The hydrophilic derivatives are the methylated and hydroxyalklyated derivatives (Szente and Szeijtli, 1999). These CDs are particularly useful as their aqueous solubility is very high and such a property may be exploited for the enhancement of dissolution and absorption of drugs. In contrast to this the hydrophobic derivatives, which are ethylated, are useful for the sustained-release carriers of water-soluble drugs and peptides (Uekama et al., 1989). The ionizable derivatives e.g. Sulfobutyl-β-cyclodextrin (SBE_7CD) can be used for site-specific targeting and modified release systems (Uekama et al., 1994). The CDs of particular interest are the hydrophilic derivatives 2,3-dimethyl-β-cyclodextrin (DMCD) and 2-hydroxylpropyl-β-cyclodextrin (HPCD) and the anionic derivative sulfobutyl-β-cyclodextrin (SBE_7CD). The toxicity profiles of CDs have been extensively reviewed (Irie and Uekama, 1997). A number of safety evaluations demonstrated that the three natural CDs (α-, β- and γ-cyclodextrin) and some chemically modified CDs (SBE_7CD, HPCD, DMCD and sulphated CDs) might be useful in the oral formulations development.

5.4 Effect of CDs on bioavailability of drugs

CDs by their very nature form inclusion complexes with drug molecules. The formation of these inclusion complexes have been utilised in the pharmaceutical, foods, cosmetics and agricultural industries (Szeijtli, 1982; Rajewski and Stella 1997).

The formation of these inclusion complexes have been used in pharmaceutical dosage forms for the prolonging of activity, increasing dissolution and hence bioavailability of the drug. The mechanisms of association and disassociation have been recently reviewed in great detail (Stella et al., 1999). In short the complete process is very rapid and if the drug is weakly bound dilution is sufficient for the break-up of the drug: CD complex, for more strongly bound drugs then the release profile of the drug may be altered. The complexation with hydrophobic drugs which are bound into the cavity has been widely reported and because of this their dissolution rates and absorption rates are enhanced

examples include a series of thiazide diuretics (Corrigan and Stanley, 1982), 17-β-estradiol (Schipper et al., 1990), prednisolone (Stella et al., 1995) and antimycotics (Jacobsen et al., 1999). CDs have been used for the enhanced delivery of peptide compounds, insulin (Merkus et al., 1991; Shao et al., 1992), growth hormone (Agerholm et al., 1994), ACTH (Schipper et al., 1993, 1994) and octreotide (Haeberlin et al., 1996). From preliminary studies it was suggested that a complex was being formed between the hydrophilic compound and the CDs. Haeberlin et al., (1996) suggested that the hydrophobic amino acid residues were forming complexes with the CDs. Matsubara et al., (1997b), demonstrated using ultraviolet absorption and circular dichroism, that the aromatic side chains of buserelin acetate, L-tryptophan and L-tyrosine residues, are incorporated into the hydrophobic environment of DMCD.

Hydrophilic CDs can solubilise and stabilise biomedically important peptides. Brewster et al., (1991) established that HPCD enhanced the solubility of ovine growth hormone, stabilised, prevented the formation of aggregates of lyophilised interleukin-2 and also prevented the formation of insulin aggregates. Other studies showed that the incorporation of CDs prevented the enzymatic degradation of peptides/ proteins; buserelin acetate degradation by chymotrypsin was prevented by maltosyl-β-cyclodextrin (Matsurbara et al., 1997a). The CDs decreased the rate of degradation by directly interacting with the protease and forming a non-productive complex with the substrate. Haeberlin et al., (1996) verified the stabilising effect that CDs had on the powder formulations of calcitonin and octreotide. The stability of modified calcitonin (msCT) was shown to be most enhanced in the presence of β- cyclodextrin and HPCD, where only 2.5% degraded was observed as compared to 6.4% for msCT alone after storage at 30^{0}C for 3 months. *In-vitro* stability studies with pepsin and α-chymotrypsin indicated that DMCD afforded the greatest protection against the enzymes. The CDs that resulted in the most pronounced intestinal absorption of msCT were HPCD and DMCD for *in-situ* rat perfusion studies and assumed that these CDs affected the paracellular route.

The enhancement potential of the modified CDs are reflected in their toxicity assessment using red blood cells and MTT with CaCo-2 cells. It is clear that DMCD> HPCD>

SBE_7CD with regards toxicity and enhancement effects in rat intestinal perfusion model (Krishnamoorthy et al., 1995). The exact mechanism of enhancement for these enhancers is not completely understood but because these systems are capable of solubilizing cholesterol from membranes and in addition they are known to affect the tight junction (Irie et al., 1992; Haeberlin et al., 1996), would suggest that they are capable of enhancing the transcellular and paracellular pathways.

CHAPTER 6

Calcitonin (CT)

6.1 General properties of calcitonin

Calcitonin (CT) is a single chain peptide hormone consisting of 32 amino acids (Table 6.1). Different sources of calcitonin are available including, bovine, eel, salmon, human, porcine and chicken. Although the amino acid sequence varies from one species to another, two common features essential for biological activity are 1) the disulphide bond between Cys^1 and Cys^7 which forms a free amino ring and 2) the proline amide group at the C-terminus.

Table 6.1 Primary amino acid sequences of calcitonin from man and salmon (Wimalawansa, 1997)

Residue No.	Man	Salmon
1	Cys	-
2	Gly	Ser
3	Asn	-
4	Leu	-
5	Ser	-
6	Thr	-
7	Cys	-
8	Met	Val
9	Leu	-
10	Gly	-
11	Thr	Lys
12	Tyr	Leu
13	Thr	Ser
14	Gln	-
15	Asp	Glu
16	Phe	Leu
17	Asn	His
18	Lys	-
19	Phe	Leu
20	His	Gln
21	Thr	-
22	Phe	Tyr
23	Pro	-
24	Gln	Arg
25	Thr	-
26	Ala	Asn
27	Ile	Thr
28	Gly	-
29	Val	Ser
30	Gly	-
31	Ala	Thr
32	Pro	-

In humans, calcitonin is secreted mainly by the parafollicular cells (C-cells) of the thyroid, which are of neural crest origin. In addition, a small number of calcitonin-producing cells have also been localized in the branchial and bronchiolar Kulchitsky (K) cells in the lungs. The K cells have been shown to be the origin of branchial carcinoid and small cell carcinoma of the lung, both of which secrete calcitonin.

Receptors for calcitonin are located in the osteoclasts and also in the kidney (Marx et al., 1972, 1973). The biological potency of calcitonin is usually assessed by its ability to reduce plasma Ca^{2+} levels in growing rats. It has been shown that the fish calcitonins are more potent that the mammalian calcitonins (Epand, 1983; Sinko et al., 1999).

The physiological role of calcitonin is to maintain skeletal mass during periods of calcium stress, as during growth, pregnancy and lactation, see Figure 6.1. CT also plays a central role in controlling calcium homeostasis and maintaining serum calcium without significant fluctuations. It affects a variety of tissues and organs including bone, intestine, kidney, breast and the hypothalamopituitary axis (Azria, 1989). Calcitonin is the most potent inhibitor of osteoclasts-mediated bone resorbtion, it is therefore highly effective in conditions associated with increased bone turnover, such as Paget's disease, osteoporosis, Sudecks atrophy and hypercalcaemia (Wimalawansa, 1993; Martindale, 1996). Osteoclasts are the main target cell for calcitonin and in the presence of CT they stop bone resorbtion and shrink in size. The efficacy of CT in preventing and treating osteoporosis is well documented (Schneyer, 1991). It has also been suggested that CT has an analgesic effect (Lyritis et al., 1991). Minor effects on the gastrointestinal tract and the kidney have also been reported (Azria, 1989).
Prolonged and continuous administration of calcitonin may result in a loss of potency (Wimalawansa and MacIntyre, 1991). The loss is associated with the formation of antibodies with non-human CTs or an increase in the catabolism of CT and perhaps the down-regulation of receptors. Non-human calcitonins have been shown to cause allergic-type reaction within 1-3 years of use in 50% of cases (Reginster et al., 1992b). However the actual clinical resistance to sCT varies considerably and it has been demonstrated as a safe drug (Wimalawansa, 1993).

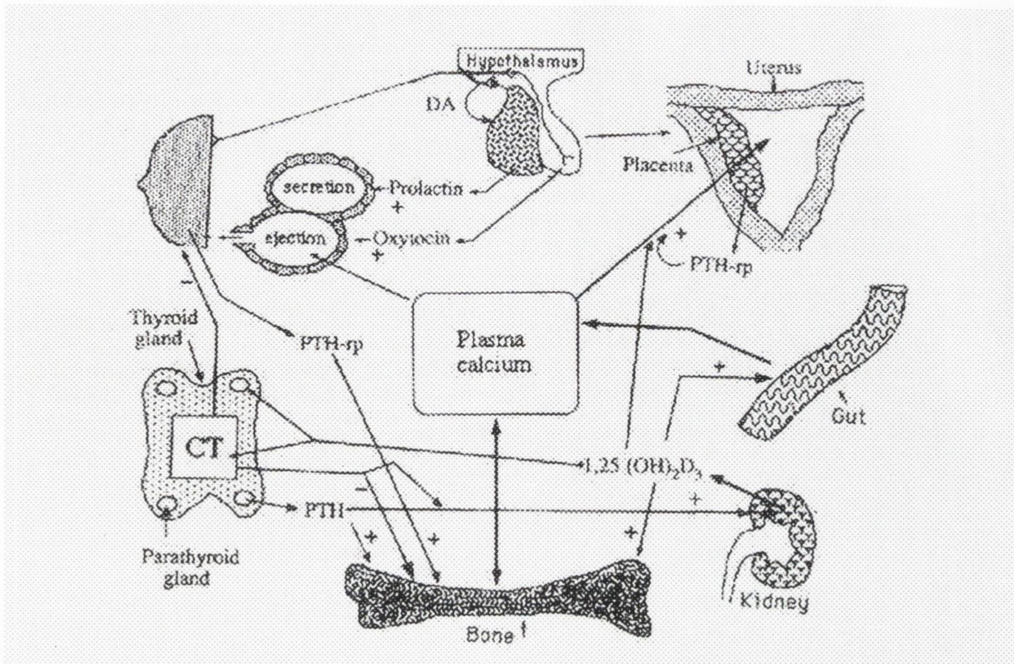


Figure 6.1 Role of calcitonin in calcium stress. CT takes part in maintaining Ca^{2+} homeostasis in conjunction with other calcium-regulating hormones and factors including $1,25(OH)_2D_3$, parathyroid hormone and parathyroid related protein (Wimalawansa, 1997)

Currently CT is administered via the parenteral or nasal routes (Reginster et al., 1992a). The oral route is a preferred route of administration considering the chronic nature of CT therapy. However, due to extensive proteolytic degradation in the GI lumen and low intrinsic intestinal membrane permeability, insufficient oral bioavailability of CT necessitates the use of high doses of CT. sCT is primarily used for formulation studies because it is 20-30 times more potent than human calcitonin (hCT) (Sinko et al., 1993) and unlike hCT does not aggregate (Baudyš and Kim, 1994; Lu et al., 1999). It also has been shown that *in-vitro* that sCT binds extensively to plastics and glass, therefore the inclusion of a surfactant in the formulation is required to prevent adsorption (Duncan et al., 1994). In this study the isolelectric point was approximately 9 implying that the peptide is positively charge at physiological pH.

A number of recent studies have investigated the factors that may influence the transport of sCT from the intestinal lumen (Tozaki et al., 1998; Sinko et al., 1999; Lee et al., 1999). Tozaki et al., (1998) clearly indicated the need for protease inhibitors in

an oral calcitonin formulation. In that study the use of bacitracin, soybean trypsin inhibitor, camostat mesilate or aprotinin enhanced human calcitonin absorption from *in-situ* rat ileum, jejunum and colon, with the greatest effects being observed in the colon. In our laboratory a range of protease inhibitors were assessed and it was shown that potato carboxypeptidase inhibitor had the most stabilising effect on sCT *in-vitro* rat intestinal homogenates (O'Donnell et al., 1996). Sinko et al., (1999) in an attempt to orally deliver sCT in a site –specific manner used surgically implanted access ports in dogs. This system allowed for evaluating the sites of absorption of sCT and the effect that co administration of citric acid had on the permeability and stability of sCT. The data showed that ileal absorption of sCT was higher than in other regions of the intestine. Citric acid enhanced the bioavailability of sCT. This may be due to the acidification of the microenvironment at the site of administration thereby stabilising the peptide, since sCT is most stable at pH 3.3 (Lee et al., 1992) and inactivating of the pancreatic serine protease trypsin, as it is most active at pH 5-6. Lee et al., (1999) further demonstrated that modulation of the intestinal pH might affect the absorption profile of sCT in the dog model. Therefore it is clear that the absorption of sCT is enhanced in the presence of protease inhibitors and when the pH is decreased to acidic conditions. Lee et al., (1999) in a further attempt to increase the bioavailability of sCT prepared a series of sCT pro-drugs. In this study the sCT was mono-PEGylated which produced three positional isomers of N-terminus, Lys^{18}- and Lys^{11}-residue. It was shown that the stability of these pro-drugs in rat kidney homogenates was significantly increased compared to the parent, sCT. The stability of the Lys^{18}-residue modified mono-PEG-sCT was the highest with a 58-fold increase observed.

CHAPTER 7

Materials and Experimental Methods

7.1 Solutes used in this work

Salmon calcitonin, Bachem, UK

^{125}I salmon calcitonin, Amersham, IM250

7.2 Solvents, Reagents and Excipients used

-Hank's Balanced Salt Solution (HBSS) Powder (1L), without Phenol Red, without Sodium Bicarbonate, Gibco, Catalogue No. 11201-019

-Dulbecco's Phosphate Buffered Saline (PBS) Powder (1L), without Calcium, without Sodium Bicarbonate, Gibco, Catalogue No. 21300-017

-Trypsin-EDTA (1X) Liquid, Gibco, Catalogue No. 45300-019

-Dulbecco's Modified Eagle's Medium (DMEM), 1X, with Non Essential Amino Acids and without L-Glutamine and Sodium pyruvate, Gibco, Catalogue No. 12501-029

-{N-(2 hydroxyethyl) piperazine-N-(2-ethanesulphonicacid)} sodium salt, HEPES, Sigma, H-0763

-Sodium Bicarbonate, $NaHCO_3$, Sigma, S-5761

-D-(+)-Glucose, Sigma, G-7021

-Gentamycin 10mg/ml, Gibco, Catalogue No. 15710-031

-L-Glutamine 200mM, (100X), Gibco, Catalogue No. 25030-024

-Transferrin, Gibco, Catalogue No. 13008-016

-Foetal Bovine Serum, Gibco, Catalogue No. 10106-078

-Trypan Blue Solution 0.4%, Sigma, T-8154

-Dimethylsulfoxide, DMSO, Sigma, D-5534

-Collagen, Type 1 from Rat Tail, Sigma, C-7661

-Transwell® 0.4μm, 4.71cm^2, Costar, Product No. 3412

-Trans-Col®0.4μm, 4.71cm^2, collagen coated, Costar, Product No. 3425

-Snapwell® 0.44μm, 1cm^2, Costar, Product No. 3407

-PTFE Filter Inserts, 0.4μm, 4.71cm^2, Falcon.

- ^{14}C Polyethylene glycol (PEG) 4,000, Amersham, Product No. CFA408

- ^{3}H Mannitol, Sigma, M0781

-^{3}H Polyethylene glycol (PEG) 900, NEN Products, Product No. NET-404

-Scintillation Cocktail, Ultima Gold, Canberra Packard, Cat. No. 6013329

-Cholic acid, sodium salt from sheep or ox bile, Sigma, C-1254

-Taurocholic acid, sodium salt, Sigma, T-4009

-Glycocholic acid, sodium salt, Sigma, G-7132

-Deoxycholic acid, sodium salt, Sigma, D-5-670

-Linoleic Acid (sodium salt) approximately 99% by capillary GC, Sigma, L-Linoleic, L-8134

-2,3-dimethyl-β-cyclodextrin, Janssen, Prod. Code 30, 239, 72

-2-hydroxylpropyl-β-cyclodextrin, Pharmatec, Florida, USA

-Sulfobutyl-β-cyclodextrin, CyDex Inc., L.C., Kansas, USA

-Novel cyclodextrins synthesised under the supervision of Dr. Raphael Darcy, Dept of Chemistry, University College Dublin: Heptakis (6-O-Sulphonatophenyl) -β-cyclodextrin sodium salt, Heptakis (6-deoxy-6-Aminopyridyl)-β-cyclodextrin and Heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin

-Trichloroacetic acid, Ultra-pure grade, Sigma, T-4009

-ELISA salmon calcitonin kit, Cortecs Diagnostics, Deeside, Clwyd, UK

-Citric acid, Reidel-de-Häen. Code No. 33114

-Trizma Base, Sigma, T-1410

-Calcium Chloride, anhydrous, Sigma, C-4501

-Sodium Chloride, Sigma, S-9888

-Sodium azide, Sigma, S-2002

-EDTA, Sigma, E1644

-MTT (Thiazolyl Blue), Sigma, M5655

-Sodium dodecyl sulphate, Sigma, L-6026

-N-Acetylcysteine, Sigma A-9165

-Propidium Iodide (PI), Molecular Probes, P-3566

-Partially purified porcine mucin, Type III purified from porcine stomach, Sigma, H-1778

-Bovine serum albumin, Sigma, A7030

-Bovine serum albumin, RIA grade, Sigma, A7888

-*Helix pomatia* (Edible Roman Snail) peroxidase labelled lectin, Sigma, L-6387

-Gelatin B, Sigma, G-9391

-Tween 20, Sigma, P-1379

-di-Sodium Hydrogen Ortho-Phosphate, Reidel-de-Häen. Code No. 04273

-Hydrogen peroxide solution 30%, BDH, Product No. 285194F

-OPD tablet, Sigma, P8287

-DHEP, Texas Red®, Molecular Probes, T1395

-Para-formaldehyde, Sigma,

-Triton-X, Sigma, X-100

-Glycine, Sigma, G-8898

-Polyclonal salmon calcitonin antibody, host rabbit, Biogenisis, UK.
Cat. No. 1720-8055

-FITC-protein labelling kit, Molecular probes, Cat No. F-6434

-Gelman Sterile Acrocap Filter unit, 0.2μm, Product No. 4480

-Gelman Sterile Acrodiscs syringe filters, 0.2μm, Product No. 4192

-Gelman Supor 200 membrane filters, 13mm, 0.2μm, Product No. 60298

-Gelman Plastic Filter Holders, 13mm, Product No. 4317

-All tissue culture plastics were obtained from Gibco or Costar.

7.3 Instrumentation Used

-Nuaire water-jacketed infrared automatic CO_2 Incubator

-Nuaire Class 11 Type A/B3 Laminar Flow Hood

-Techne, Tempette Junior, TE-8J Water Bath

-Balance, Mettler AE 240

-Orion pH Meter Model 520A

-EVOM, Volt ohmmeter, WPI

-Sigma 203 centrifuge

-Watson Marlow peristaltic pump

-Tri-Carb 2100 series liquid scintillation counter

-Wilovert 1 Light Microscope

-Fluorescent Microscope

-Hitachi H7000 transmission electron microscope

-Leica Stereoscan scanning electron microscope

-Dynatec Plate reader

-Cobra auto γ-scintillation counter, Canberra Packard

-Transferpettes, 10-50μl, 50-250μl and 200-1,000μl, Brand

-Gilsen pipettes 10-50μl, 50-250μl and 200-1,000μl

-Sealpette, 0-10μl, Jencons

-Portable Pipet-Aid, Drummond

-Priorclave Autoclave

-The Advanced ® Osmometer Model 3D3, AGB

-BioRad Confocal scanning Laser Microscope, Model MRC1024

-Viscometer, Carri-Med Rheometer CSL^2, TA Instruments

-Water purification and deionisation, Purite labwater RO100 HP

7.4 Cell Culture

7.4.1 Cell Lines

CaCo-2 cells (passage 30) were obtained from ECACC (Porton Down, UK). Ht29GlucH cells (passage 12) were a generous gift from Dr. Daniel Louvard, Pasteur Institute, Paris, France. Cells of passage 30-50 (CaCo-2) and passage 12-32 (Ht29GlucH) were used throughout.

7.4.2 Maintenance Medium

Both CaCo-2 and Ht29GlucH cells were cultured in supplemented Dulbecco's modified Eagle's medium (DMEM).

Prior to use the medium was supplemented with 10% v/v bovine serum (FBS), L-Glutamine 1% w/v, and Gentamycin 100mg/ml for culturing CaCo-2 cells. Medium being used for the maintenance of Ht29GlucH and CaCo-2: Ht29GlucH co-culture cells were also supplemented with 5μg/ml of transferrin.

7.4.3 Phosphate buffered saline (PBS)

Cells were washed with PBS prior to trypsinisation to aid in loosening of the tight junctions. One stock container (Gibco) was dissolved in one litre of sterile water and the pH was adjusted to 7.35. The PBS was then aseptically filtered using a 0.2μm filter in the LFH.

7.4.4 Hank's Balanced Salt Solution (HBSS)

Transport studies were carried out in HBSS containing 25mM Glucose and 10mM HEPES during the course of this work. This was prepared as outlined below:

HBSS powder	
Glucose	3.7g/l
HEPES (10mM sodium salt)	2.6g/l
Sterile water to volume	
pH to 7.35	

The appropriate quantities of ingredients were dissolved in sterile deionised water, and the pH adjusted to 7.35 before being aseptically filtered using a 0.2μm filter in the LFH.

7.4.5 Passaging of Cells

The cells were routinely grown in 75cm^2 culture flasks in DMEM maintenance medium and maintained at 37^{0}C in an atmosphere of 5% CO_2 and 90% relative humidity in the incubator. Before reaching confluency the culture medium was removed from the flasks and the cells were rinsed with 10ml of PBS for approximately 5 min to remove any residual medium. 5ml of trypsinising solution (0.25% trypsin and 0.02% EDTA) was added to cover all the cells in the flask. These flasks were then incubated in the trypsin solution at 37^{0}C for 5-10min after which the flask(s) were repeatedly agitated to ensure rapid and complete removal of all the cells. The trypsinising solution was

inactivated by adding culture medium (5ml) to the flask(s) and repeatedly pipetting the cell suspension, which was subsequently centrifuged at 1,000rpm for 5min. The cell pellet was resuspended in 1ml of culture medium. If the cells were passaged into flasks counting was unnecessary and the cell suspension was divided between three or four flasks containing 15ml culture medium (1:3 or 1:4 passage). Cells were passaged every 4-7 days and a maximum of 20 passages was observed.

7.4.6 Freezing of cells

Freezing medium was prepared by adding 10% v/v dimethylsulfoxide (DMSO) to foetal bovine serum (FBS). After trypsinising and resuspension of the cell pellet in 1ml of culture medium the number of viable cells present was determined using a haemocytometer and trypan blue exclusion test as follows: 20µl of cell suspension was mixed with 20µl of Trypan Blue and a drop of the mixture was placed on the haemocytometer. The cells occupying the grids in the centre were counted and the number of cells per ml calculated using the following equation;

$$\text{No. of cells/ml} = \text{No. of cells in 25 grids} \times 2 \times 10^4 \qquad \text{Eqn. 7.1}$$

The cell suspension in culture medium was mixed 50:50 with freezing medium to give a solution containing 3×10^6 cells/ml. 1.5ml of this solution was then placed in 2ml cryo vials (Costar), which in turn were placed in a polystyrene container and placed in an -80^{0}C freezer for 4 hours. Then the cryo vials were placed in liquid nitrogen for long-term storage.

7.4.7 De-freeze protocol

Due to the toxic nature of DMSO to the cell membrane, cells were thawed rapidly at 37^{0}C and the contents of one cryo vial transferred immediately to a universal tube containing 10ml of pre-warmed culture medium. The cells were pelleted in a centrifuge at 1,000rpm for 5min and finally resuspended in fresh culture medium and

placed in a cell culture flask. The cells were fed the following day to remove any cell debris.

7.4.8 Mycoplasma Testing

Standard direct and indirect tests for the detection of mycoplasma infection were carried out at the National Cell and Tissue Culture Centre, Dublin City University, Dublin on samples of antibiotic free medium from CaCo-2 and Ht29GlucH cells. The cell lines had been cultured in antibiotic free DMEM medium for at least three passages prior to mycoplasma testing. Both cell lines were shown to be mycoplasma free.

7.4.9 Collagen Coating of Snapwells

A 3:1 ratio of rat-tail collagen to 60% v/v sterilized ethanol was prepared. A 100μl aliquot of this mixture was added to each Snapwell insert and then dried overnight in the LFH under UV light.

7.4.10 Seeding of Transwells

CaCo-2

Following resuspension of the cell pellet after trypsinisation the number of cells present was determined using a haemocytometer. CaCo-2 cells were plated at a density of 63,000 cells/cm^2 (Hidalgo et al., 1989a) in the culture medium onto Transwell® polycarbonate membranes.

The Caco-2 cells were fed with the culture medium every other day for the first seven days and then daily up to day 18-21, by replacing 1.5ml of the medium in the apical (AP) chamber and 2.6ml of the medium in the basolateral (BL) chamber of the Transwell®.

Ht29GlucH

Ht29GlucH cells were seeded at a density of 8.5 x 10^5 cells/cm^2 (Wikman et al., 1993) onto Trans-Col®.

CaCo-2: Ht29GlucH Co-Culture

The two cell lines were trypsinised as described and resuspended in Ht29GlucH medium. Cell numbers of each line were counted and adjusted. The two cell populations were then mixed in the ratio, 3:1, CaCo-2: Ht29GlucH, and seeded at a density of 4.25 x 10^5 cells/cm^2 (Allen et al., 1992) onto TransCol® or collagen coated Snapwell®.

7.5 Cell Morphology

7.5.1 Transmission Electron Microscopy (TEM)

The cells were fixed in a 0.1M sodium cacodylate buffer pH 7.4 containing 3% v/v glutaraldehyde and 5% w/v sucrose for 1 hour at 25^0C or overnight at 4^0C. Using 0.1M sodium cacodylate buffer the cell monolayers were washed six times over a 30min period to remove the glutaraldehyde. Post-fixation was carried out in 2% osmium tetraoxide in 0.1M sodium cacodylate buffer (pH 7.4) for 1-2 hours at room temperature. The cell monolayers were dehydrated from cacodylate buffer through an ascending ethanol series as follows:

10% alcohol in H_2O	10min
30% alcohol in H_2O	10min
50% alcohol in H_2O	10min
75% alcohol in H_2O	10min
95% alcohol in H_2O	10min
100% alcohol in H_2O	10min
100% alcohol in H_2O	15min
100% alcohol in H_2O	20min

Dehydrated cell monolayers were then transferred into propylene oxide for 15min. This was replaced with fresh propylene oxide and the cell monolayers were exposed for a further 30 min. Then the cell monolayers were placed in a 50:50 solution of Epon resin and propylene oxide for 1-2 hrs with continuous agitation before being

transferred into the resin alone for a further 2 hrs. Finally, the samples were placed in pans containing freshly poured resin and placed in the oven, which was initially placed under vacuum to remove any air bubbles. The samples were then incubated at 60^0C for 24hr to allow the resin to harden. Thin sections were cut with an ultramicrotome and stained with uranyl acetate and lead citrate. The specimens were studied using a Hitachi H7000 electron microscope.

7.5.2 Scanning Electron Microscopy (SEM)

The cell monolayers were fixed, washed, post-fixed and dehydrated as described for TEM. Subsequently they were exposed to Freon 113 transitional fluid for 30 min at room temperature. Specimens were then quickly transferred to a critical point dryer (Balzers CPD 020), and the Freon 113 exchanged for liquid CO_2. After flushing the dryer several times to ensure complete removal of the Freon 113, the dryer was heated up until the critical temperature (32^0C) and critical pressure (1,150 psi) of CO_2 was exceeded. The CO_2 was vented off slowly and the samples allowed dry. Then these samples were mounted onto SEM stubs using double sided cello tape or silver dag. A fine conducting layer of gold was applied using a sputter coating unit (Emscope SC 500) to leak away to earth any build up of electrons on the specimen surface. Samples were then viewed using a Leica Stereoscan scanning electron microscope.

7.5.3 Light Microscopy (LM)

Samples for embedding in paraffin wax were fixed for 1hr as described under TEM. Following fixation the cells were transferred to 0.1M cacodylate buffer, encased in embedding grids and equilibrated in 50% ethanol for 10min, before being permeated with wax according to the following protocol:

30% Ethanol	2hr
50% Ethanol	2hr
70% Ethanol	2hr
95% Ethanol	2hr
Absolute Alcohol	2hr

Absolute Alcohol	2hr
Chloroform	2hr
Chloroform	1hr
Wax 1	1hr
Wax 2	1hr

Samples were then transferred to a cold table for embedding following the final wax stage. Embedded samples were trimmed and 5mm sections cut on a microtome. The freshly cut sections were floated onto a water bath at 50^0C and collected on glass slides. Finally the mounted sections were dried on the slides for 16hr at 37^0C prior to staining.

Samples for staining were cleared and then rehydrated through a descending ethanol series (100-75-50%), 5min in each solution before being transferred to distilled water.

7.5.3.1 Alcian Blue Staining

Alcian Blue stain was used as a 1% solution in distilled water, adjusted to pH 2.5 with 1M HCl. Samples were first stained according to the following protocol:

Procedure	Time (min)
Stain with Haemotoxylin	0.5- 1
Wash under cold tap water	5
Stain with Alcian Blue	10
Rinse with distilled water	5
Expose to phosphomolybdic acid 1% in distilled water	10
Rinse with distilled water	5
Stain with Eosin	0.5- 1
Rinse with distilled water	5

7.5.3.2 Periodic acid Schiffs Staining (PAS)

Periodic acid was used as a freshly prepared 1% v/v solution in distilled water. Schiff's reagent was fuschin based and stored at 40^{0}C. Where necessary samples for PAS staining were first treated with a human salivary amylase solution for 30min at 37^{0}C to clear glycogen. The protocol for PAS staining was as follows:

Procedure	**Time (min.)**
Stain with 1% Periodic acid	5
Rinse with distilled water	5
Stain with Schiff's Reagent	10
Rinse with distilled water	5
Stain with Haemotoxylin	0.5- 1
Differentiate using Acid Alcohol (0.01%v/v HCl in 100% EtOH)	5
Stain with Haemotoxylin	0.5- 1
Rinse with distilled water	5

Following staining the samples were then dehydrated through an ascending ethanol series (50-75-100%), cleared and mounted on the slides. Samples were then viewed under the light microscope.

7.5.4 Fluorescence Microscopy

7.5.4.1 Propidium iodide staining

The intercalating dye, propidium iodide (PI), was used to discern cells with damaged membranes. Propidium iodide does not permeate intact cell membranes. The monolayers were rinsed twice with PBS and incubated with 30μg/ml propidium iodide in PBS for exactly 3 min. They were rinsed twice with PBS and fixed for 10min with 4% formaldehyde in PBS on ice before being rinsed again in PBS (four times). Finally the filters were mounted on glass slides in a 1:1 solution of PBS and glycerol and viewed using a fluorescent microscope.

7.6 Confocal scanning laser microscopy

7.6.1 Preparation of the glass slides

On a new glass slide the corners of a coverslip were marked for its approximate position for placement of the sample. On the side opposite to where the slide had been marker, 'posts' were constructed. These were prepared by sweeping diagonally on the marked area with clear nail polish. The coverslip was then placed on these four corner posts to prevent the sample from being crushed when being viewed under the microscope.

7.6.2 Preparation of the anti-sCT antibody

The polyclonal antibody for sCT (host rabbit) was purified on a Protein A column and then FITC conjugated using the FITC-protein labelling kit as described by Molecular Probes.

7.6.3 Sample preparation

The protocol was taken and subsequently modified from The Handbook of Biological Confocal Microscopy, Chapter 18. The formulation being examined was placed in the apical chamber of day 21 cultures grown on Transwell® inserts for 4h. After which the system was removed from the apical chamber and the monolayer was washed three times with pre-warmed PBS. Then the counter stain, DHEP Texas Red ® was placed on the apical and basolateral chambers at a concentration of 1:1000 in PBS from a stock solution of 1mg/ml in absolute ethanol. This was left in contact with the cell monolayer for 10min at $37^{0}C$ in the dark. This was used to specifically stain the cell walls giving the orientation of the cell when viewed by the microscope. Then the apical and basolateral chamber were rinsed with 5% BSA in PBS three times. The monolayer was then fixed for 10 min at room temperature with 3% para-formaldehyde and 0.1% Triton-X in PBS at pH 7.4. Again it was washed three times with pre-warmed PBS. To ensure that all the unreacted formaldehyde was removed the

monolayer was subjected to a series of glycine washes. Firstly with a 1M solution in PBS for 30min, which was followed by three PBS rinses. Then exposed to glycine: BSA (1M: 5%w/v) in PBS for a further 45 min, which was followed by a further three washes with PBS. Then the membrane was cut into four pieces for further processing whilst minimising volumes of solutes required. A drop of anti-salmon calcitonin FITC labelled probe was placed in a suitable container and the membrane was then placed in the droplet overnight at 4^0C in the dark. This was then washed three times with 5% BSA in PBS which was followed by a further two washes with PBS. The membrane samples were then ready for placement on slides. The sample was placed centre with a droplet of DABCO in PBS of the previously prepared 'posts'. The coverslip was carefully placed over the sample ensuring that no air bubbles were trapped underneath and allowed rest its corners on the 'posts'. The samples were then sealed at the edges using clear nail varnish to prevent the loss of DABCO, which prevents bleaching of the fluorescent probes. The samples were stored at 4^0C protected from light until viewed using a BioRad Confocal scanning laser microscope.

Slides were viewed and scanned as described by the manufacturing company, with the laser intensity retained at a minimum. A series of control specimens were viewed to ensure that thre was no cross-reactivity with the cell culture systems and the confocal laser microscope. Manipulation of a series of X-Y scans allowed for the intensity measurement to be assessed using the associated computer packages on the system. Sakai et al., 1997, had previously described this method of analysis.

7.7 Enzyme linked lectin assay for Mucins

It has been demonstrated by Rhodes et al., (1993) that peroxidase labelled lectins may be used for quantifying mucin glycoprotein. It was shown that these lectins were also suitable for testing on intestinal mucus glycoprotein homogenates. This assay was kindly modified assay was kindly supplied by Nageen Hasheen, Dept. of Pharmaceutics, School of Pharmacy, Kings College, London. An apical sample of 300μl was taken and tested in triplicate for mucin glycoproteins using peroxidase labelled Roman snail (edible) (*Helix pomatia*) (HPA). HPA a α/β–D-Galactosyl-

specific lectin, binds specifically to the epitopes α-GalNAc and GalNAcβ4Gal. This specifically associates with mucus-producing cells in adult mice (Falk et al., 1994). The assay was calibrated using freshly prepared partially purified mucus (PPPM) in the range 100- 0 ng/ml in HBSS. The DMEM was used as background levels and all samples were blanked with this when apical samples containing DMEM were examined otherwise HBSS was used.

Five main solutions for the assay

1. Washing solution = PBS+ 0.5%v/v Tween 20+ 0.5% w/v Gelatin type B
2. Blocking solution = PBS+1% w/v Gelatin Type B
3. Lectin solution = PBS+ 0.1% w/v Gelatin Type B + 5μg/ml *Helix pomatia* lectin (HPA) peroxidase labelled.
4. Substrate solution = Equal volumes (10ml) of di-sodium hydrogen orthophosphate (0.2M) and Citric acid (0.1M) are mixed in amber container then 0.05% v/v hydrogen peroxide is added and pH adjusted to pH 5. Then 0.05% w/v o- phenlyene- diamine (OPD) was added with vigorous shaking. The substrate solution was prepared just prior to use.
5. Stop solution = 20% v/v Sulphuric acid.

The procedure for carrying out the ELLA is as follows:

1. Incubate 100μl sample i.e. apical media or ng/ml standards in a 96 well plate at 4^0C overnight to allow the plate well to be coated.
2. Remove samples/standards and wash wells three times with 200μl /well of washing solution.
3. Incubate with 100μl of blocking solution for 1hr at 37^0C.
4. The well were again washed three times with 200 μl /well.
5. Incubate with 100μl of lectin solution for 1hr at 37^0C.
6. Wash plate six times with 200μl /well
7. Incubate with substrate solution for 10min at room temperature
8. Stop reaction with 20μl addition of stop solution.
9. Read plates within 10min of stop solution addition at 492nm

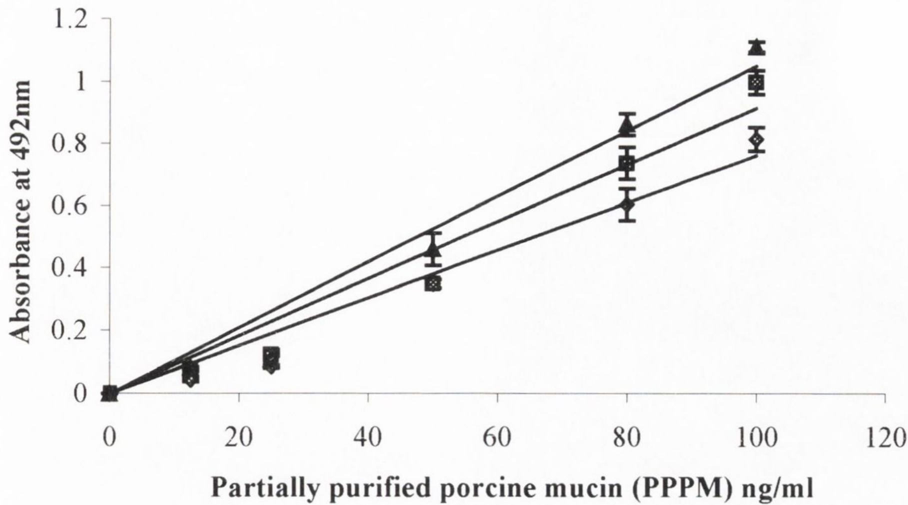


Figure 7.1 Calibration curve for partially purified porcine mucus for the ELLA. Values are expressed as mean ± SD for a minimum of at least 3 observations. Background has been accounted for.

Figure 7.1 represents the calibration curves obtained for PPPM with this assay which each gave an R^2 of 0.97. These plots were used to calculate the amount of mucin in a sample from the absorbance reading at 492nm. The calibration curve has been constructed to account for any background reading associated with the diluent.

7.8 Measurement of Transepithelial Electrical Resistance (TEER)

The integrity of the membrane was evaluated by measuring the transepithelial electrical resistance (TEER) using a WPI voltmeter/ohmmeter fitted with 'chopstick' electrodes. In order to obtain reproducible results the electrodes were maintained in a constant position, using a retort stand. In addition, readings were always taken prior to feeding at room temperature in an effort to standardize measurement conditions. The electrical resistance values were expressed as transmembrane resistance ($\Omega.cm^2$) by multiplying by the area of the membrane ($4.71cm^2$). TEER values obtained in the absence of cells, i.e. using the uncoated polycarbonate membrane was considered as background giving a value of 200 $\Omega.cm^2$.

7.9 Transport experiments

On the days of the transport experiment, the culture medium was replaced with Hank's balanced salts solution (HBSS) containing 25mM glucose and 10mM HEPES buffer, pH 7.35 (transport medium) and the cell monolayers seeded in the Transwell ® were allowed to equilibrate for 30-60min. The TEER was measured before and following equilibration as previously described in section 7.8. All wells in 6 well clusters received 2.6ml of marker free transport medium equilibrated at 37^{0}C. Inserts were placed in the first well of a cluster so that the outer surface of the insert was immersed in the medium. At time zero, the test solution containing the system being examined, radiolabelled hydrophilic compounds, ^{3}H mannitol, ^{14}C PEG 4000 and ^{125}I salmon calcitonin (sCT) and 10μg/ml cold sCT was added to the inserts (apical cell surface) dissolved in 1.5ml of the transport medium. At each sampling time the inserts were rapidly transferred to another well containing 2.6ml of fresh transport medium (basolateral cell surface). The amount of radionuclides transported at each time interval was determined in an appropriate scintillation counter.

The cumulative % transported was then plotted against time and the slope of this line, dQ/dt, was substituted into Eqn. 7.2 to determine the Papp (Adson et al., 1994). Equation 7.2 assumes sink conditions i.e. $<10\%$ of the compound has been transported. In the case of $>10\%$ of the marker being transported over the time course of the study dQ/dt was determined from the initial linear portion of the cumulative % transported versus time graph.

Papp = dQ/dt.1/(AC$_0$)	**Eqn. 7.2**

Papp	=Apparent permeability coefficient (cm/sec)
dQ/dt	=steady state flux (mol/sec)
A	=surface area of the membrane (cm^{2})
C_0	=initial concentration in the donor chamber (mol/ml)

7.10 Recovery Experiments

Initially the monolayers were exposed to HBSS (for 20-60 min) containing the radiolabelled paracellular markers, mannitol and PEG 4000 to equilibrate. The medium was then changed to HBSS containing the test system and radiolabelled markers for 1 or 4h. Finally, the test medium was removed and replaced by DMEM culturing medium. The Papp of the marker molecules were measured throughout the experiments. Similar experiments where the addition of the bile salt system had been excluded were used as controls.

7.11 MTT Assay

Intracellular dehydrogenase activity was determined by the MTT method (Mossman (1983); Tada et al. (1986)). It has previously been shown that exposure to surfactant concentrations that caused reversible effects on epithelial permeability demonstrated a decrease in mitochondrial dehydrogenase (Anderburg et al 1992, 1993). MTT is a tetrazolium salt that is cleaved by mitochondrial dehydrogenase in living but not dead cells to give a dark blue product.

Briefly, the MTT assay was carried out as follows; CaCo-2 cells were seeded at a density of 35,000 cells/100μl/well onto 96 well plates and cultured for 24-48hr in DMEM medium until confluent. Subsequently, 100μl of the test substance in HBSS was added to the wells and incubated for 10min at 37^0C. MTT solution (20μl) was then added and the assay completed as described under the calibration procedure. The concentration of reagent that produced a 50% inhibition of the dehydrogenase enzyme activity was obtained from the concentration-absorbance curves.

7.12 Preparation of micellar and cyclodextrin systems

7.12.1 Preparation of simple micellar systems

Weighing accurately the appropriate quantity of the salt and dissolving in a small volume of HBSS pH 7.4 prepared bile salt simple micellar systems. Dissolution of the

salt was achieved by constant stirring on a magnetic stirrer. After dissolution the system was made up to volume in a volumetric flask.

7.12.2 Preparation of mixed micellar systems incorporating fatty acid

The preparation of mixed micellar systems incorporating fatty acid involved preparing initially the simple bile salt system. The fatty acid was weighed carefully and added incrementally. All systems were prepared with vigorous stirring on a magnetic hot plate at 37^0C. When all the fatty acid had been added, the system was made up slowly to volume using HBSS.

7.12.3 Preparation of commercially available and novel cyclodextrins

The appropriate amount of cyclodextrin was accurately weighed and dissolved in a small volume of HBSS pH 7.34. All systems were prepared with vigorous stirring on a magnetic plate. When all the CDs had gone into solution the system was made up to volume in a volumetric flask.

7.13 Assay of samples

7.13.1 Assay of ^{14}C PEG 4,000 and ^{3}H Mannitol

Samples were assayed by micropipette 1ml of sample into a scintillation vial and adding 10ml of Ultima Gold® a liquid scintillation cocktail. Samples were then read on a Tri-Carb® liquid scintillation system. The DPM reading (disintegrations per minute) of the sample was compared to the DPM reading of the initial solution prepared to determine the percentage of the marker absorbed. Quench correction was carried out be the method of external standardisation.

7.13.2 Assay of salmon calcitonin (sCT)

7.13.2.1 Enzyme linked immunoassay

The sCT immunoassay kit (Cortecs Diagnostics, Deeside, Clwd, UK) employed a non-competitive sandwich-type enzyme immunoassay technique utilising biotin-avidin enhancement and a high activity (TMB) substrate system. Samples were added to plastic microwells as provided and the assay followed according to manufacturers instructions. Samples for ELISA were stabilised by the addition of a concentrated acetate buffer pH 2 to the sample, a 1:100 dilution. It has been shown that the iso-electric point (pI) of sCT was around pH 4.7 and this was where the peptide was most stable (Duncan et al., 1995). The kit came provided with a series of standards providing a calibration curve for the plate facilitating calculations of the concentration of sCT in the sample assayed.

7.13.2.2 Trichloroacetic acid precipitation of ^{125}I sCT (TCA)

Trichloroacetic acid precipitation was used to separate and quantify the percentage intact and degraded iodinated protein (Zapf et al., 1994; Fath et al., 1994). At the appropriate sampling times 1ml was withdrawn from the basolateral chamber and placed in an eppendorf, to which ice cold TCA was added yielding a final concentration of 10% v/v. These vessels were kept on ice. The samples were vortexed and centrifuged (10,000 rpm for 5min). This resulted in the supernatant (degraded peptide) separating from the pellet (intact peptide). Detection of ^{125}I sCT was by γ scintillation counting.

To ensure that this assay only precipitated intact sCT it was compared to the assay results obtained from the more specific ELISA. In table 7.1 it was clear that there was no statistically significant difference between samples analysed by ELISA or TCA for sCT alone or in the presence of NaGC 10mM. From theses results it was concluded that using ^{125}I sCT TCA precipitation assay was suitable for the detection of salmon calcitonin.

Table 7.1 Comparison of the apparent permeability of sCT across the CaCo-2 cell monolayer when analysed by ELISA and TCA in the presence and absence of NaGC 10mM. Values are represented as mean ± SE of at least 10 observations.

System	**Papp X 10^6 (cm/sec) ± SD ($n \geq 10$)**	
	ELISA	**TCA**
sCT	0.03 ± 0.02	0.05 ± 0.01
NaGC (10mM)	0.01 ± 0.005	0.02 ± 0.01

These results were reflected on analysis of *in-situ* exiting rat intestinal perfusate and *in-vitro* rat intestinal mucosal homogenates, which showed that the detection level of sCT was similar for both ELISA and TCA. However, it appeared that ELISA was more variable compared to TCA precipitation and HPLC (O'Donnell, 2000).

7.14 Detection of %sCT degraded for stability studies

The concentration of sCT was analysed using TCA precipitation (section 7.13.2.2) that was placed in the apical chamber at the beginning of the experiment and again after 4h exposure to the cell monolayer in the apical chamber. The test solutions were heated to 37^0C in a water-bath for 10min prior to initiation of the experiment. 100μl of sample was removed and TCA precipitation carried out resulting in a pellet, intact sCT and supernatant, degraded sCT. The % degraded was calculated by expressing the amount degraded over the total count. These values were done in triplicate.

7.15 Viscosity determination

The apparent viscosity (η_{app}) of partially purified porcine mucin (PPPM) combined with simple and mixed micellar systems of NaGC: LA were measured using a CSL^2 500 rheometer. PPPM demonstrated Newtonian-type behaviour.

7.16 Molecular Modelling

The cross sectional diameter of salmon calcitonin was determined by molecular modelling using Silicone Graphics 02 Workstation with MacroModel V6.5 simulation software.

7.17 Statistical Analysis

Unpaired Student's t-test (two tailed, $\alpha=0.05$) was used to test the significance of differences between two mean values. When comparisons of more than two mean values were made, the data was analysed with one-way ANOVA ($\alpha=0.05$).

CHAPTER 8

The effects of bile salt simple micelles on the apparent permeabilities of the hydrophilic compounds, mannitol, PEG 4000 and sCT and on the integrity of the CaCo-2 cell monolayer

8.1 Introduction

The effect of bile salts on the absorption of compounds has previously been extensively studied producing results that are conflicting. It has been shown that the co administration of drugs with micelles causes a decrease in absorption (Feldman et al., 1973; Poelma et al., 1989, 1990). In contrast to this the absorption of some molecules have been increased in the presence of the bile salt micelle. Proposed mechanisms of action include disruption of the lipid matrix of the cell (Feldman et al., 1973), an affect on the integrity of the tight junction causing dilation (Yamashita et al., 1990; O'Reilly et al., 1994; Werner et al., 1996), an alteration in the viscosity or production of mucus (Martin et al., 1978; Poelma 1990) or by enzymatic inhibition (Hirai et al., 1981b). It is therefore easy to conclude that the precise mechanism of action of bile salts is complex.
In this study the CaCo-2 cell culture model was used to assess and compare the permeability enhancing and drug stabilizing potential of bile salt simple micellar systems. This was determined by observing the effects that these systems had on the apparent permeability (Papp) and stability of salmon calcitonin (sCT), a therapeutic peptide molecule (MW 3413) and on the Papp of mannitol (MW 182) and polyethylene glycol (PEG) (MW 4000), both of which are hydrophilic paracellular marker compounds (Meaney 1997). The bile salts investigated were the trihydroxy-unconjugated bile salt, sodium cholate (NaC), the conjugated bile salts, sodium taurocholate (NaTC) and sodium glycocholate (NaGC) and the unconjugated dihydroxy bile salt, sodium deoxycholate (Deoxy).

8.2. Examining the Toxicity of Simple Micellar Systems

Prior to transport and stability studies cytotoxicity was examined using the MTT assay and fluorescent microscopy. The purpose of these assays was to provided information regarding a suitable concentration range of bile salts for transport studies where damage to the cell monolayer was minimal.

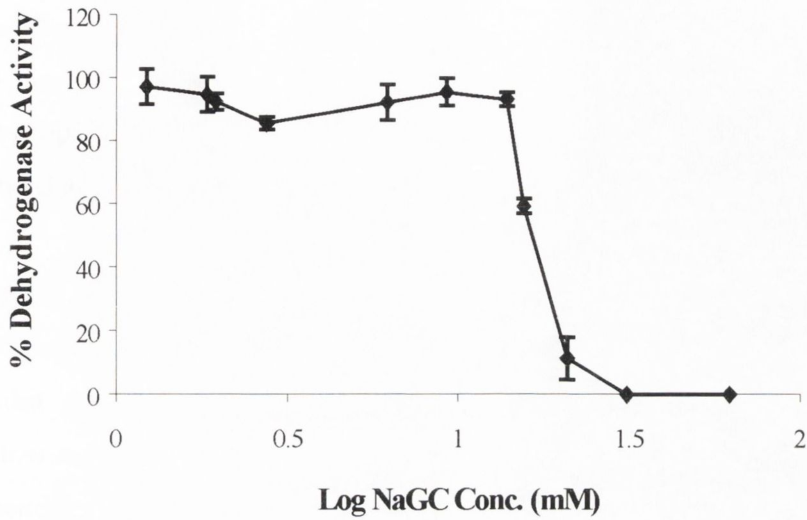
8.2.1 Intracellular Enzyme Activity

Intracellular dehydrogenase activity was determined by the MTT method (Mossman (1983) and Tada et al. (1986)). This assay was used to determine concentration of bile salt that caused a 50% decrease in enzyme activity (IC_{50}) and has been quoted as an indicator of cell toxicity. It has previously been shown that exposure to surfactant concentrations that caused reversible effects on epithelial permeability demonstrated a decrease in mitochondrial dehydrogenase (Anderburg et al 1992, 1993). The effect of NaGC and Deoxy on the intracellular enzyme activity was determined using the MTT method. A concentration dependant decrease in enzyme activity was noted for both systems. The IC_{50} was calculated from the slope of the linear portion of the profile for each bile salt as shown in Figure 8.1. The IC_{50} value for NaGC was 18.05 ± 2 mM which compares well with the value reported by Jørgensen et al (1993) of 24.2 mM. The value for Deoxy was 2mM. The IC_{50} value for NaTC (10mM) and NaC (6mM) were previously established in our laboratory and are in agreement with literature values, see Table 8.1 (Anderburg et al. (1992); Meaney (1997)).

Table 8.1 Concentration of bile salt causing 50% inhibition of intracellular dehydrogenase activity (IC_{50}) in CaCo-2 cells and critical micellar concentration (CMC).

System	**CMC (mM) (Small, 1971)**	**IC_{50} (mM)**
NaGC	3.1	18.05
NaC	3.3	6
NaTC	3.4	10
Deoxy	1	2

(a)



(b)

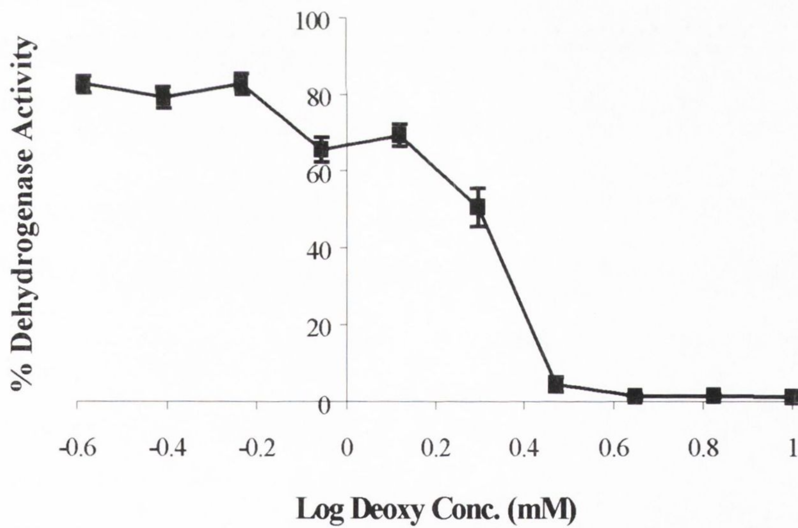


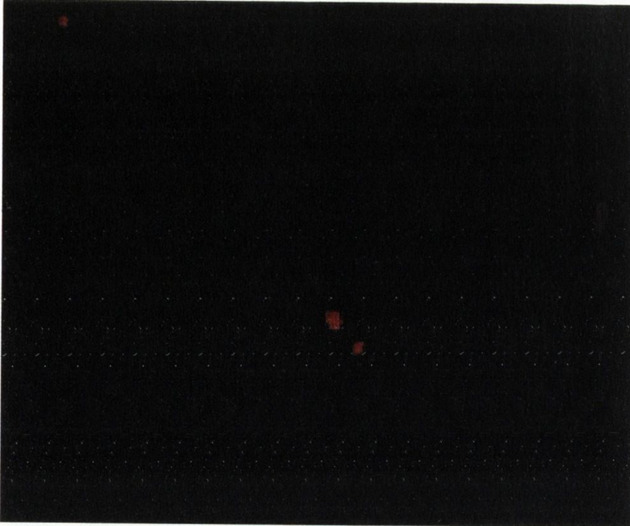
Fig. 8.1 The effects of (a) sodium glycocholate (NaGC) and (b) sodium deoxycholate (Deoxy) on the intracellular dehydrogenase activity. Values are expressed as mean ± SE ($n \geq 6$).

8.2.2 Fluorescence Microscopy

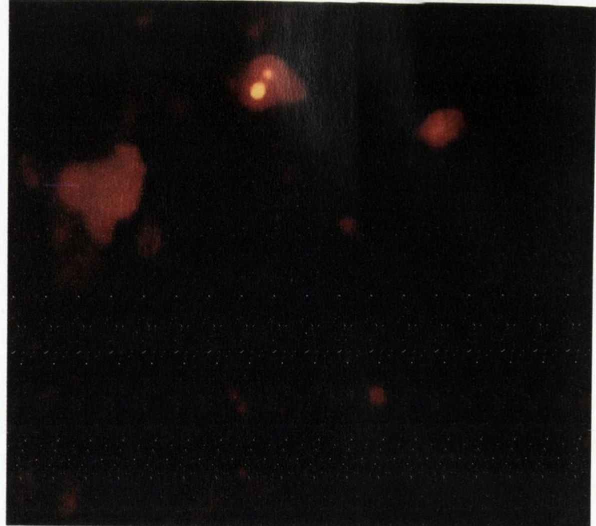
The effect of bile salts at concentration approximate to that used in the MTT assay on the cell membrane integrity of CaCo-2 cells was further investigated using the fluorescent dye propidium iodide (PI). This is an intercalating dye, which permeates damaged cells to yield a red fluorescence.

After 60 minutes exposure of the CaCo-2 cell monolayers to the bile salt systems, each of the monolayers was examined for fluorescence. The untreated control monolayers exhibit no fluorescence and indicated that the cell membrane is intact (Fig 8.2 (a)). Following exposure to NaGC (10, 15 and 20mM) there appears to be a concentration dependant increase in the intensity and distribution of fluorescence as the concentration of bile salt is increased indicating increasing deterioration in the integrity of the cell membrane (Fig 8.2 (b-d)), with NaGC 20mM> NaGC 15mM >NaGC 10mM. NaC (5mM) and NaTC (20mM) were examined as previous work suggested that concentrations of NaC greater than 5mM adversely affected the integrity of the monolayer and NaTC 20mM was shown to cause a reversible enhancement in the transport of paracellular markers across the CaCo-2 cell monolayer (Meaney 1997). Deoxy (1mM) was chosen as it was approximately its' CMC. The fluorescence for these systems can be seen in figure 8.2 (e-g), the fluorescence observed for these systems is less intense and less widespread than for NaGC (15mM). The values obtained from the MTT toxicity test and the observation made from the intensity and localization of PI fluorescence provided a further indication as to the suitable concentration range for use in the transport and the stability experiments. These qualitative results support the quantitative results as previously shown by the MTT assay for the bile salt systems and suggest that NaGC is less toxic than Deoxy. This finding was corroborated by previous finding that indicated that unconjugated bile salts i.e. Deoxy were more lytic than conjugated bile salts i.e. NaGC (Carey 1985).

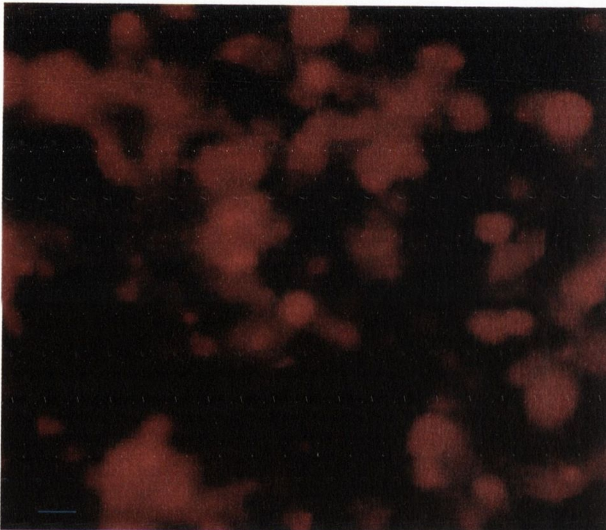
(a)



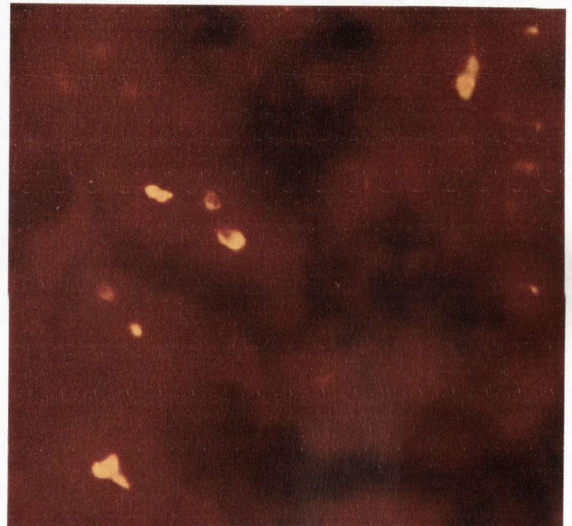
(b)



(c)



(d)



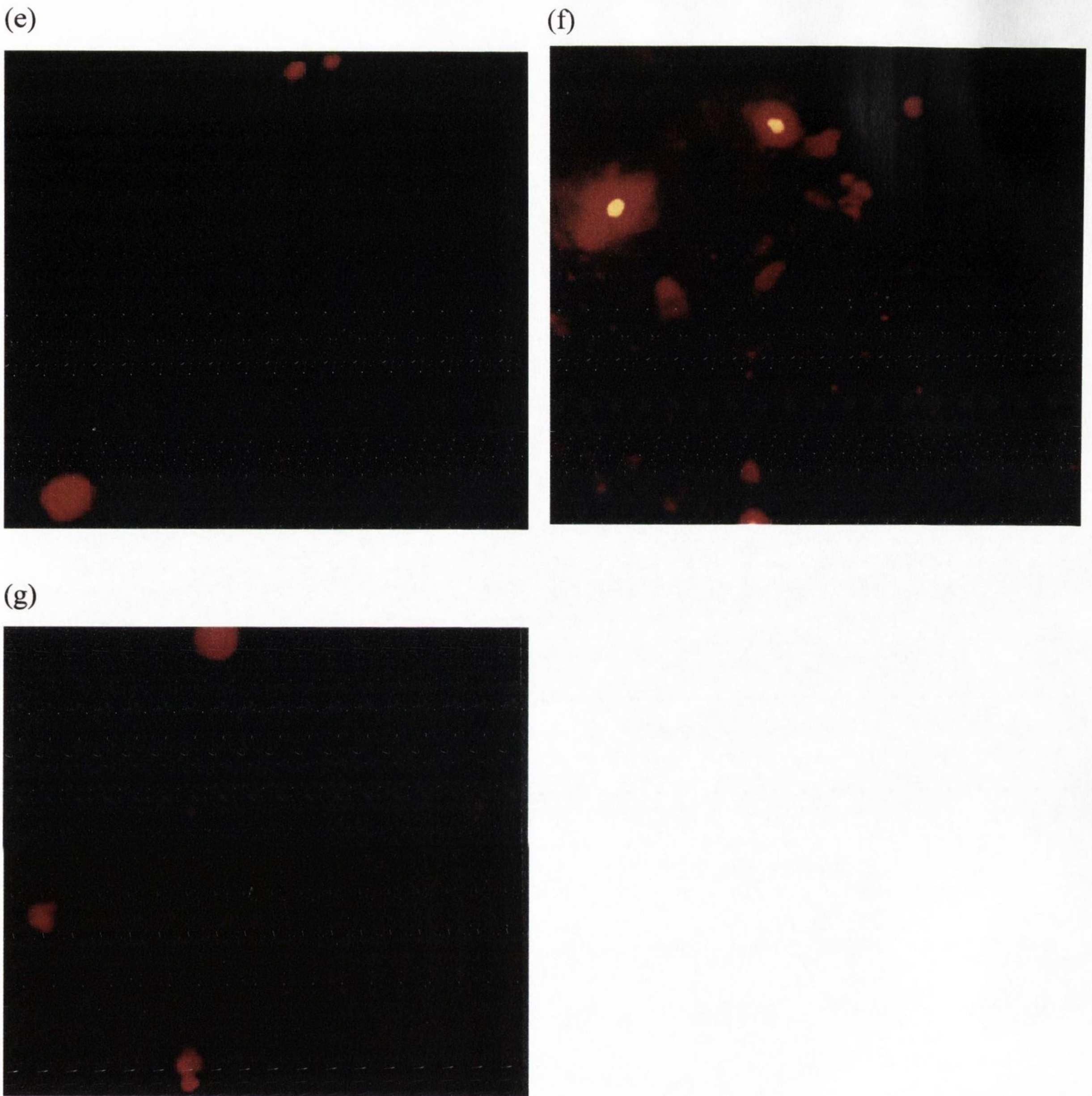


Fig. 8.2 Photographs of CaCo-2 cells incubated with

(a) Control HBSS
(b) NaGC (10mM)
(c) NaGC (15mM)
(d) NaGC (20mM)
(e) NaC (5mM)
(f) NaTC (20mM)
(g) Deoxy (1mM)

for 4h respectively and then stained with propidium iodide.

8.3 Stability of sCT in the presence of simple micelles

Due to the labile nature of peptides the stability of salmon calcitonin (sCT) was investigated in the presence of the various bile salt systems. The concentration range studied was based on the IC_{50} values (see Table 8.1) and the propidium iodide staining (figure 8.2). To detect intact sCT an assay involving the precipitation of the peptide using TCA as outlined in materials and methods see Chapter 7, was used.

Sodium glycocholate (NaGC)

The stability of the peptide calcitonin (sCT) sample in the apical chamber was investigated immediately after preparation and following warming to 37^0C, time = 0min and the end of a transport experiment, time = 240min (appendix 1). The concentrations of NaGC investigated ranged from 10-20mM thus incorporating the value for the IC_{50} for this bile salt as estimated by the cytotoxicity experiments. With all concentrations of NaGC studied the percentage of sCT degraded at time 0 min was similar to what was seen for the drug alone, with approximately 4.04- 8.80% sCT degraded (Table 8.2). This implies that the NaGC over the concentration range studied does not have an adverse effect on the stability of the peptide sCT (table 8.2).

Sodium taurocholate (NaTC)

In the presence of NaTC 20mM it is apparent that there is a significant increase in the quantity of degraded sCT compared to control ($p < 0.01$) (Table 8.2). At the initial sampling point 83.29 ± 1.63% of sCT was degraded in the presence of NaTC (20mM).

Sodium cholate (NaC)

The concentration of 5mM NaC used in the stability and transport experiments was chosen as similar concentrations were used in our laboratory without any irreversible adverse effect on the integrity of the CaCo-2 cell monolayer (Meaney 1997). From table

8.2 there appears to be no difference in the amount of sCT degraded as compared to the control at the beginning of the experiment. From appendix 1 it was apparent that after 4h exposure of sCT to the NaC the degradation was significantly increased ($p < 0.05$).

Sodium deoxycholate (Deoxy)

The stability of the peptide sCT was unaffected relative to the control at the onset and at 4h in the presence of Deoxy (1mM), see Table 8.2 and Appendix 1.

Table 8.2: The % of salmon calcitonin (sCT) degraded in the apical chamber in the presence of the CaCo-2 cell monolayer at the beginning of a 4h transport experiment for each of the bile salt systems, ($p < 0.05$ * compared to sCT alone)

System	% sCT Degraded ± SE of the Initial Starting Conc. at onset of experiment ($n \geq 3$)
sCT	7.92± 4.09
NaGC (10mM)	4.03 ± 3.62
NaGC (12.5mM)	3.76 ± 0.64
NaGC (15mM)	3.68 ± 1.11
NaGC (20mM)	8.80 ± 1.71
NaTC (20mM)	83.29 ± 1.63*
NaC (5mM)	4.93 ± 1.04
Deoxy (1mM)	7.34 ± 0.18

The results from the stability experiment indicate that at the concentrations studied NaTC accelerated the instability of the peptide salmon calcitonin (sCT) whereas this was not seen in the presence of NaGC, NaC and Deoxy at the onset of the experiment.

8.4 Transport of hydrophilic compounds in the presence of simple micelles

Following these initial studies, the apparent permeability coefficients (P_{app}) of the hydrophilic compounds, mannitol (MW 182), sCT (MW 3413) and PEG 4000 across the CaCo-2 cell monolayer were estimated in the presence of the bile salt simple micellar systems.

8.4.1 Sodium glycocholate (NaGC)

Salmon calcitonin (sCT)

The concentrations of NaGC investigated ranged from 10-20mM the data indicated a concentration dependant increase in the transport of the peptide sCT (Fig 8.4 and Table 8.3). The apparent permeability of salmon calcitonin (sCT) across the CaCo-2 cell monolayer was $0.05 \pm 0.01 \times 10^{-6}$ cm/sec. In the presence of NaGC 10mM and 12.5mM the permeability of sCT was not significant relative to the control, but at concentrations of 15mM and 20mM significant differences were observed ($p < 0.05$), see Figure 8.4.

The apparent permeability in the presence of NaGC 15mM increased by approximately 12-fold to $0.60 \pm 0.10 \times 10^{-6}$ cm/s, $p < 0.05$. In the presence of NaGC 20mM the apparent permeability for sCT increased 16-fold to $0.83 \pm 0.06 \times 10^{-6}$ cm/s, $p < 0.05$, see Table 8.3. The apparent permeability coefficients (Papp) were calculated in each case using equation 2.1 as previously described.

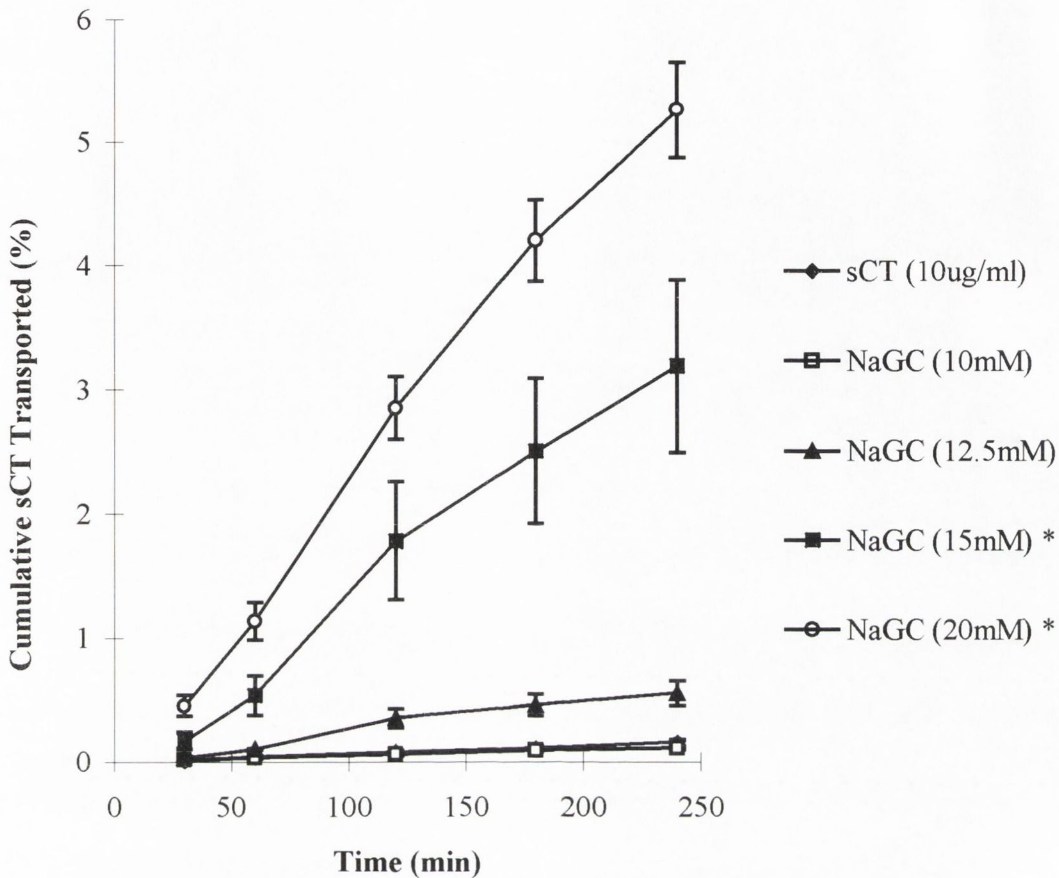


Fig. 8.4 Transport profile of salmon calcitonin (sCT) across the CaCo-2 cell monolayer in the presence of different concentrations of sodium glycocholate (NaGC). (*, $p < 0.05$). Values are expressed as mean ± SE of at least $n \geq 4$ observations.

Mannitol and PEG 4000

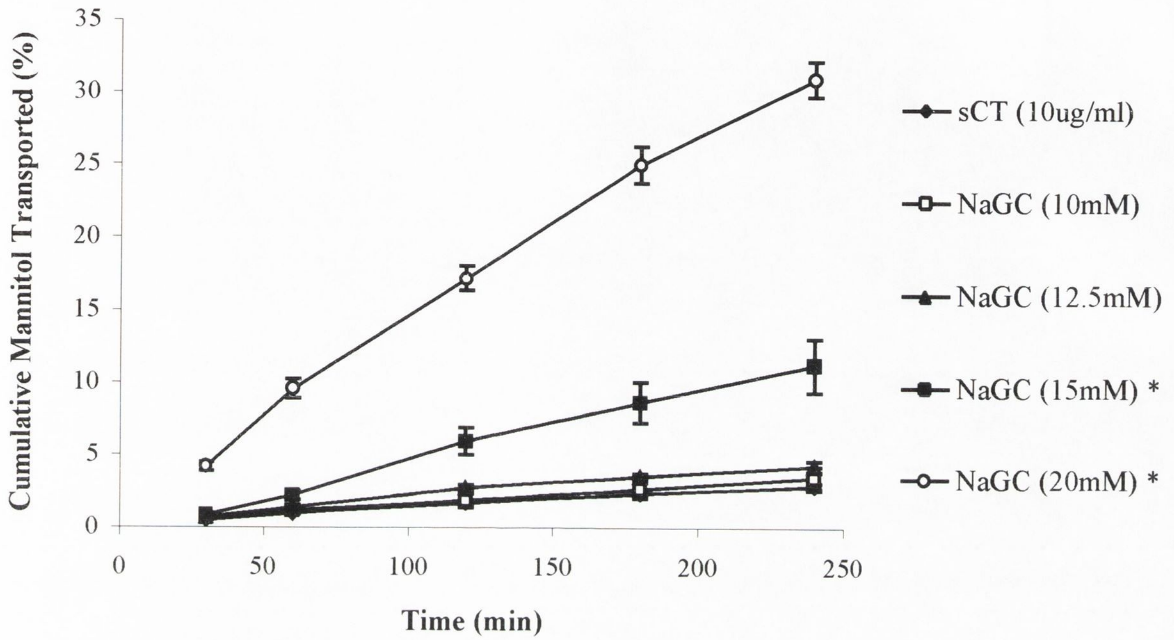
The transport of the paracellular markers, mannitol and PEG 4000 in the presence of a range of concentrations of NaGC (10-20mM) gives a constant transport rate over 4 hours represented in figure 8.5 (a) and (b). When the apparent permabilities (Papp) of the hydrophilic compounds are calculated using the cumulative amount transported over the time of the experiment, 4 hours, it was obvious that there was a concentration dependent increase in the transport of these compounds across the CaCo-2 cell monolayer. For systems of NaGC 15mM and 20mM there is a statistically significant increase in the

apparent permeability of mannitol and PEG 4000. The apparent permeability coefficient (Papp) of mannitol increased almost 4-fold to 1.78 ± 0.31 X 10^{-6} cm/sec from a control of 0.46 ± 0.02 X 10^{-6} cm/sec in the presence of NaGC 15mM. At the higher concentration of NaGC 20mM the increase in mannitol transport is 10-fold with a Papp of 4.48 ± 0.16 X 10^{-6} cm/sec. For PEG 4000 similar fold enhancements may be seen, see Table 8.3 and Figure 8.5 (b). The Papp for NaGC 15mM and 20mM are significantly increased from control (sCT alone), 0.05 ± 0.01 X 10^{-6} cm/sec, to 0.40 ± 0.07 X 10^{-6} cm/sec and 0.84 ± 0.12 X10^{-6} cm/sec respectively.

Table 8.3 The apparent permeability (Papp) and the percentage transported across the CaCo-2 cell monolayer over 4 hours of the hydrophilic compounds, mannitol, PEG 4000 and sCT for each of the simple micellar systems for sodium glycocholate (NaGC).

System	**Papp ± SD (X10^6) (cm/sec) (n ≥ 3) ($p<0.05$, *) (Total Percentage transported after 4hr)**		
NaGC (mM)	**Mannitol**	**PEG 4000**	**sCT**
0	0.46 ± 0.02 (2.9 ± 0.17%)	0.05 ± 0.01 (0.34 ± 0.04%)	0.05 ± 0.01 (0.33 ± 0.05%)
10	0.57 ± 0.10 (3.46 ± 0.82%)	0.05 ± 0.04 (0.34 ± 0.09%)	0.02 ± 0.01 (0.10 ± 0.06%)
12.5	0.60 ± 0.05 (4.28 ± 0.37%)	0.09 ± 0.01 (0.61 ± 0.10%)	0.09 ± 0.02 (0.55 ± 0.10%)
15	1.78 ± 0.31* (11.24 ± 1.83%)	0.40 ± 0.07* (2.53 ± 0.48%)	0.60 ± 0.10* (3.60 ± 0.59%)
20	4.48 ± 0.16* (30.95 ± 1.24%)	0.84 ± 0.12* (5.79 ± 0.84%)	0.83 ± 0.06* (5.27 ± 0.38%)

(a) Mannitol



(b) PEG 4000

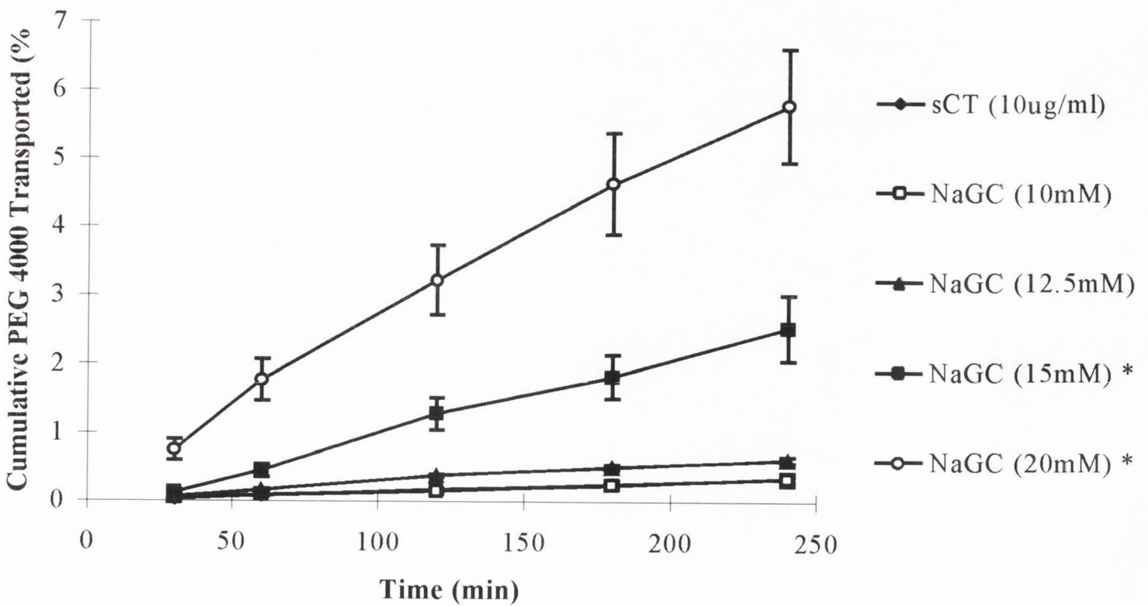


Fig. 8.5 The effect that NaGC simple micelles have on the transport of the paracellular markers (a) mannitol and (b) PEG 4000 across the CaCo-2 cell monolayers.

Generally it may be summarised that in the presence of NaGC 15mM and 20mM that the relative enhancement of the Papp of sCT≥ PEG 4000> mannitol when compared to control (Figure 8.6).

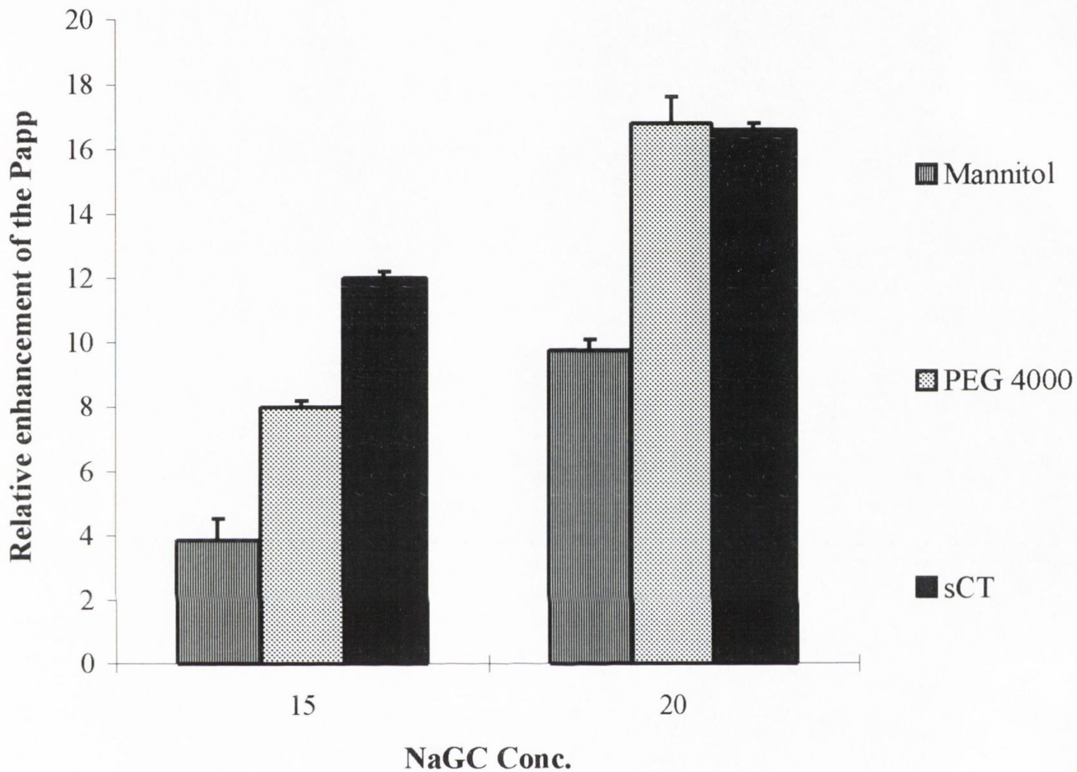


Figure 8.6 The relative enhancement of the apparent permeability (Papp) of the peptide salmon calcitonin (sCT), and the paracellular markers, Mannitol and PEG 4000 in the presence of simple micellar systems of sodium glycocholate (NaGC) compared to control after 4h.

8.4.2 Transport of hydrophilic compounds in the presence of NaTC, NaC and Deoxy simple micellar systems

The apparent permeability (Papp) of the hydrophilic compounds were also determined in the presence of the other simple micelles of sodium taurocholate (NaTC), sodium cholate (NaC) and sodium deoxycholate (Deoxy).

NaTC and NaC have previously been investigated in our laboratory and have shown an enhancing effect on the transport of compounds of paracellular marker compounds, mannitol, PEG 900 and PEG 4000 and the lipophilic compound, dextropropoxyphene (Meaney 1997).

Salmon calcitonin (sCT)

The stability data, see section 8.2.1, indicated that both NaTC (20mM) and NaC (5mM) had a destabilizing effect on the peptide sCT and therefore it was expected that the transport of the peptide would consequently be affected. The transport of the intact peptide compound was investigated and it was noted that both bile salts, NaTC and NaC caused a statistically significant increase in its transport relative to the sCT alone (control) $p < 0.05$, see figure 8.7.

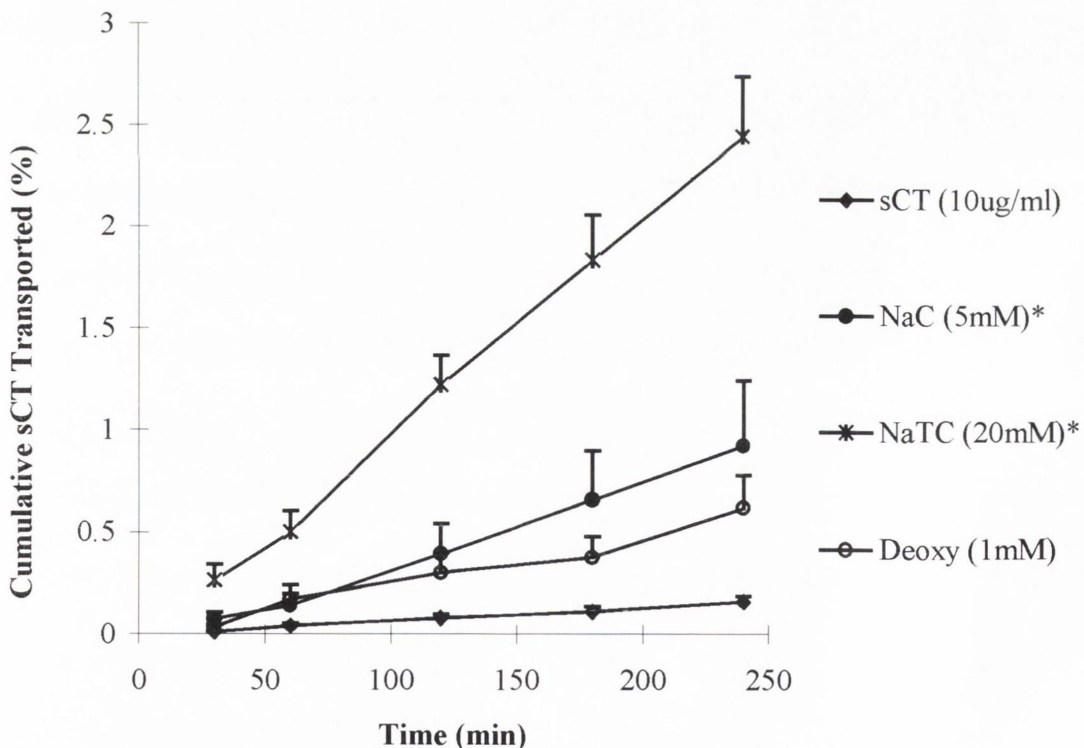


Fig. 8.7 Transport profile of salmon calcitonin (sCT) across the CaCo-2 cell monolayer in the presence of sodium taurocholate (NaTC), sodium cholate (NaC) and sodium deoxycholate (Deoxy) (*, $p < 0.05$)

The apparent permeability of sCT in the presence of NaTC 20mM across the CaCo-2 monolayer was 0.37 ± 0.04 cm/sec which is an approximate 7.4-fold increase compared to control, 0.05 ± 0.01 X 10^{-6}. This enhancement was substantial; as 83.3 % of sCT in the apical chamber was degraded at time zero of the experiment, see Table 8.2. Because of the enhanced degradation of sCT and the relatively poor permeability enhancement compared to NaGC (15mM) NaTC 20mM had limited use as an enhancer for sCT.

A similar trend was seen for sodium cholate (NaC) 5mM with a Papp of 0.20 ± 0.06 X 10^{-6} cm/sec which is an approximate 3-fold increase in the transport of sCT across the Caco-2 monolayer. The stability of the peptide was also detrimentally affected in the presence of NaC (5mM) after a 4h period with less than 65% of sCT left intact in the apical chamber (Appendix 1). It was demonstrated that NaC (5mM) was not a suitable enhancer for sCT because it destabilised the peptide and the resultant increase in the transport of sCT was significantly lower than for NaGC 15mM ($p < 0.05$).

Sodium deoxycholate (Deoxy) (1mM) does not cause a significant enhancement in the apparent permeability of sCT, 0.08 ± 0.02 X 10^{-6} cm/sec. Increasing the concentration of this bile salt could result in damage to the cell membrane as the IC_{50} was 2mM (Table 8.1) and this would limit the use of Deoxy as an enhancer.

Mannitol and PEG 4000

The apparent permeability of mannitol and PEG 4000 is increased in the presence of NaC 5mM and NaTC 20mM. There was a 1.72-fold increase in the transport of mannitol in the presence of NaC 5mM with the apparent permeability increasing from 0.46 ± 0.02 X 10^{-6} cm/sec to 0.79 ± 0.13 X 10^{-6} cm/sec. In the presence of NaTC 20mM the increase was greater with a 3.3-fold increase in the Papp. The greater enhancement was seen in the case of PEG 4000, with the apparent permeability in the presence of NaC 5mM and NaTC 20mM increasing 4 and 7.4-fold respectively (table 8.4 and figure 8.8).

Table 8.4 The apparent permeability (Papp) and the % transported across the CaCo-2 cell monolayer over 4h of the hydrophilic compounds, mannitol, PEG 4000 and sCT for the simple micellar systems for sodium taurocholate (NaTC), sodium cholate (NaC) and sodium deoxycholate (Deoxy).

System	Papp ± SD (X10^{6}) (cm/sec) (n ≥ 3) (Total Percentage transported after 4hr)		
	Mannitol	**PEG 4000**	**sCT**
sCT	0.46 ± 0.02 (2.9 ± 0.17%)	0.05 ± 0.01 (0.34 ± 0.04%)	0.05 ± 0.01 (0.33 ± 0.05%)
Deoxy (1mM)	0.56 ± 0.08 (3.96 ± 0.55%)	0.08 ± 0.04 (0.56 ± 0.28%)	0.08 ± 0.02 (0.62 ± 0.16%)
NaC (5mM)	0.79 ± 0.13* (5.34 ± 0.88%)	0.22 ± 0.06* (1.07 ± 0.32%)	0.20 ± 0.06* (1.25 ± 0.42%)
NaTC (20mM)	1.51 ± 0.07* (9.71 ± 0.55%)	0.40 ± 0.03* (2.57 ± 0.21%)	0.37 ± 0.04* (2.44 ± 0.29%)

As previously observed for NaGC (10-20mM) the apparent permeability coefficients (Papp) for sCT and PEG 4000 were enhanced to a greater extent than mannitol in the presence of NaTC (20mM), NaC (5mM) and Deoxy (1mM) (Figure 8.8).

The increasing enhancement potential of the bile salt simple micellar systems on the Papp of mannitol, PEG 4000 and sCT was as follows: Control (sCT alone) = NaGC (10mM) = Deoxy (1mM) < NaC (5mM) < NaTC (20mM)< NaGC (15mM) < NaGC (20mM).

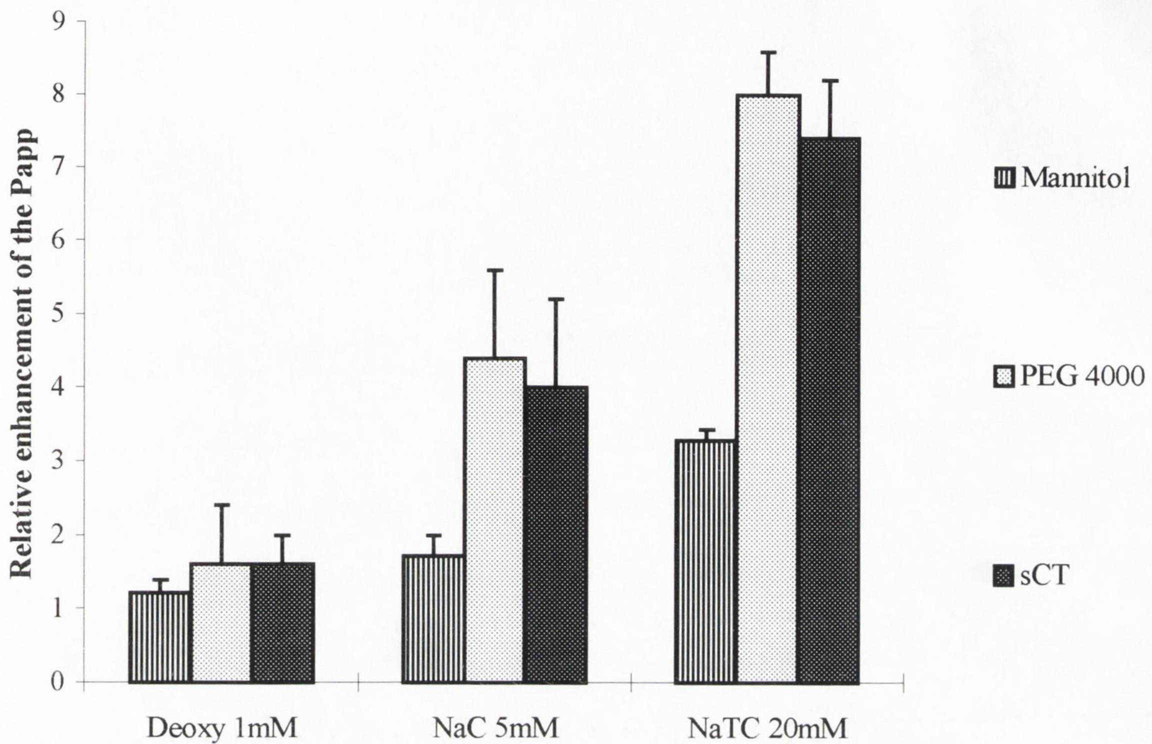


Figure 8.8 The relative enhancement of the apparent permeability (Papp) of the peptide salmon calcitonin (sCT), and the paracellular markers, mannitol and PEG 4000 in the presence of the simple micellar systems of sodium deoxycholate 1mM (Deoxy), sodium cholate 5mM (NaC) and sodium taurocholate 20mM (NaTC) as compared to control after 4h.

8.5 Influence of apical pH on the absorption enhancing action of NaGC

Previous results have been published regarding the effect of pH on the absorption enhancement potential of bile salts (Meaney, 1997). Meaney, (1997) showed that by decreasing the pH there is an increase in the absorption of paracellular markers, mannitol and PEG 4000 in the presence of micellar systems. Further to this it has been suggested that sCT stability was enhanced at low pH (O'Donnell et al., 1996; Sinko et al., 1999). The apical side of CaCo-2 cell monolayers were exposed to HBSS with 10mM Citric acid, pH 4.5 for 240 min and the transport of sCT, and the two paracellular markers,

mannitol and PEG 4000 was monitored in the absence and presence of NaGC micellar systems. The integrity of the CaCo-2 cell monolayer has been shown to be unaffected by shifting the apical pH from 7.4 to 4.5 (Takanaga et al., 1994; Meaney, 1997).

Salmon calcitonin (sCT)

Initially the effect of apical pH on sCT stability was investigated. The stability of sCT at pH 4.5 was not enhanced compared to pH 7.4 in the apical chamber in the presence of the CaCo-2 cell monolayer, with 8.5 ± 3.2% degraded at the beginning and 19.2 ± 6.0% degraded at the end of the transport experiment which is similar to sCT alone at pH 7.4 (table 8.2 and appendix 1). However, in the presence of NaGC 10mM a statistically significant decrease in sCT degradation was obvious at the end of the 4h transport experiment ($p < 0.05$). At the beginning of the experiment in the presence of NaGC (10mM) at pH 4.5, 4.8 ± 0.2% of sCT was degraded and only 12.1 ±1.2% degraded at the end compared to apical pH 7.4 of 4.03 ± 3.62% and 17.98 ± 1.60% degraded at the beginning and the end of the transport experiment respectively.

The CaCo-2 cell monolayers were exposed to HBSS pH 4.5 at the apical side and HBSS pH 7.4 at the basolateral side. The Papp for sCT at pH 7.4 in the apical chamber is 0.05 ± 0.01 X 10^{-6} cm/sec and at pH 4.5 the apparent permeability was 0.02 ± 0.01 X 10^{-6} cm/sec which was not statistically significantly different ($p > 0.05$). When the CaCo-2 monolayer was exposed to NaGC (10mM) at pH 4.5 in the apical chamber there was a statistically significant ($p < 0.05$) increase in the apparent permeability compared to NaGC 10mM at pH 7.4, see figure 8.9.

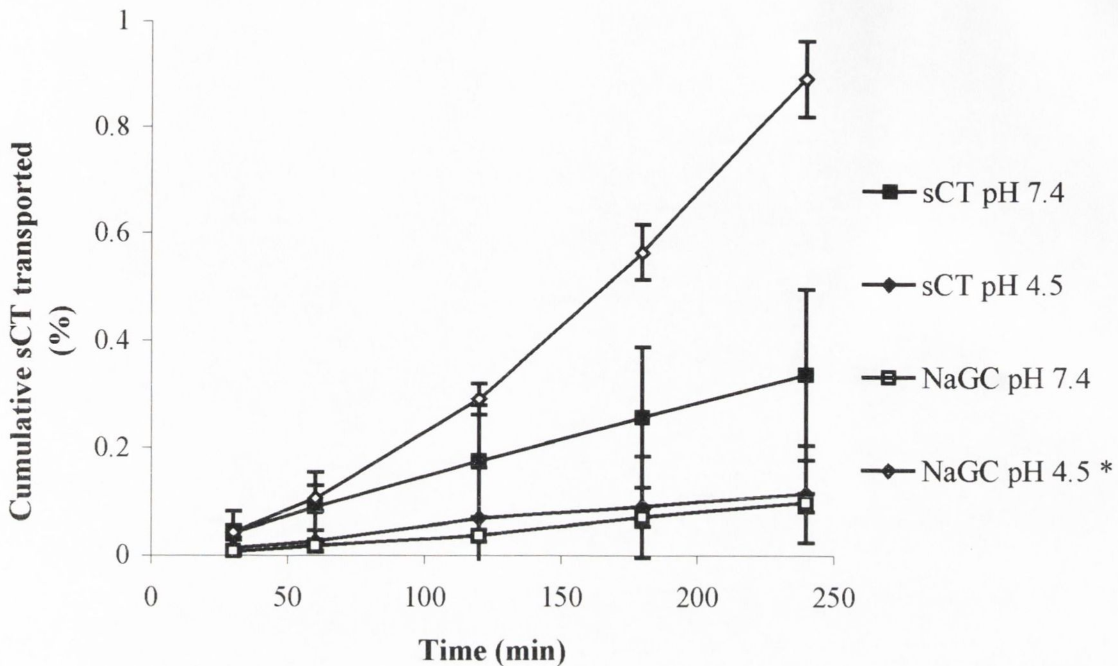


Figure 8.9 Effect of NaGC (10mM) simple micelle systems on the Papp of sCT across the CaCo-2 monolayers at apical pH 7.4 and 4.5. Values represent mean ± SE of at least n= 4 observations.

Mannitol and PEG 4000

The influence that apical pH had on the apparent permeability of the paracellular markers can be seen in table 8.5. The Papp of both mannitol and PEG 4000 were unaffected by the change in apical pH in the presence of sCT alone (control). This intimates that the monolayers integrity is unaffected at pH 4.5. However, on exposure to NaGC 10mM at pH 4.5 there is a significant increase, $p<0.05$, in the transport of mannitol and PEG 4000 compared to that observed at pH 7.4.

Table 8.5 The influence of apical pH on the Papp of mannitol and PEG 4000 across CaCo-2 monolayers in the presence of NaGC (10mM), $p < 0.05$, *.

System	Papp ± SE ($X10^6$) (cm/sec) ($n \geq 6$)			
	Mannitol		PEG 4000	
	pH 7.4	pH 4.5	pH 7.4	pH 4.5
SCT/Buffer	0.46 ± 0.02 (2.9 ± 0.17%)	0.59 ± 0.06 (4.2 ± 1.2%)	0.05 ± 0.01 (0.33 ± 0.05%)	0.02 ± 0.01 (0.11 ± 0.04%)
NaGC 10mM	0.57 ± 0.10 (3.46 ± 0.82%)	1.92 ± 0.14* (12.2 ± 2.2%)	0.05 ± 0.04 (0.33 ± 0.09%)	0.14 ± 0.01* (0.89 ± 0.22%)

8.6 The Effect of Bile Salt Simple Micellar Systems on CaCo-2 Cell Monolayer Integrity

In preceding sections simple micellar systems have enhanced the transport of the peptide, salmon calcitonin (sCT) and both paracellular markers mannitol and PEG 4000. It has been previously reported that this absorption enhancement may be associated with damage to the monolayer or alteration of the tight junction permeability (Anderburg et al., 1992). It is necessary to investigate the effects of bile salt on membrane integrity and to determine if these effects were transient and therefore reversible. The methods employed to determine the effect that these micellar systems had on the CaCo-2 cell monolayer were transepithelial electrical resistance (TEER) and transmission electron microscopy (TEM). In addition experiments were carried out to study the reversibility of the absorption enhancing effect following removal of the micellar system.

8.6.1 Transepithelial Electrical Resistance (TEER)

The TEER was monitored throughout the transport experiment as a measure of membrane integrity. The initial TEER value was determined following 30 min equilibration in HBSS prior to beginning of the transport experiments, at room temperature (25^{0}C). In all cases TEER values were expressed as a percentage of this initial value i.e. 100%.

8.6.1.1 Sodium glycocholate (NaGC)

There was a concentration dependent decrease in TEER values for sodium glycocholate (NaGC) as the concentration increased (Table 8.9). The changes in the TEER correlate with the increase in the transport of the hydrophilic compounds, sCT and the paracellular markers, mannitol and PEG 4000, see section 8.4.1. Following 1hr exposure of the CaCo-2 cell monolayer to NaGC 15mM the TEER was reduced to 79.74 ± 3.5 % of the original value. At the end of the transport experiment 4hr later the TEER value had dropped to 68.38 ± 2.62. However, on exposure of the monolayer to NaGC 20mM a greater decrease was seen between 1 hr and 4 hr compared to NaGC 15mM. In figure 8.10 one can see that a good correlation (R^2 = 0.99) exists between the apparent permeability of the paracellular markers mannitol and PEG 4000 and the decrease in the TEER values. In the case of sCT the correlation is not as good with the R^2 = 0.91, however this may be attributed to the more variable absorption of the peptide compared to the paracellular markers.

8.6.1.2 Other bile salt simple micellar system: NaTC, NaC and Deoxy

The reductions in TEER values for the other simple bile salt micellar systems of sodium taurocholate (NaTC) 20mM and sodium cholate (NaC) 5mM were similar to those previously been reported (Meaney 1997).

Table 8.6 The effect of simple micelles of NaGC, NaTC, NaC and Deoxy on TEER of CaCo-2 cell monolayers after 1hr and 4 hr exposure.

System (n)	% TEER ± SD 1 hr	4 hr
sCT (33)	94.56 ± 10.6	83.59 ± 15.62
NaGC 10mM (9)	89.07 ± 3.0	84.80 ± 2.40
NaGC 12.5mM (3)	82.34 ± 0.93	81.52 ± 7.25
NaGC 15mM (10)	79.74 ± 3.5	68.38 ± 2.62
NaGC 20mM (9)	59.53 ± 0.76	36.76 ± 1.12
NaTC 20mM (12)	50.37 ± 2.20	37.98 ± 1.92
NaC 5mM (6)	87.25 ± 7.27	48.63 ± 6.52
Deoxy 1mM (6)	96.88 ± 7.27	61.55 ± 2.33

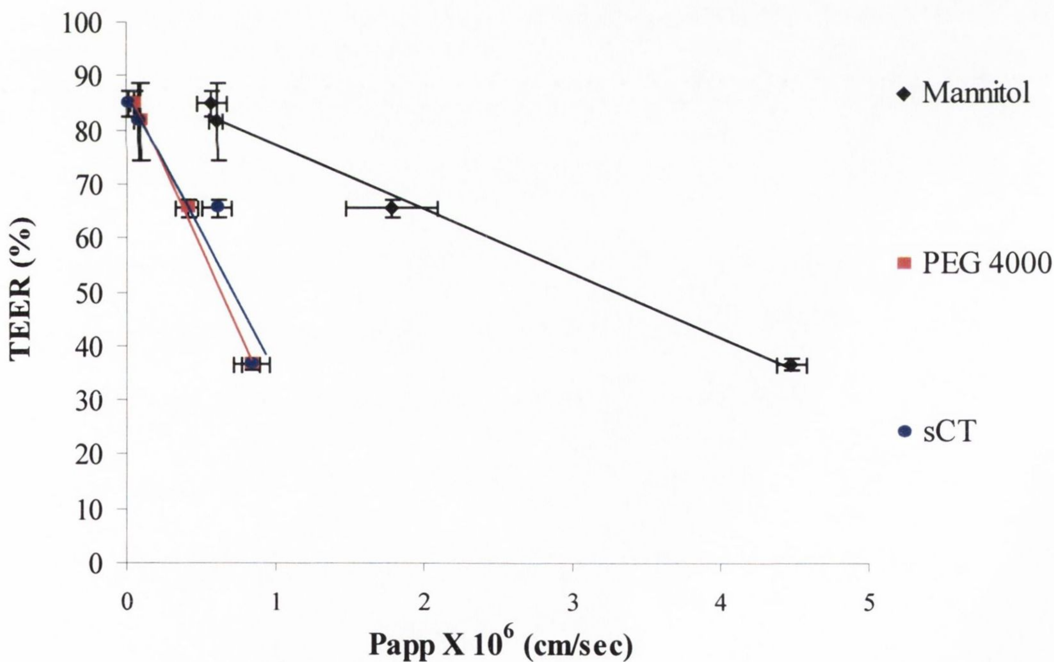


Figure 8.10 The correlation between the apparent permeabilities (Papp) of the hydrophilic compounds, salmon calcitonin (sCT) mannitol and PEG 4000 and the TEER value at 4hr in the presence of the simple micelles of NaGC (10- 20mM).

8.6.1.3 Consequences on TEER when pH 4.5 is in the apical chamber

Changing the apical pH from 7.4 to 4.5 did not affect the TEER values in the presence of sCT. This is consistent with no statistical difference in the apparent permeabilities of the hydrophilic compounds, sCT, mannitol and PEG 4000 when the apical pH was varied from 4.5 to 7.4 as previously reported (see section 8.5). In the presence of the simple micelle NaGC 10mM the reduction in TEER is significant at apical pH 4.5 compared to pH 7.4 at 1h and 4h intervals, see table 8.7. This decrease in the TEER value for the simple micelle is reflected in the increase in the transport of the hydrophilic compounds across the CaCo-2 monolayer, see section 8.5.

Table 8.6 The effect of apical pH on TEER of CaCo-2 monolayers after exposure to HBSS and NaGC 10mM for 1h and 4h.

System	% TEER ± SD (n ≥ 3)	
	1h	4h
sCT pH 7.4	94.56 ± 10.6	83.59 ± 15.62
sCT pH 4.5	96.25 ± 1.55	94.62 ± 1.56
NaGC 10mM pH 7.4	89.07 ± 3.0	84.37 ± 1.0
NaGC 10mM pH 4.5	63.46 ± 1.61	52.92 ± 1.40

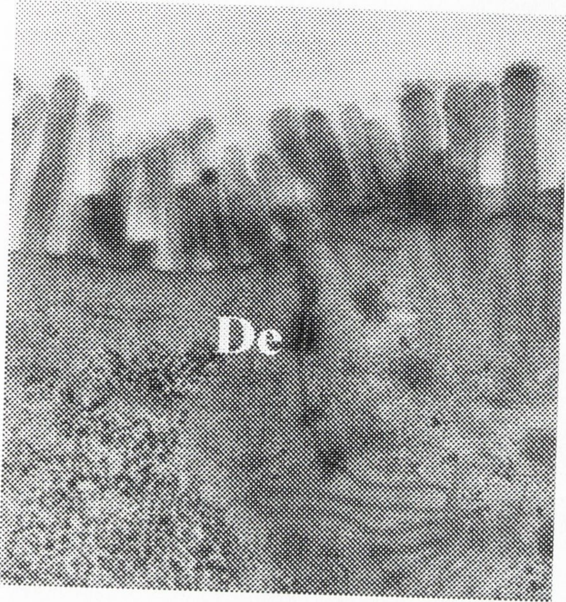
8.6.2 Transmission electron microscopy (TEM)

The TEER values indicate that there is some disruption to the integrity of the tight junctions. Transmission electron microscopy (TEM) was used to complement and extend the TEER studies. This technique enables one to view the effect that simple micelles have on the structure of the CaCo-2 cell membrane and tight junction.

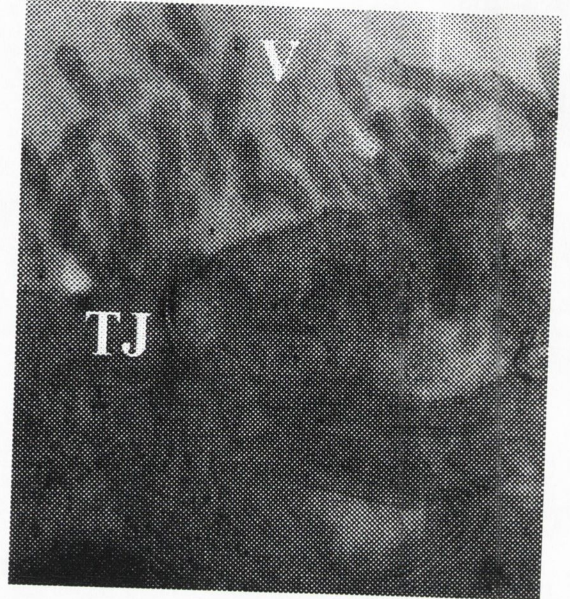
8.6.2.1 Influence of pH 7.4 in the presence of simple micelles on cell morphology

From figure 8.11 it is clear that sCT in buffer alone has no detrimental effect on the CaCo-2 cell membrane or tight junctions after 4h. The villi (V) are intact and the junction remains undisrupted. The desmosomes are clearly visible and are indicated by label (De). With sodium glycocholate (NaGC) there appears to be a concentration dependent effect on the integrity of the CaCo-2 cell monolayer after 4h exposure to the simple micelle. At NaGC 10mM there is little to no effect on the monolayer, however when increased to 15mM there is an increase in denudation of the villi and some dilation of the tight junction, a further increase to 20mM and it is obvious that there was greater dilation of the tight junctions and almost complete loss of the villi, see figure 8.11 (b, c, d).
In figure 8.11(e) with NaC 5mM, it was apparent that after 4h exposure that there was some dilation and denudation of the villi. Similarly, with NaTC 20mM there was also dilation and damage to the surface villi (Figure 8.11(f)). It appeared that Deoxy 1mM had little effect on the integrity on the monolayer with the tight junctions remaining intact and no damage to the villi, see figure 8.11(g). These results for simple micelles are supported by the previously reported data for the transport of sCT, mannitol and PEG 4000 and the TEER (Section 8.4 and 8.6.1).

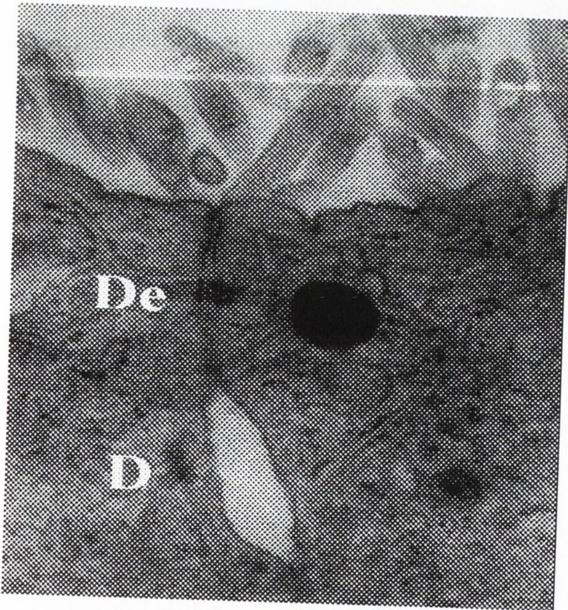
a)



b)



c)



d)

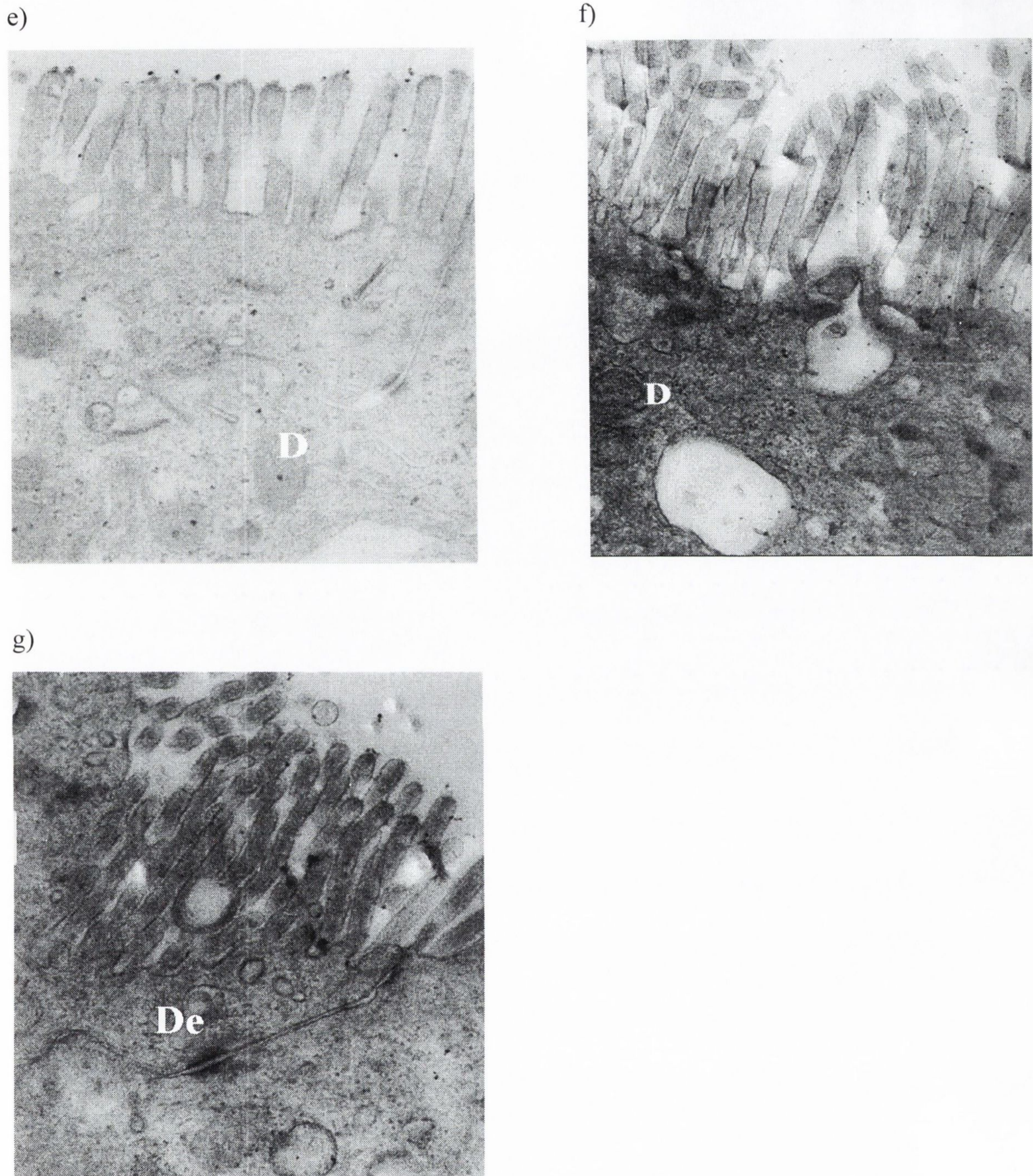


Figure 8.11 Transmission electron micrographs of CaCo-2 cell monolayers exposed to a) sCT, b) NaGC (10mM), c) NaGC (15mM), d) NaGC (20mM), e) NaC (5mM), f) NaTC (20mM), and g) Deoxy (1mM) for 4 h pH 7.4 in the apical chamber (X 40,000).

8.6.2.2 Influence of pH 4.5 in the apical chamber in the presence of NaGC simple micelles on cell morphology

Exposure of the CaCo-2 cell monolayer to pH 4.5 in the apical chamber did not appear to have a detrimental effect on the integrity of the cell monolayer, see figure 8.12 (a). This supports the preceding results, which showed no significant difference seen in the transport of the peptide sCT and both paracellular markers, mannitol and PEG 4000 when the apical pH was either 4.5 or 7.4. It is further supported by the similar TEER values obtained when the CaCo-2 cell monolayer was exposed to either pH. However after exposure to NaGC 10mM for 4hr at pH 4.5 there is a distinct change in the cell morphology with some denudation of the villi and dilation of tight junction, figure 8.12 (b).

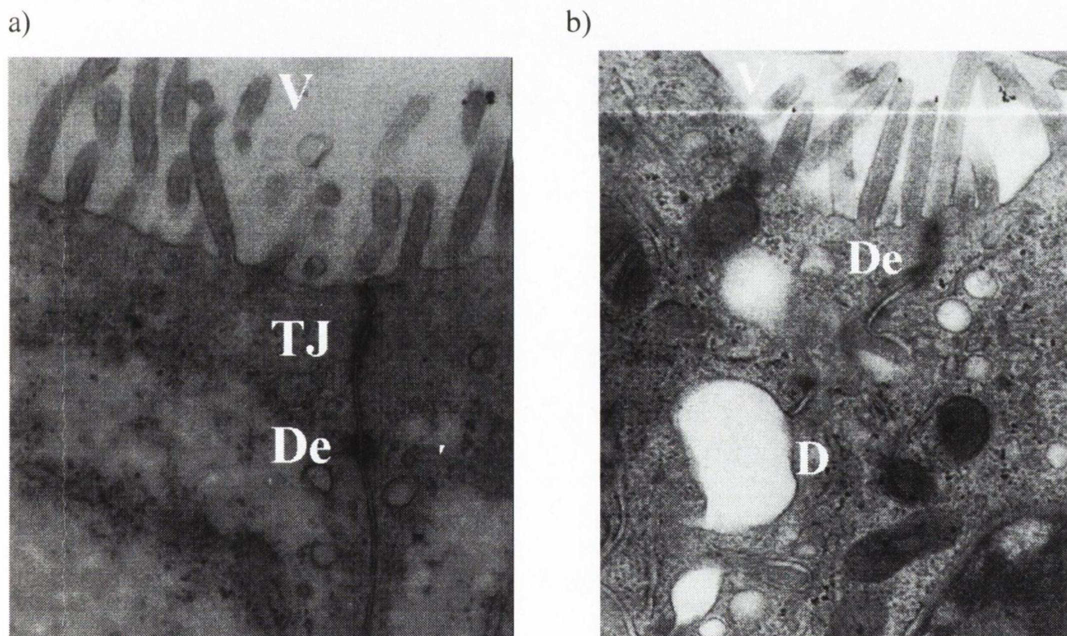


Figure 8.12 Transmission electron micrographs of CaCo-2 cell monolayers exposed to a) sCT and b) NaGC 10mM for 4 h pH 4.5 in the apical chamber (X 40,000).

8.6.3 Recovery experiments

It has been previously demonstrated that absorption enhancers may alter the ultra-structure of the cell. Therefore, it is important to consider the reversibility of these systems. The ability of the CaCo-2 cell monolayers to recover from simple micelle NaGC 15mM after 4h exposure was investigated. Recovery of the monolayer was assessed by monitoring the transport of the hydrophilic paracellular markers, mannitol and PEG 4000 and the TEER during exposure (4h) and following removal of the enhancers (up to 50h).

8.6.3.1 Transport of the hydrophilic markers

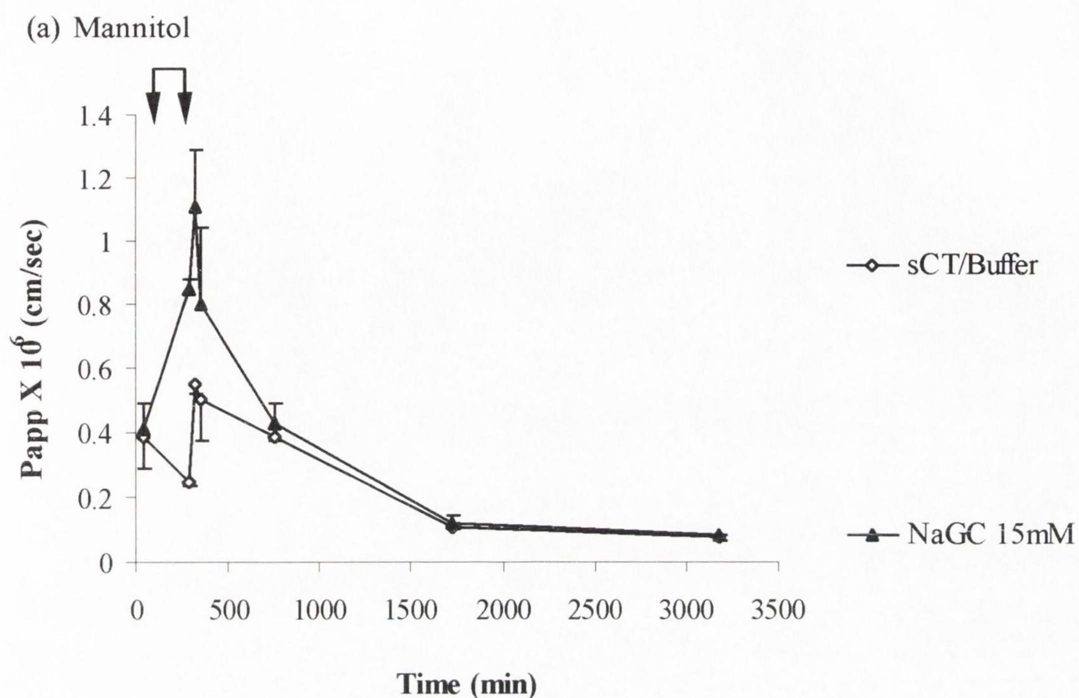
In the NaGC 15mM system there was an increase in the absorption of the paracellular marker compounds, mannitol and PEG 4000. The Papp for mannitol across the CaCo-2 cell monolayer exposed to sCT (control) over 4h was $0.25 \pm 0.02 \times 10^{-6}$ cm/sec this increased to a maximum of $1.11 \pm 0.18 \times 10^{-6}$ cm/sec in the presence of NaGC 15mM 30min post bile salt exposure. The PEG 4000 permeability also increased in the presence of NaGC 15mM from $0.05 \pm 0.01 \times 10^{-6}$ cm/sec to $0.16 \pm 0.04 \times 10^{-6}$ cm/sec, see figure 8.13 (a) and (b). This increase was maintained for 2h post exposure for both markers and returned to control levels within 24h. The absorption enhancement was quantified using the area under the curve (AUC) for each treatment with the trapezoidal rule as previously described by Anderberg et al 1993 (b), see Equation 8.1. This permits the comparison of the control to the treated sample.

AUC = Papp X t	**Eqn. 8.1**

Papp = apparent permeability coefficient (cm/sec)
t = time (s)

The AUC for mannitol after 4h exposure to NaGC 15mM was approximately 1.41 times greater than for the untreated monolayer. In the case of PEG 4000 the increase was greater resulting in a 2.6-fold difference between the treated and untreated monolayers.

(a) Mannitol



(b) PEG 4000

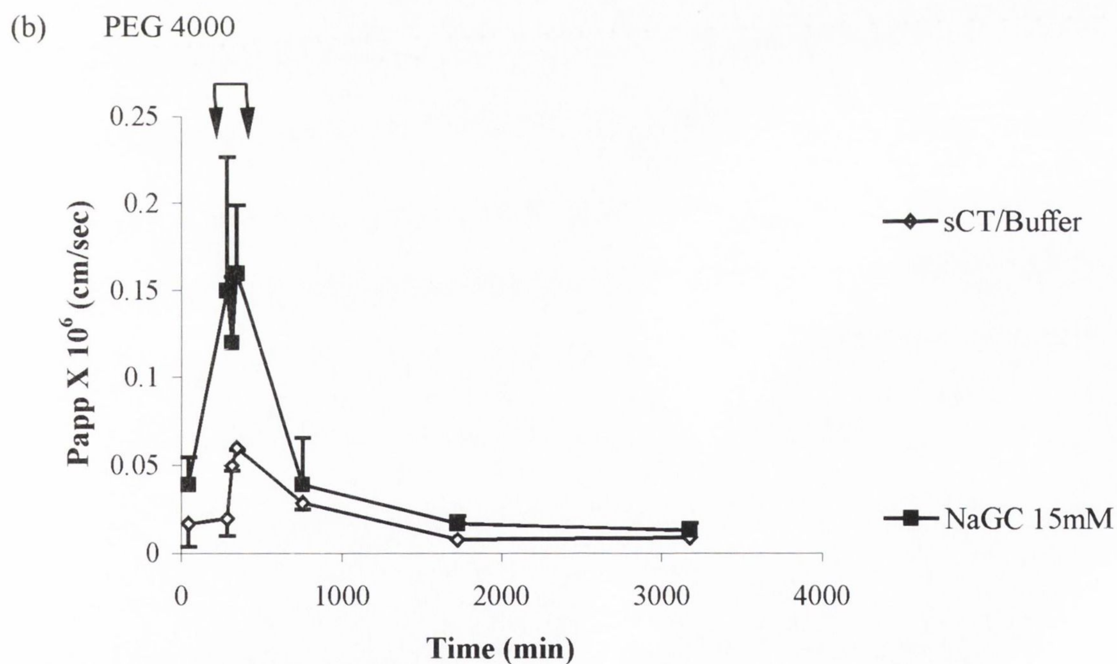


Figure 8.13 The effect of 4h exposure to simple micelle NaGC 15mm on (a) mannitol and (b) PEG 4000 transport. The arrows indicate the time that the monolayer was exposed to the micelle. Values are expressed as mean ± SD (n =3).

8.6.3.2 Transepithelial electrical resistance

The TEER was monitored throughout the recovery experiment. In figure 8.14 the effect that the simple micelle of NaGC 15mM was apparent. With NaGC 15mM the TEER drops to 64% and returns to control values within 24h post exposure to this system. These results are consistent with the previous marker data.

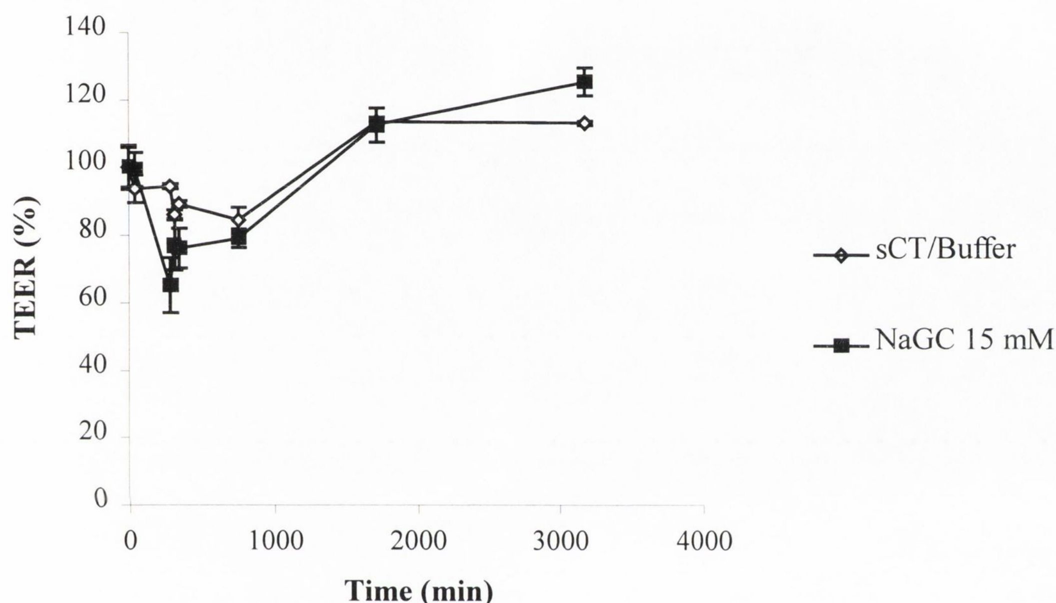


Figure 8.14 The effect of 4h exposure to the simple (NaGC 15mM) on the TEER pre-, during and post-exposure. Values are expressed as mean ± SD (n= 3- 4).

8.7 Confocal laser scanning microscopy

To examine the route of transport of sCT and the enhancing mechanism of NaGC (15mM) across the CaCo-2 monolayer confocal scanning laser microscopy (CFSLM) was used. This technique allows for the qualitative visualisation of sCT crossing the membrane.

sCT was detected using an anti-sCT FITC label. The results from this are shown in fig 8.15. The red labelling is the cell wall and the yellow/green is sCT, which is directly under the red fluorescence. A measure of the intensity of fluorescence across three adjacent cells indicates that the FITC peaks of sCT correspond with the Texas Red ® located specifically at the cell wall. This implies that sCT a large protein was transported across the CaCo-2 cell monolayer mainly via the paracellular route.

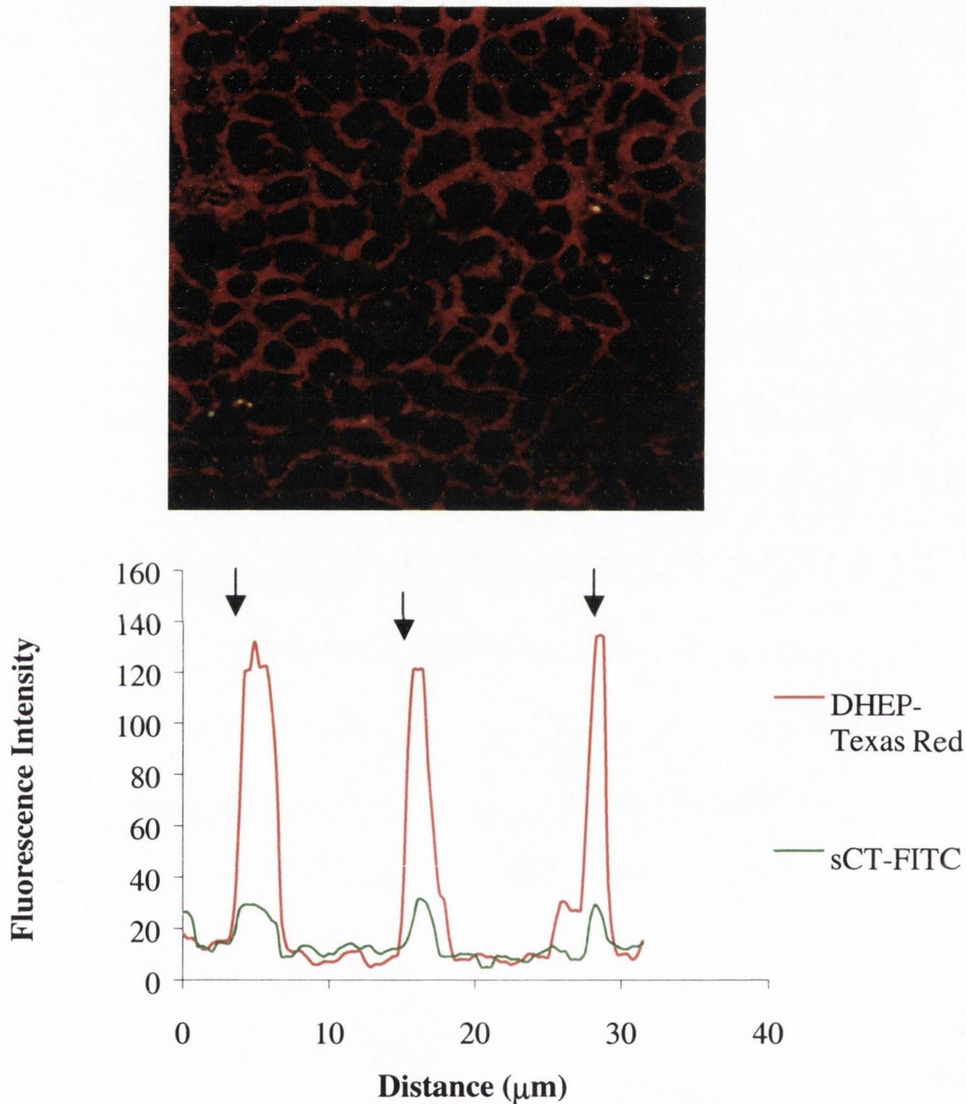
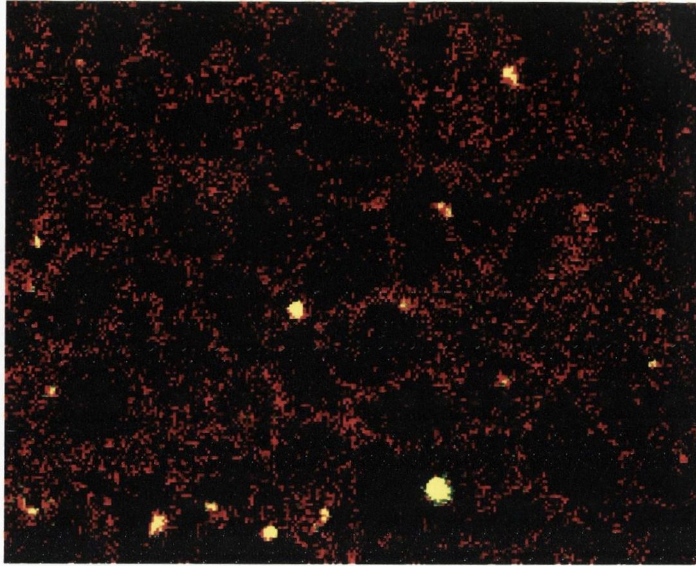


Figure 8.15 Confocal scanning image of the X-Y cross-section and fluorescence intensity of CaCo-2 cell monolayer after the transport experiment with sCT only. Arrows indicate cell walls. This scan was taken at an approximate depth of 5μm with the laser intensity set at 30%.

(a)



(b)

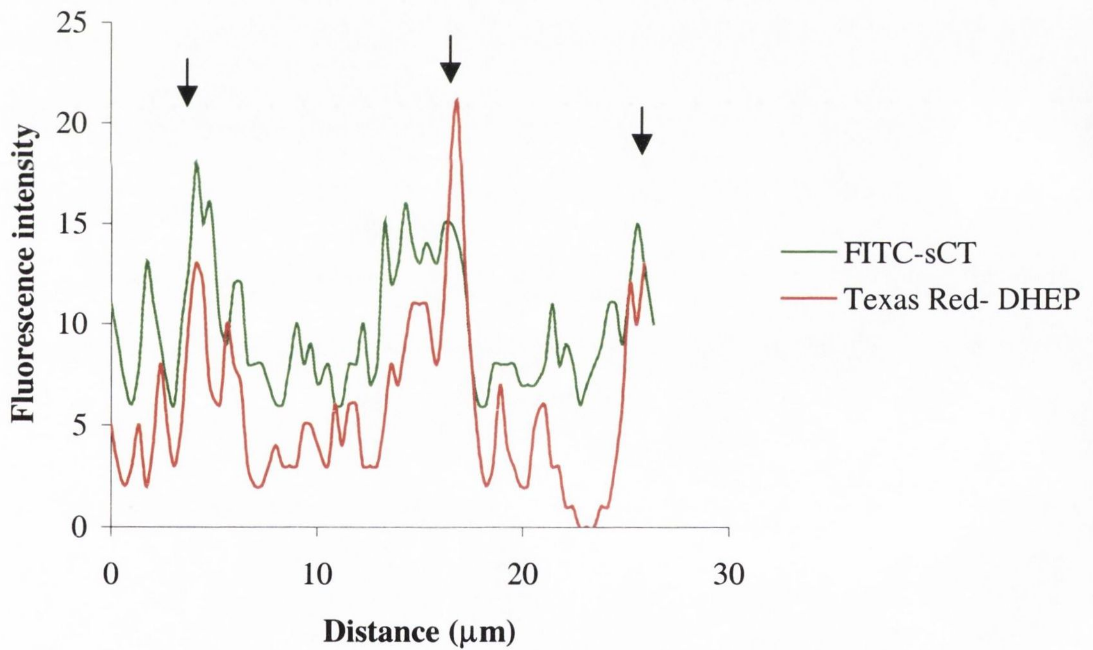


Figure 8.16 (a) Confocal scanning image of the X-Y cross-section and (b) fluorescence intensity of CaCo-2 cell monolayer after the transport experiment with NaGC (15mM). Intensity 10%

It also appears from CFSLM that the predominant pathway of absorption of sCT in the presence of NaGC (15mM), was the paracellular route as the greatest fluorescence was seen at the cell walls (Figure 8.16 (a) and (b)). Previously it had been shown in our laboratory that bile salt simple micelles enhanced the paracellular route by affecting the tight junctional complex in the rat intestinal model (Lane et al., 1996) and in the CaCo-2 cell model (Meaney, 1997). There is a positive charge associated with sCT, (pI $\approx$ 9), at pH 7.4 and this also may affect it route of transport. As the paracellular route is know to have a negative charge it is a possible that sCT is attracted to traverse the membrane via this route.

8.8 Discussion

The use of bile salts as absorption enhancers has limitations particularly associated with their toxicity (Latta et al., 1993). In this present study, the aim was to assess the effect of simple micelles on the stability and transport of sCT.

Initially prior to transport and stability experiments the toxicity of the simple micellar system was determined using MTT assay. This provided information on a suitable working concentration. Werner et al., (1996) have shown that as the concentration of bile salt, NaTC, increased that there was a concentration dependent decrease in the mitochondrial dehydrogenase activity which was associated with the bile salts cytotoxic effects. From previous work in our laboratory the IC_{50} for sodium taurocholate (NaTC) (10mM) and sodium cholate (NaC) (6mM) had been established (Meaney (1997)). The IC_{50} for sodium glycocholate (NaGC) was18.5mM and sodium deoxycholate (Deoxy) was 1mM which are in agreement with Jørgensen et al., (1992) and Carey, (1985), respectively. The validity of this test as a suitable indicator for cell toxicity has been amply demonstrated (Sakai et al., 1998 and Anderburg et al., 1992). It is interesting to note that the IC_{50} values decrease with increasing hydrophobicity of the bile salt; taurine conjugates $\leq$ glycine conjugates < free tri-hydroxy bile salts < dihydroxy bile salts. That is to say the IC_{50} for NaTC is 10mM, NaGC is 18.2mM, NaC 5mM and Deoxy 1mM. Although the IC_{50} for NaTC is 10mM concentrations of 20mM have shown a reversible

absorption enhancement effect in the CaCo-2 cell model (Meaney (1997) and Werner et al., (1996)).

Having determined suitable working concentrations for the simple micelles the stability of the therapeutic peptide salmon calcitonin (sCT) and the apparent permeabilities of sCT and both paracellular markers, mannitol and PEG 4000 across the CaCo-2 cell monolayer were determined. The stability of sCT was established at the beginning and the end of the 4h experiment. It has previously been shown that NaGC had enzyme inhibitory effects in rat nasal homogenates containing insulin (Hirai et al., 1981(a) and (b)) and therefore had a dual role as an absorption promoter and a stabilizing entity. In the CaCo-2 cell monolayer apical chamber the concentration range of NaGC 10-20mM caused no further degradation of sCT as compared to sCT alone. However, in the presence of NaTC 20mM the stability of sCT was severely compromised with almost 80% of the peptide degraded at the onset of the experiment. With NaC 5mM peptide instability was seen after 4h exposure with 60% of the peptide degraded (appendix 1).

As the stability was being monitored the transport of sCT and the paracellular markers, mannitol and PEG 4000 was also assessed. It is clear from the results that there is a concentration dependant increase in the transport of sCT, mannitol and PEG 4000 across the CaCo-2 cell monolayer in the presence of increasing concentrations of NaGC micellar systems (Jørgensen et al., 1993). The rank order of enhancement of the peptide sCT and both paracellular markers was NaGC 20mM> NaGC 15mM> NaTC 20mM> NaC 5mM> Deoxy 1mM= NaGC 10mM=sCT. The relative enhancement in Papp observed following exposure to the simple micellar systems was in general PEG 4000 =sCT> mannitol.

In varying the pH of the apical chamber from 7.4 to 4.5 there was no evidence to suggest that the monolayers integrity had been adversely affected, as the transport of sCT and both paracellular markers were not significantly different, this was further supported by the TEER and TEM data. In this study the transport of the markers, mannitol and PEG 4000 and the peptide sCT were significantly increased in the presence of NaGC 10mM at pH 4.5 relative to 7.4. It has been reported that at low pH there was dissociation of the

micelle thereby increasing the bile salt free monomer (Shiau and Levine, 1980). These alterations in the bile salt physical-chemical properties may be factors to consider in understanding the effect of pH. Meaney (1997) reported that decreasing the pH to 4.5 in the presence of NaTC 20mM was related to an increase in toxicity as indicated by TEER and TEM.

The use of bile salts as absorption enhancers has limitations primarily as they are know to cause some toxic effects. By monitoring the TEER, TEM and CFSLM which provided more information on the mechanism of action of these simple micellar systems.

There was a concentration dependant decrease in TEER for increasing concentrations of NaGC. It would appear that the unconjugated tri-hydroxy bile salt NaC caused a greater reduction in TEER than the corresponding conjugated glycine derivative (Martin et al., 1992). The TEM data complements the TEER values, as it was apparent that there was dilation of the tight junctions in the presence of these micellar systems. It has been demonstrated that the bile salt NaTC up to concentrations of 20mM caused reversible changes to the tight junctions of CaCo-2 cell monolayers (Werner et al. 1996). In this study results show that the effects of NaTC on the monolayer integrity were minor.

The relationship between TEER and the Papp was demonstrated in this study. The exposure of the monolayer to increasing concentrations of simple and mixed micelles had effects on the permeability of the paracellular marker compounds, mannitol and PEG 4000 and sCT and a concomitant effect on the TEER. There appears to be a good linear correlation between the decrease in TEER and the increase in the Papp of the hydrophilic compounds, mannitol and PEG 4000 and sCT in the presence of simple micelles of NaGC. Thwaites et al., (1993) also noted that for mannitol and thyrotropin-releasing hormone (TRH) their permeabilities were linearly correlated to the electrical conductance of the CaCo-2 cell monolayer above 1mS/cm. The relationship was complex and was dependant on a number of factors such as the solute characteristics, molecular size and charge.

The relationship between the decrease in TEER and the increase in the Papp in the presence of simple micelles indicates an effect on the tight junctional complex. This alteration in the tight junctions was further supported by the TEM and the CFSLM results. The CFSLM suggested that sCT alone and in the presence of NaGC (15mM) was transported via the paracellular route. From this preliminary screening of these simple micelles an appropriate candidate was chosen as suitable for the studies on the effects of mixed micelles on the CaCo-2 cell monolayer

CHAPTER 9

The effects of sodium glycocholate (NaGC) mixed micelles on the apparent permeabilities of the hydrophilic compounds, mannitol, PEG 4000 and sCT and on the integrity of the CaCo-2 cell monolayer

9.1 Introduction

Previously in Chapter 8, the effects that simple micelles had on the CaCo-2 cell monolayer were investigated. Following the initial studies with the simple micelles of sodium cholate (NaC), sodium taurocholate (NaTC), sodium deoxycholate (Deoxy) and sodium glycocholate (NaGC), it was clear that the most appropriate simple micelle for future studies was NaGC. Sodium glycocholate (NaGC) was chosen on the basis that it promoted the greatest enhancement in the transport of hydrophilic compounds without affecting the stability of the peptide sCT. NaGC was then combined with various concentrations of linoleic acid (LA), a long-chain unsaturated fatty acid, to determine if it would further promote the absorption of the two paracellular markers, mannitol and PEG 4000 and the peptide salmon calcitonin (sCT). O' Reilly (1991) previously investigated the absorption enhancing potential of a number of bile salt: fatty acid mixed micellar systems in the *in-situ* rat gut model and reported maximum absorption was obtained using the long-chain unsaturated fatty acid, linoleic acid (LA).

In this study the CaCo-2 cell culture model was used to assess and compare the permeability enhancing and stabilizing potential of mixed micelles of NaGC: linoleic acid (LA). This was determined by observing the effects that these systems had on the apparent permeability (Papp) and stability of salmon calcitonin (sCT), and the Papp of the paracellular markers, mannitol and PEG 4000.

9.2 Examining the toxicity of the mixed micelles

Similarly to simple micelles the cytotoxicity of the NaGC: LA mixed micelles was examined initially using the MTT assay. This assay provided information regarding the concentration of bile salt: fatty acid mixed micelle that causes a 50% decrease in enzyme activity (IC_{50}) and therefore indicated a suitable working concentration range.

Intracellular dehydrogenase activity was determined by the MTT method as previously described in Chapter7 and Chapter 8, section 8.2.1.

Fixed concentrations of NaGC, 10mM, 12.5mM and 15mM were combined with increasing concentrations of LA, 0.25-2mM. The IC_{50} value for different NaGC: LA was then calculated. A concentration dependant decrease in enzyme activity was apparent as the fatty acid loading increased in the bile salt: fatty acid micelles. For a set concentration of bile salt the fatty acid loading in the mixed micellar system that caused a 50% decrease in enzyme activity (IC_{50}) was calculated from the slope of the linear portion of the profile. In figure 9.1 the IC_{50} for the NaGC: LA mixed micelle systems are shown. In the case of 10mM NaGC the IC_{50} was approximately 1.06mM LA, for 12.5mM NaGC it was approximately 0.6mM LA and for 15mM NaGC it was approximately 0.7 mM.

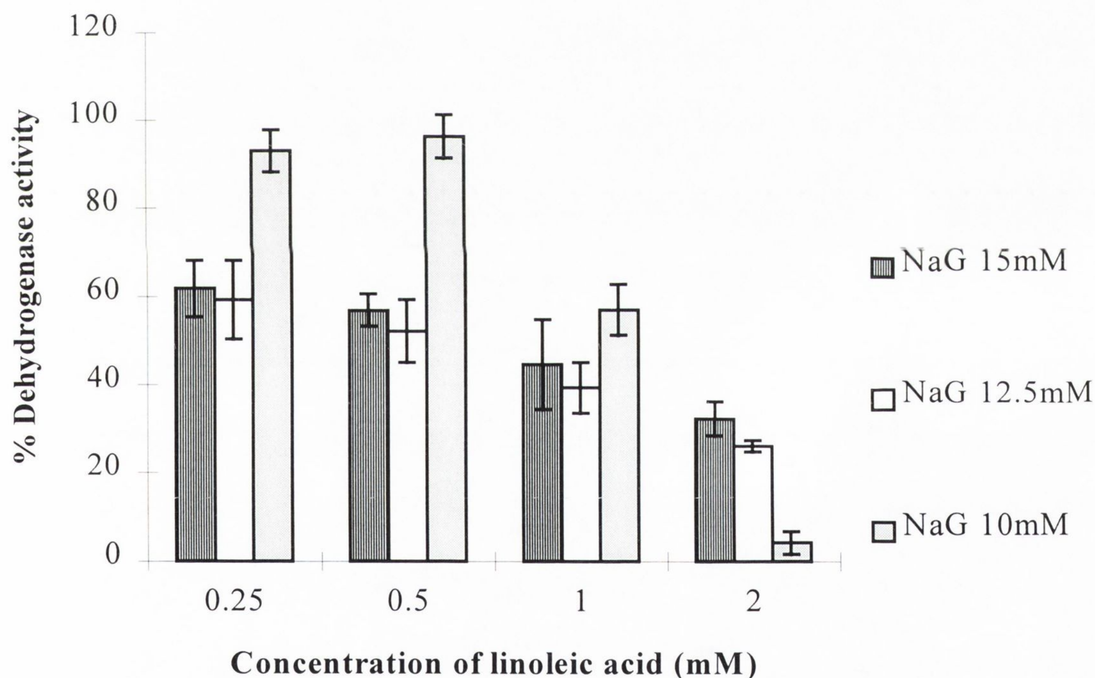


Fig. 9.1 The effect of sodium glycocholate (NaGC) and linoleic acid (LA) mixed micelle systems on the intracellular dehydrogenase activity. Values are expressed as mean ± SE ($n \geq 6$).

9.3 Stability of sCT in the presence of mixed micelles

The stability of sCT in the apical chamber in the presence of CaCo-2 cell monolayer was investigated in the presence of various NaGC: LA loading. The IC_{50} value was used as a guide to the suitable proportion of bile salt: fatty acid mixed micelle systems examined. At the onset of the experiment there appears to be a trend for mixed micelles particularly for NaGC 15mM, that as the fatty acid concentration increased the stability of the peptide, was enhanced. This was similar to the trends that have previously been reported for *in-vitro* rat intestinal homogenates (O'Donnell et al 1996). After 4h the mixed micelles do not accelerate the degradation of the peptide relative to the control (Appendix 1).

Table 9.1: The percentage of degraded salmon calcitonin (sCT) at the beginning of a 4 hour transport experiment for each of the mixed micelle systems of NaGC: LA.

System NaGC: LA (mM)	**% sCT Degraded ± SD of the Initial Starting Conc. at onset of experiment (n ≥ 3)**
0: 0	7.92± 4.09
10: 0	4.03 ± 3.62
10: 1	2.49 ± 0.22
10: 1.25	3.45 ± 0.43
12.5: 0	3.76 ± 0.64
12.5: 1	2.89 ± 0.25
15: 0	3.68 ± 1.11
15: 0.5	2.89 ± 0.62
15: 0.75	2.73 ± 0.73
15: 0.85	5.69 ± 2.72
15: 0.9	3.62 ± 0.30
15: 1	2.89 ± 1.59

9.4 Transport of hydrophilic compounds in the presence of mixed micelles

Following these initial studies, the apparent permeabilities (P_{app}) of the hydrophilic compounds, mannitol (MW 182), sCT (MW 3413) and PEG 4000 across the CaCo-2 cell monolayer were studied in the presence of mixed micellar systems.

9.4.1 Salmon calcitonin (sCT)

The apparent permeability of the peptide salmon calcitonin (sCT) was investigated in the presence of the following proportion of bile salt: fatty acid mixed micelles, bile salt NaGC, 10mM: LA 1mM and 1.25mM; NaGC 12.5mM: LA 1mM and NaGC 15mM: LA 0.5mM, 0.75mM, 0.85mM, 0.9mM and 1mM.

In figure 9.2 the various loadings of LA with NaGC 10mM can be seen, as the loading increases from 0 to 1mM to 1.25 mM there is an increase in the transport of sCT across the CaCo-2 cell monolayer. The Papp from NaGC 10mM is $0.02 \pm 0.01 \times 10^{-6}$ cm/sec, when a mixed micelle is formed with LA 1mM there is a 9-fold increase in the transport of sCT, with the Papp being $0.18 \pm 0.03 \times 10^{-6}$ cm/sec. When the concentration of LA is increased to 1.25mM there is almost a 68-fold enhancement compared to NaGC 10mM alone. This indicates that the formation of mixed micelles of LA with NaGC 10mM causes a statistically significant increase in the transport of sCT, ($p < 0.05$).

When the concentration of bile salt NaGC is increased to 12.5mM, the Papp for NaGC 12.5mM: LA 1mM is $1.74 \pm 0.34 \times 10^{-6}$ cm/sec. This is 9.7 times greater than the mixed micelle of NaGC 10mM: LA 1mM, thus indicating that as the bile salt concentration was increased the potency of the mixed micelle was also increased.

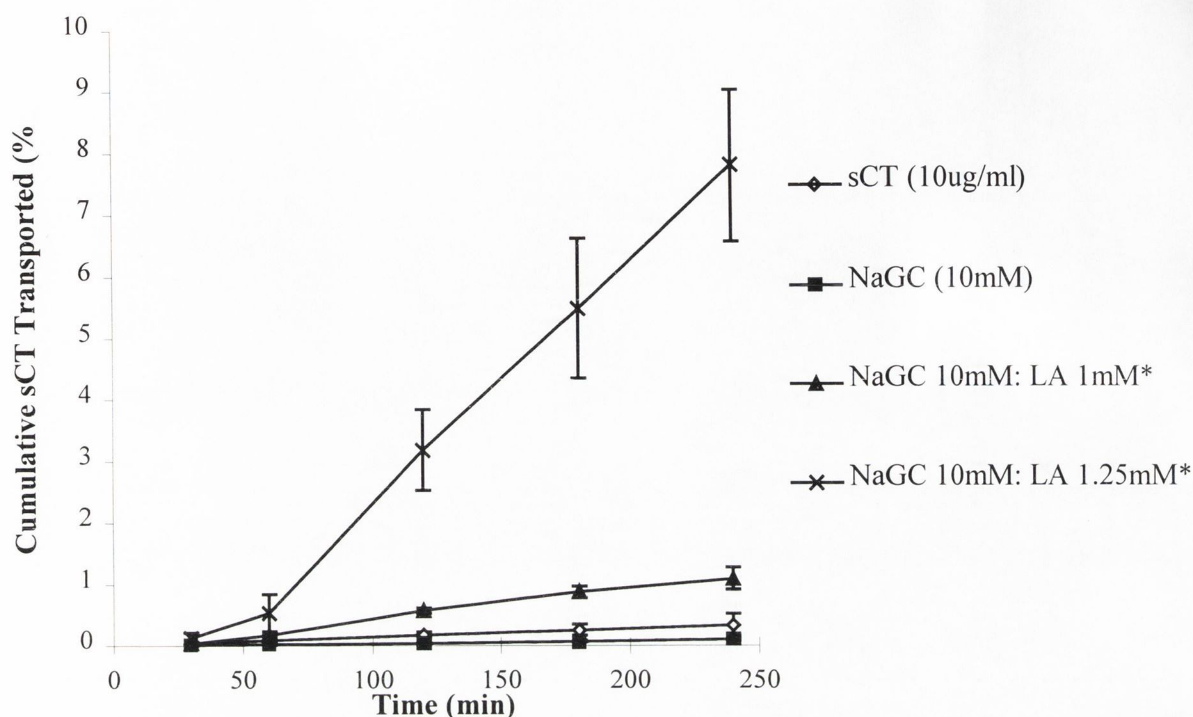


Figure 9.2 The transport profile of sCT across the CaCo-2 cell monolayer in the presence of simple and mixed micelles of NaGC (10mM) and LA. (* $p < 0.05$).

Mixed micelles containing 15mM NaGC with varying concentrations of LA were also investigated. The trend was as expected as the concentration of fatty acid loading increased the amount of sCT transported also increased. From figure 9.3 there appears to be an optimal fatty acid loading between 0.75 and 0.85mM LA in that the Papp for NaGC 15mM: LA 0.75mM is $0.30 \pm 0.07 \times 10^{-6}$ cm/sec and for the latter mixed micelle the value is almost 16-fold higher at $4.77 \pm 0.40 \times 10^{-6}$ cm/sec. There is no statistical difference between mixed micelles that are prepared with concentrations of LA greater than 0.85mM, $p > 0.05$. Similarly no statistical difference exists between concentrations of LA less than 0.75mM and NaGC 15mM alone, $p > 0.05$.

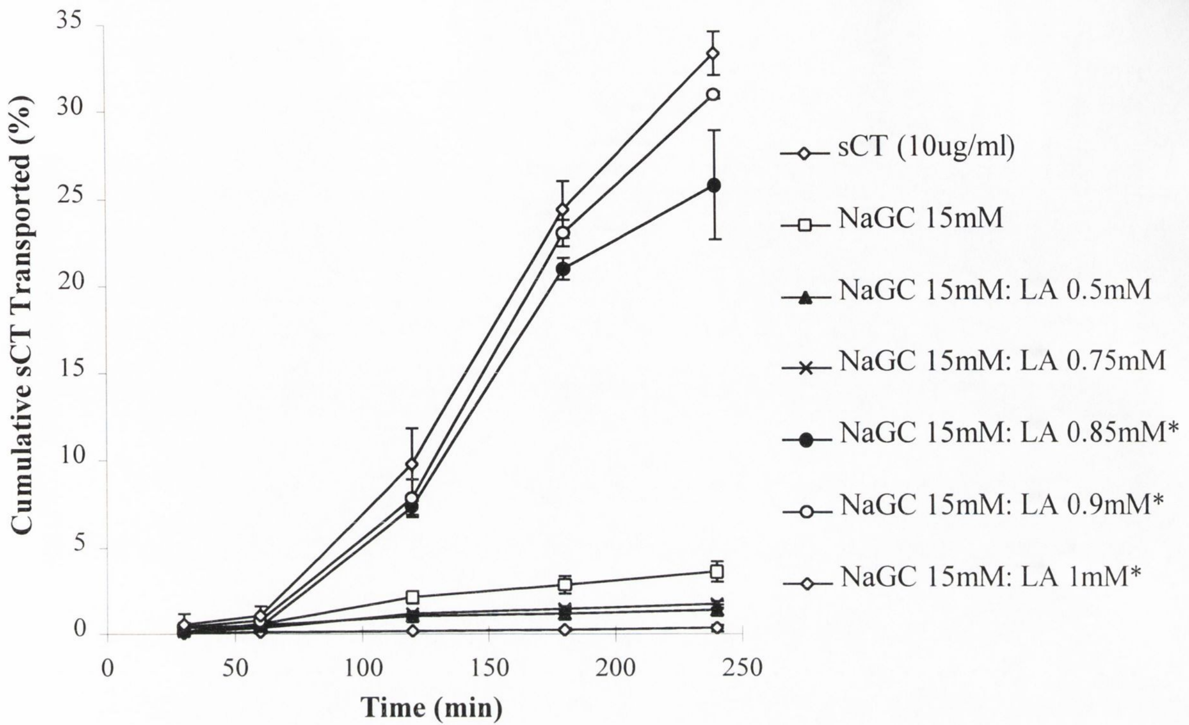


Figure 9.3 The transport profile of sCT across the CaCo-2 cell monolayer in the presence of simple and mixed micelles of NaGC (15mM) and LA. (* $p < 0.05$).

Results indicate that formation of mixed micelles can increase the transport of sCT relative to the corresponding simple micelle and that fatty acid loading is critical to optimising this effect. The effect of increasing the concentration of bile salt with a fixed fatty acid loading of 1mM is shown in Figure 9.4.

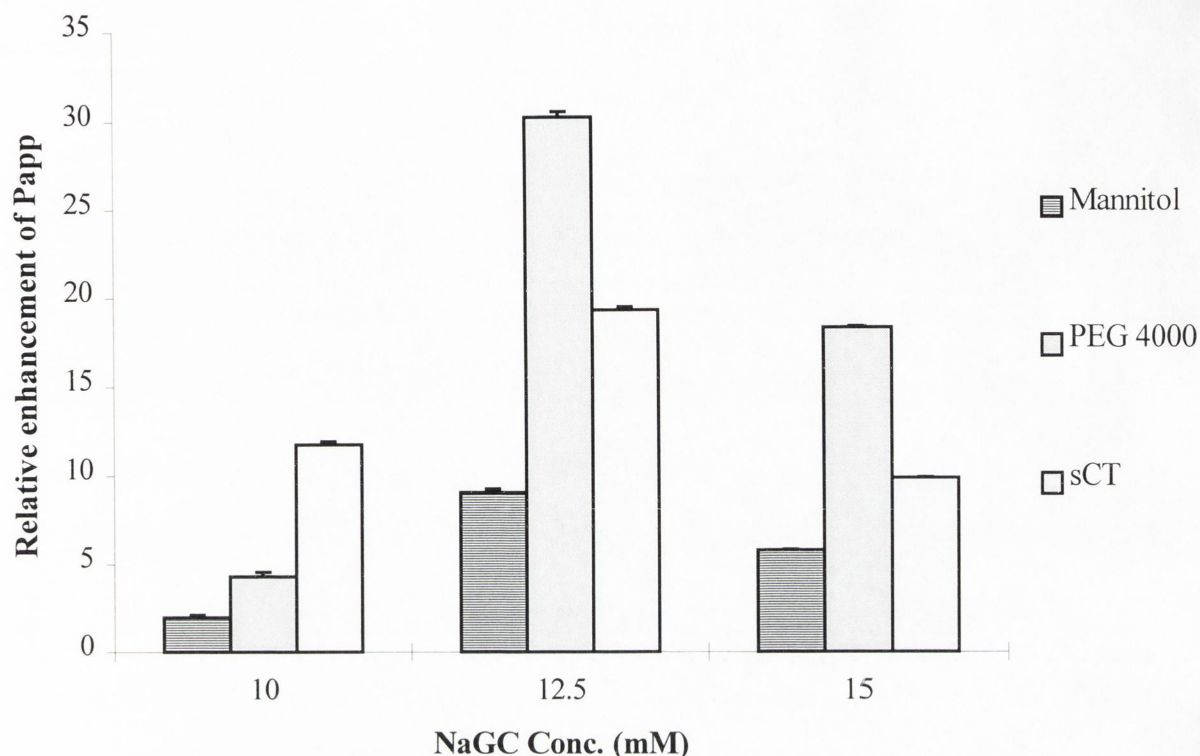


Figure 9.4 Relative absorption enhancement of the Papp $_{(30\text{-}240min)}$ to the corresponding simple micelle systems for ^{3}H Mannitol, ^{14}C PEG 4000 and ^{125}I salmon calcitonin (sCT) across the CaCo-2 cell monolayers by NaGC: LA (1mM) mixed micellar systems. Values represent mean ± SD of at least n= 3 observations.

9.4.2 Paracellular markers: Mannitol and PEG 4000

The effect that the mixed micelle systems of NaGC: LA systems have on the apparent permeabilities of the paracellular markers were similar to those seen for sCT. In the presence of the mixed micelles of NaGC 10mM, 12.5mM and 15mM with 1mM LA, the apparent permeability of mannitol increased 2-fold, 9-fold and 6-fold respectively, see figure 9.5, table 9.2. As the concentration of LA increased above LA (0.75mM) in the presence of NaGC 15mM the apparent permeabilities of the peptide, sCT and both paracellular markers, mannitol and PEG 4000 significantly increased, see table 9.2. The

permeabilities of PEG 4000 and sCT were enhanced to a greater extent than that of mannitol in all the NaGC: LA mixed micelles studied, see figure 9.4 and 9.5.

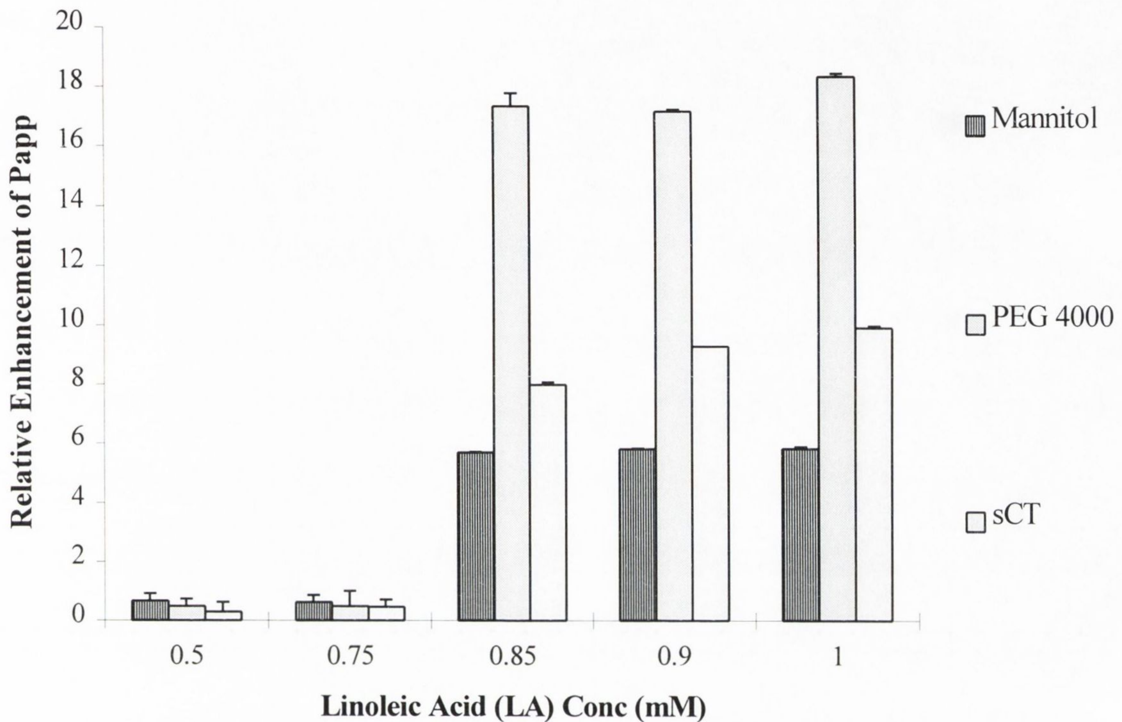


Figure 9.5 Effect of NaGC 15mM: LA mixed micelle systems on the apparent permeability relative to the simple micelle NaGC 15mM on the peptide sCT and the two paracellular markers, mannitol and PEG 4000.

Table 9.2 The apparent permeability (Papp) and the percentage transported across the CaCo-2 cell monolayer over 4 hours of the hydrophilic compounds, mannitol, PEG 4000 and sCT for each of the mixed micellar systems for sodium glycocholate (NaGC). ($p < 0.05$ statistically significant relative to the corresponding simple micellar system).

System	**Papp ± SD (X10^6) (cm/sec) (n ≥ 3) (p<0.05, *) (Total Percentage transported after 4hr)**		
NaGC: LA (mM)	**Mannitol**	**PEG 4000**	**sCT**
0:0	0.46 ± 0.02 (2.9 ± 0.17%)	0.05 ± 0.01 (0.34 ± 0.04%)	0.05 ± 0.01 (0.33 ± 0.05%)
10: 1	1.12 ± 0.18* (7.16 ± 0.91%)	0.21 ± 0.06* (1.22 ± 0.32%)	0.18 ± 0.03* (1.09 ± 0.18%)
10: 1.25	6.87 ± 0.29* (39.89 ± 1.26%)	4.17 ± 0.57* (24.16 ± 2.25%)	1.34 ± 0.21* (7.78 ± 1.22%)
12.5: 1	5.46 ± 0.87* (31.88 ± 4.9%)	2.79 ± 0.86* (16.3 ± 4.96%)	1.74 ± 0.34* (10.14 ± 1.95%)
15: 0.5	1.20 ± 0.31 (7.88 ± 1.80%)	0.19 ± 0.05 (1.28 ± 0.25%)	0.21 ± 0.05 (1.71 ± 0.66%)
15: 0.75	1.12 ± 0.11 (7.28 ± 1.43%)	0.19 ± 0.04 (1.20 ± 0.57%)	0.28 ± 0.07 (1.73 ± 0.47%)
15: 0.85	10.1 ± 0.21* (56.74 ± 1.43%)	6.97 ± 0.20* (38.71 ± 1.04%)	4.77 ± 0.40* (25.73 ± 3.09%)
15: 0.95	10.3 ± 0.27* (58.16 ± 1.66%)	6.9 ± 0.34* (38.37 ± 1.68%)	5.54 ± 0.01* (30.83 ± 0.21%)
15: 1	10.3 ± 0.58* (58.05 ± 3.27%)	7.37 ± 0.67* (41.74 ± 3.81%)	5.9 ± 0.29* (33.24 ± 1.27%)

9.5 The effect of extracellular calcium on the absorption enhancing action of NaGC simple and mixed micelles

Yamashita et al (1990) have indicated that bile salts may alter the structure of the cell tight junction by binding to Ca^{2+}, therefore it was necessary to look at the effect that apical calcium has on the absorption potential of NaGC simple and mixed micellar systems. Conflicting results have been published regarding the role Ca^{2+} plays in relation to the mechanism of absorption enhancement of bile salts (Swenson et al 1992).

9.5.1 Salmon calcitonin (sCT)

The effect of apical calcium was investigated for sCT. The CaCo-2 cell monolayers were exposed to calcium free HBSS at the apical side and calcium containing HBSS at the basolateral side. Removal of calcium from the apical chamber did not affect the transport of sCT alone across the monolayer. The Papp for sCT in calcium containing HBSS is $0.05 \pm 0.01 \times 10^{-6}$ cm/sec and on the removal of calcium from the apical medium the permeability was $0.02 \pm 0.01 \times 10^{-6}$ cm/sec which was not statistically different ($p > 0.05$). When the CaCo-2 monolayer was exposed to simple and mixed micelles of NaGC in the absence of calcium in the apical chamber there was a statistically significant ($p < 0.05$) decrease in the apparent permeability of sCT in the presence of NaGC 15mM and NaGC 15mM: LA 1mM, see table 9.3. The decrease was more pronounced for the mixed micelle system of NaGC 15mM: LA 1 mM.

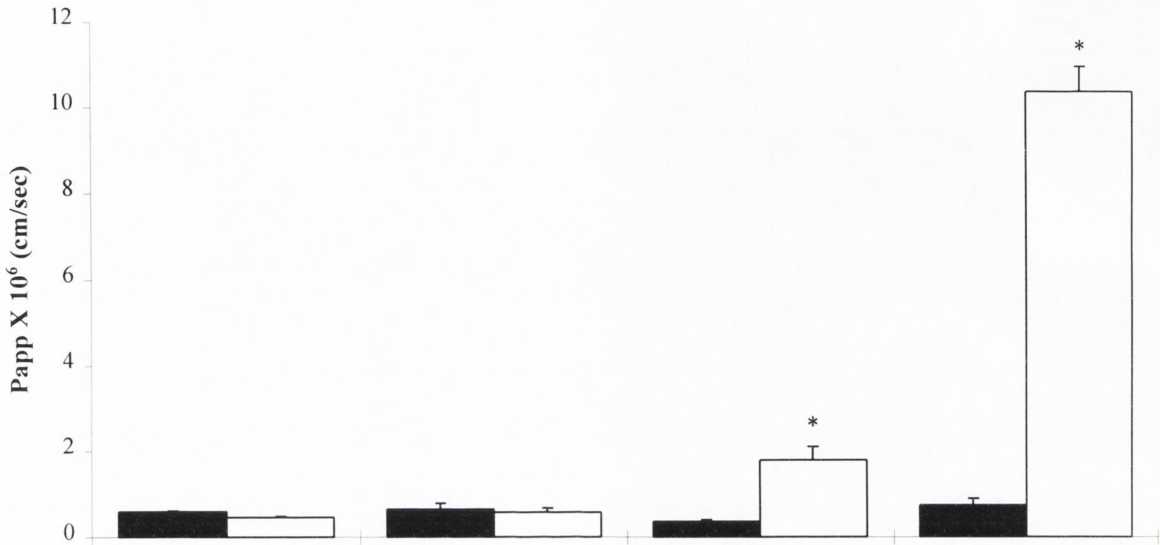
Table 9.3 Influence of apical Ca^{2+} on the Papp of sCT across CaCo-2 monolayers in the presence of simple and mixed micelle systems of NaGC and LA. p< 0.05,* when Papp is compared to HBSS with and without Ca^{2+}.

System	Papp ± SE (X10^{6}) (cm/sec) (n ≥ 3)	
	HBSS	**HBSS - Ca^{2+} Free**
sCT/Buffer	0.05 ± 0.01 (0.33 ± 0.05%)	0.02 ± 0.01 (0.11 ± 0.05%)
NaGC 10mM	0.02 ± 0.01 (0.10 ± 0.06%)	0.03 ± 0.02 (0.18 ± 0.13%)
NaGC 15mM	0.60 ± 0.10 (3.60 ± 0.59%)	0.07 ± 0.03* (0.47 ± 0.18%)
NaGC 15mM:LA 1mM	5.9 ± 0.29 (33.24 ± 1.27%)	0.22 ± 0.05* (1.24 ± 0.26%)

9.5.2 Mannitol and PEG 4000

The influence of the absence of apical Ca^{2+} had on the apparent permeability of the paracellular markers, mannitol and PEG 4000 can be seen from figure 9.6 (a) and (b) respectively. When the concentration of NaGC increases to 15mM there is a statistically significant decrease in the Papp of both compounds in the absence of Ca^{2+}. This is similar for the mixed micelle formulation of NaGC: LA (15:1 mM) but the decrease in Papp is much greater.

(a) Mannitol



(b) PEG 4000

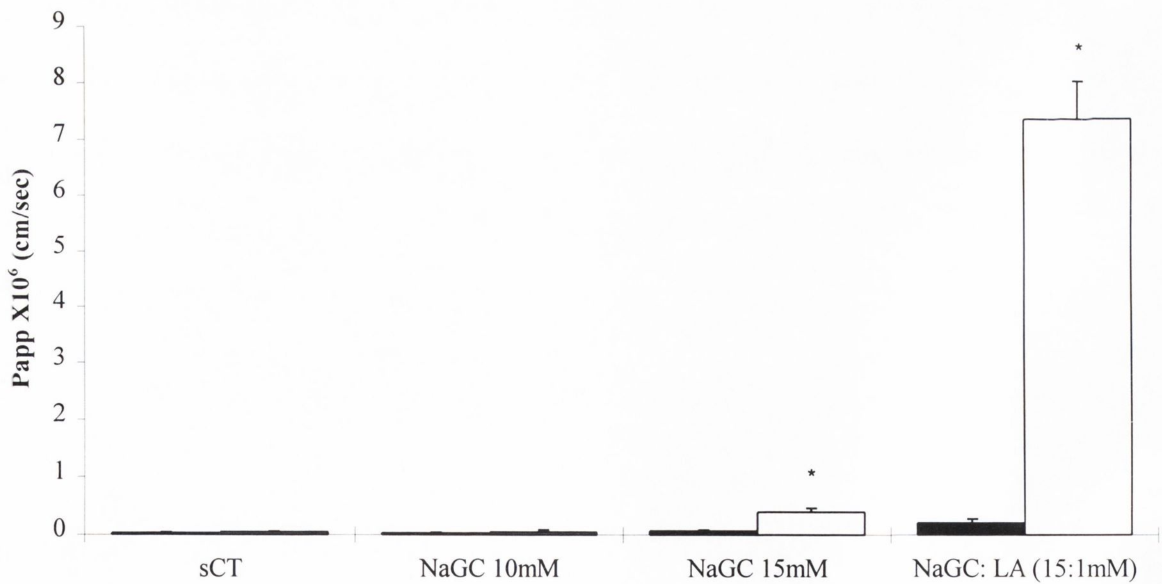


Figure 9.6 Effect of NaGC simple and mixed micelle systems on the Papp of (a) Mannitol and (b) PEG 4000 across the CaCo-2 monolayers in the presence (□) and absence (+) of apical Ca^{2+}. Values represent mean ± SE of at least n= 4 observations. ($p< 0.05$, *)

9.6 The Effect of Bile Salt Micellar Systems on CaCo-2 Cell Monolayer Integrity.

It appears from the data above that the mixed micellar systems have enhanced the transport of the peptide, salmon calcitonin (sCT) and both paracellular markers mannitol and PEG 4000. As this enhancement may be associated with damage it was necessary to investigate the effects that the mixed micelle systems had on membrane integrity and to determine if theses effect were transient and therefore reversible (Anderberg et al., 1993). The methods employed to determine the effect that these micellar systems had on the CaCo-2 cell monolayer were transepithelial electrical resistance (TEER) and transmission electron microscopy (TEM). In addition experiments were carried out to study the reversibility of the absorption enhancing effect following removal of the micellar system.

9.6.1 Transepithelial Electrical Resistance (TEER)

The TEER was monitored throughout the transport experiment as a measure of membrane integrity. The initial TEER value was determined following 30 min equilibration in HBSS prior to beginning of the transport experiments, at room temperature (25^0C). In all cases TEER values were expressed as a percentage of this initial value i.e. 100%.

9.6.1.1 Effect that mixed micelles have on TEER

The changes in TEER values in the presence of mixed micelle systems of NaGC can be seen in Table 9.4 and Figure 9.7. Generally as the fatty acid loading increased there was a further reduction in the TEER value. These results correlate well with to the observations made on the transport of the hydrophilic compounds across the CaCo-2 cell monolayer. The correlation between the Papp and the reduction in TEER for the paracellular markers mannitol and PEG 4000 were 0.95 and 0.93 and for the peptide sCT the correlation was 0.89.

Table 9.4 The effect of mixed micelles of NaGC: LA on the TEER of CaCo-2 cell monolayers after 1hr and 4 hr exposure. $p < 0.05$, *, significant when compared to the corresponding simple micellar system.

System (n)	**% TEER ± SD**	
NaGC: LA	**1 hr**	**4 hr**
0:0 (33)	94.56 ± 10.6	83.59 ± 15.62
10: 1 (3)	64.69 ± 3.0	58.26 ± 0.99*
10: 1.25 (3)	64.68 ± 0.23	44.39 ± 0.31
12.5: 1 (3)	60.92 ± 0.90	49.12 ± 0.41*
15: 0.5 (6)	78.74 ± 1.91	75.47 ± 3.84
15: 0.75 (6)	76.84 ± 1.05	76.67 ± 1.85
15: 0.85 (3)	24.30 ± 1.44	14.68 ± 2.36*
15: 0.95 (3)	23.45 ± 4.77	13.99 ± 3.66*
15: 1 (7)	32.94 ± 1.87	26.42 ± 1.40*

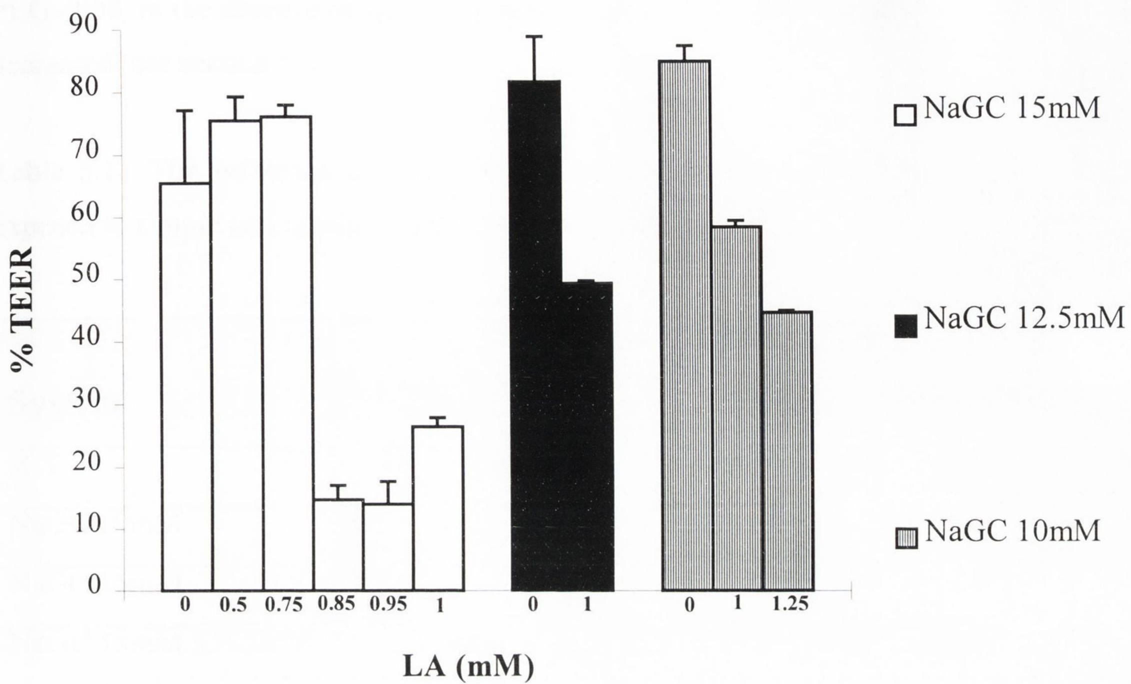


Figure 9.7 Comparison of the effect of simple and mixed micelles systems of sodium glycocholate (NaGC) on TEER of CaCo-2 monolayers after 4 hr exposure.

9.6.1.2 Effect of extracellular calcium on TEER in the presence of NaGC micellar systems

The removal of Ca^{2+} from the apical chamber appeared to have no adverse effect on the integrity of the tight junctions, see table 9.5. There was no difference in TEER values after 4 hr exposure to Ca^{2+} free HBSS and HBSS containing Ca^{2+}. This result corresponds to the transport studies, which demonstrated that there was no difference in the apparent permeability of the peptide sCT and both paracellular markers mannitol and PEG 4000 were unaffected by the absence of Ca^{2+} in the apical chamber (see section 9.5). In the presence of the simple micelle systems of NaGC the TEER value was unaffected for 10mM but for 15mM there was a significant increase in the absence of Ca^{2+}. In the case of the mixed micelle NaGC: LA (15: 1mM) the absence of calcium also increased the TEER value. These observation concur with the transport data for sCT, mannitol and

PEG 4000, in the absence of apical Ca^{2+} the apparent permeabilities for these compounds decreased, see section 5.4.

Table 5.11 The influence of apical Ca^{2+} on the TEER of CaCo-2 monolayers when exposed to simple and mixed micelles of NaGC and LA for 4h.

System	% TEER ± SD (n ≥ 3) (4h)	
	HBSS	**HBSS - Ca^{2+} Free**
sCT	83.59 ± 15.62	95.59 ± 2.46
NaGC 10mM	84.80 ± 2.40	84.37 ± 1.0
NaGC 15mM	65.18 ± 1.62	88.04 ± 0.5*
NaGC 15mM:LA 1mM	26.42 ± 1.40	63.86 ± 0.67*

9.6.2 Transmission electron microscopy

The TEER values indicate that there is some disruption to the integrity of the cell monolayer. Transmission electron microscopy (TEM) was used to complement and extend the TEER studies. This technique enables one to view the effect that the mixed micelles have on the structure of the CaCo-2 cell membrane and tight junction.

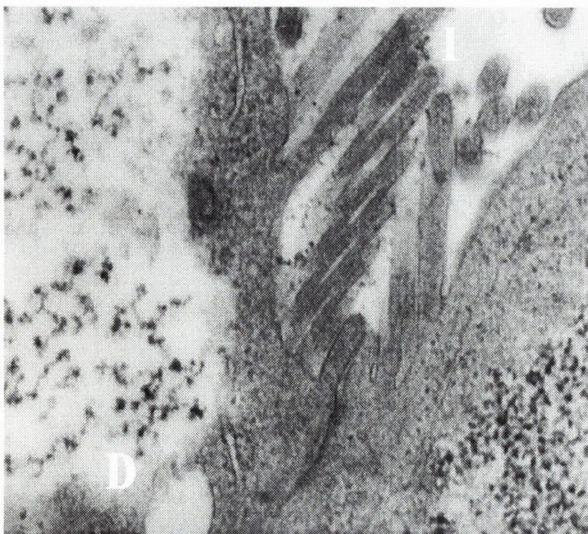
9.9.2.1 Effect mixed micelles (NaGC: LA) have on the CaCo-2 cell monolayer morphology

After treatment of the CaCo-2 cell monolayer with the mixed micelles of NaGC: LA (15:1mM) for 4h the TEM indicated a detrimental effect on the cell integrity, see figure 9.8 (a). It is apparent for the evidence that there is dilation of the tight junction to the point where the cells are almost completely separate entities, and severe denudation of the villi. It would appear that the enhancement associated with this system could be attributed to damage. However when the systems containing less NaGC are investigated the effect on the CaCo-2 cell monolayer is less pronounced. In figure 9.8 (b) the system

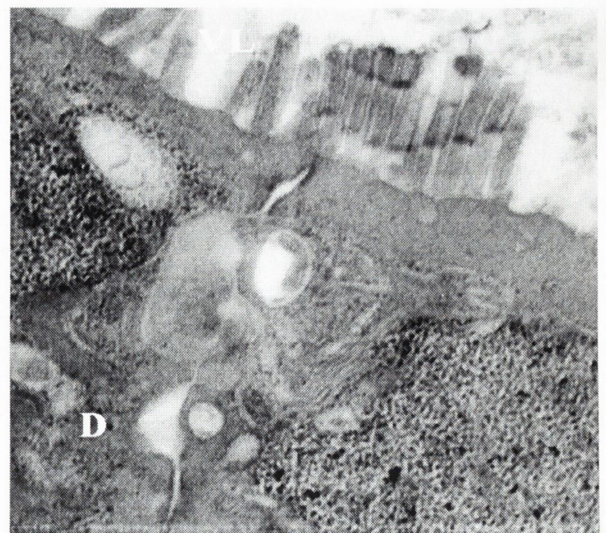
investigated is NaGC: LA (12.5:1 mM) and there is some dilation of the tight junction and villi loss but not as extreme as previously seen for NaGC: LA (15: 1 mM). When the concentration of bile salt is further reduced to 10mM in the presence of LA 1mM the effect on the monolayer is not as apparent as for the other systems of NaGC 15mM and 12.5mM see figure 9.8 (c). Therefore it appears that there is a concentration dependent effect and this is also reflected in the TEER values and the transport data for these systems.

For mixed micelle systems containing NaGC 15mM with increasing concentrations of LA there are differences in the cell morphology. Figure 9.8 (e) shows the effect that NaGC: LA (15:0.75 mM) has on the monolayer morphology demonstrating some minor dilation of the tight junctions (D) and slight sloughing of the villi (V). However, increasing the fatty acid loading to 0.85mM there were changes in the cell morphology, see Figure 9.8 (d). From figure 9.8 there appears to be an optimum fatty acid loading between 0.75 and 0.85mM LA as the cell morphology had greater dilation of the tight junction and denudation of the villi, compared the former fatty acid loading. Similar trends are observed for the transport of the hydrophilic compounds, sCT, mannitol and PEG 4000 and the TEER data (Section 9.4 and 9.6.1.1).

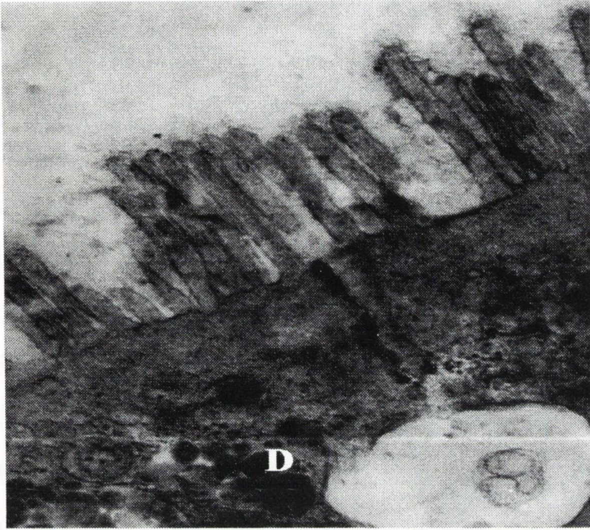
a)



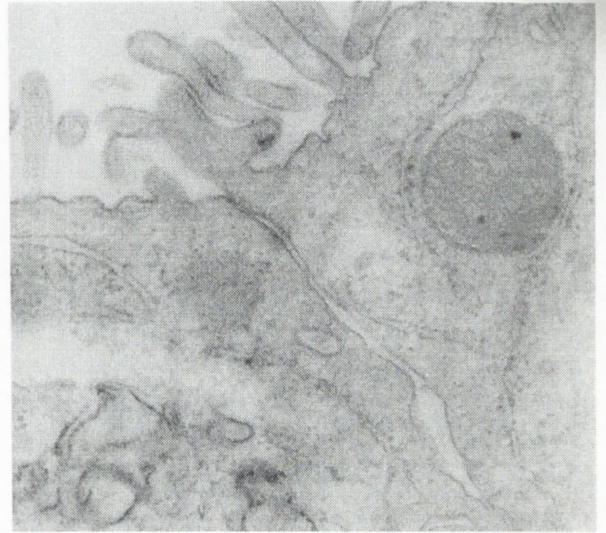
b)



c)



d)



e)

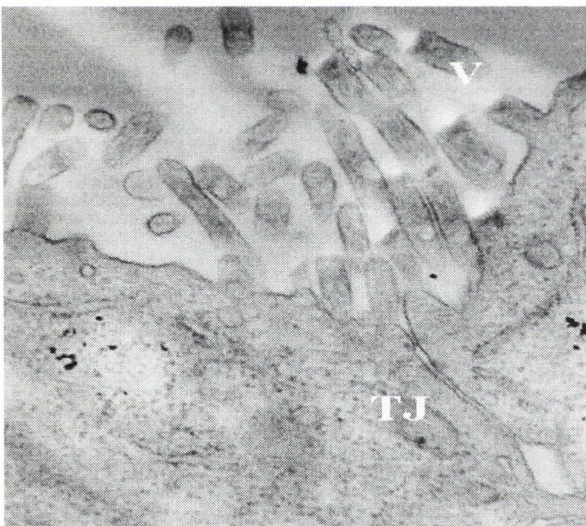


Figure 9.8 Transmission electron micrographs of CaCo-2 cell monolayers exposed to

a) NaGC: LA (15: 1mM) (X60,000)
b) NaGC: LA (12.5: 1mM)
c) NaGC: LA (10:1mM)
d) NaGC: LA (15: 0.75mM)
e) NaGC: LA (15: 0.85mM)

for 4 h in the apical chamber (all other were X 40,000).

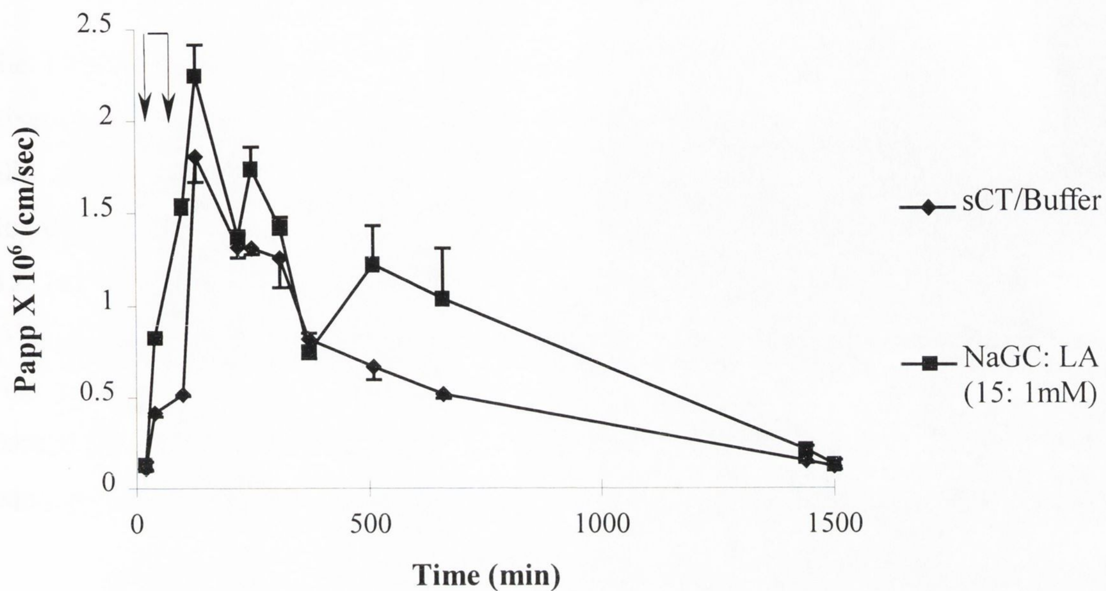
9.6.3 Recovery experiments

It has been previously demonstrated that absorption enhancers may alter the ultra-structure of the cell. Therefore, it is important that the reversibility of these systems be investigated. NaGC: LA (15: 1mM) was chosen as the systems to be investigated for their reversibility because it appeared to be the most suitable mixed micelle system in terms of absorption enhancement. The ability of the CaCo-2 cell monolayers to recover from the mixed micelle NaGC: LA (15: 1 mM) following exposure for 1h and 4h was investigated. Recovery of the monolayer was assessed by monitoring the transport of the hydrophilic paracellular markers, mannitol and PEG 4000 and the TEER during exposure (1 or 4h) and following removal of the enhancers (up to 50h).

9.6.3.1 Transport of the hydrophilic markers

When the mixed micelle NaGC :LA (15:1 mM) was only in contact with the monolayer for 1h the recovery was rapid, recovery was seen after 3h post-exposure, see figures 9.10 (a) and (b). For treated monolayers the AUC for mannitol and PEG 4000 were 1.4-fold and 1.2 fold greater than the control monolayer, calculated using equation 8.1. However, following exposure of the CaCo-2 cell monolayer to the mixed micelle NaGC: LA (15: 1mM) for 4h it appears from the paracellular marker data that a recovery is not seen. The Papp for both mannitol and PEG 4000 increase dramatically compared to the control. For mannitol at 30min post exposure the Papp was $18.71 \pm 0.59 \times 10^{-6}$ cm/sec and PEG 4000 the Papp was $4.32 \pm 0.42 \times 10^{-6}$ cm/sec. In this experiment the Papp continued to increase rapidly and the concentration of paracellular marker crossing the cell monolayer causing dramatic reduction in the concentration of marker in the apical chamber. The effect that this system had on the CaCo-2 cell monolayer appeared not to be recoverable.

(a) Mannitol



PEG 4000

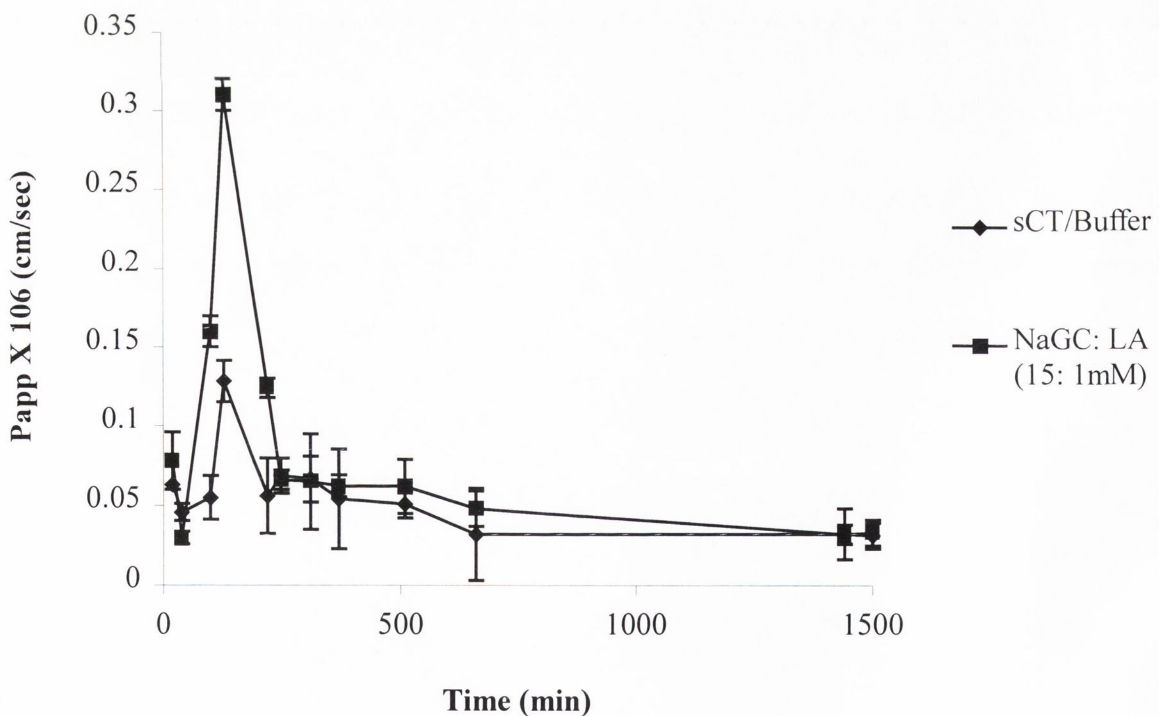


Figure 9.10 The effect of 1h exposure to mixed micelle NaGC: LA (15: 1 mM) on (a) mannitol and (b) PEG 4000 transport. The arrows indicate the time that the monolayer was exposed to the micelle. Values are expressed as mean ± SD (n =3).

9.6.3.2 Transepithelial electrical resistance

The TEER decreases to approximately 66% of its original value after exposure to the mixed micelle NaGC: LA (15: 1mM) for 4h and does not return to pre-incubation values 50h after exposure. These results are consistent with the previous marker data. However, it is clear that after only 1h of the cell monolayer being exposed to NaGC: LA (15: 1mM) there was a recovery in the TEER of the monolayer. The TEER decreased to 55%, restoring to its original value approximately six hours after exposure (Figure 9.11). This recovery was not as rapid as seen for the transport of the paracellular markers, mannitol and PEG 4000, since in the latter recovery occurred approximately 3hr after exposure.

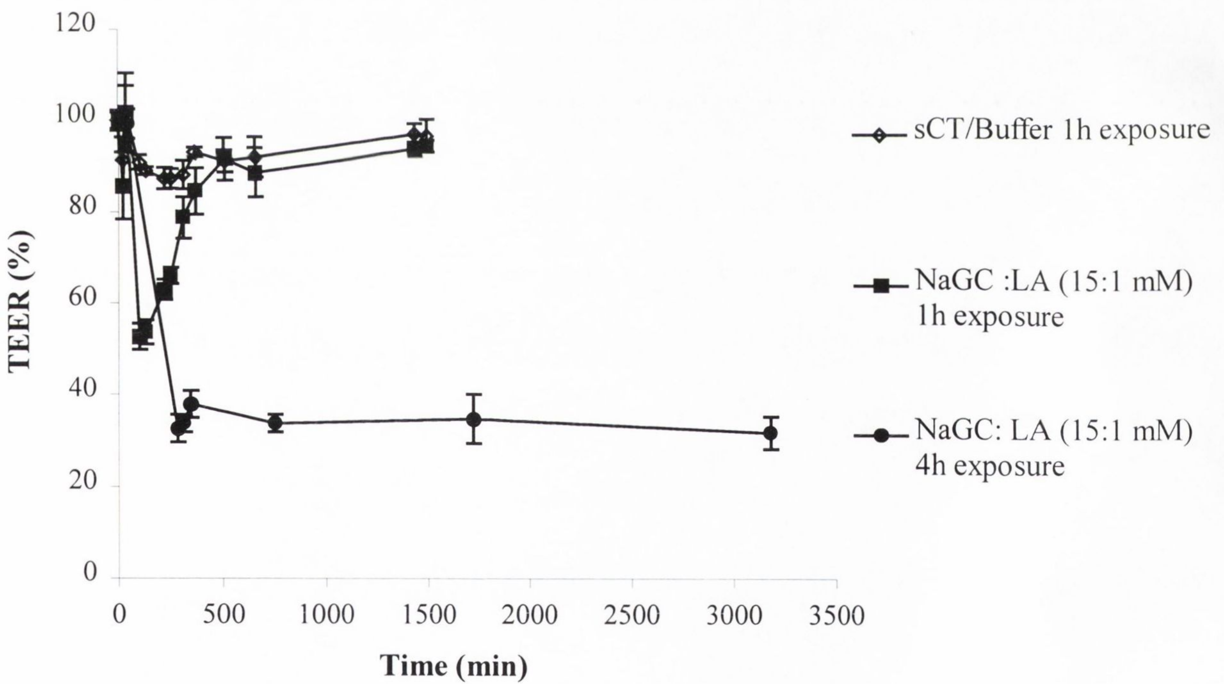


Figure 9.11 The effect of 1h and 4h exposure to the mixed micelle (NaGC: LA (15: 1mM)) on the TEER. Values are expressed as mean ± SD (n= 3- 4).

9.7 Discussion

Previously in Chapter 8, the effects of bile salt simple micelles were screened and the outcome was to choose a suitable bile salt, NaGC for further work. The formation of mixed micelles of the bile salt, NaGC and the long chain unsaturated fatty acid, linoleic acid were investigated for their ability to enhance the transport of hydrophilic markers and effects on the CaCo-2 cell monolayer integrity.

Initially, prior to transport and stability experiments the toxicity of the mixed micellar system was determined using MTT assay. This provided information on a suitable working concentration range. It appears that as the loading of fatty acid increased the enzyme activity decreased for all concentrations of NaGC investigated. It appears that the IC_{50} for the mixed micelles investigated are 10: 1.03mM, 12.5: 0.61mM and 15: 0.93mM.

Having determined suitable working concentration ranges for the mixed micelles the stability of the peptide salmon calcitonin (sCT) and the apparent permeabilities of sCT and both paracellular markers, mannitol and PEG 4000 across the CaCo-2 cell monolayer were determined. In the CaCo-2 cell monolayer apical chamber over the concentration range studied the mixed micelles appeared to reduce the amount of sCT degraded.

As the stability was being monitored the transport of sCT and the paracellular markers, mannitol and PEG 4000 was also assessed. It appears that as the fatty acid loading was increased there was an increase in the transport of the hydrophilic compounds. Another interesting trend was that as the bile salt concentration increased in the presence of 1mM LA there was a concentration dependant increase in the Papp of sCT, mannitol and PEG 4000. Previously mixed micelles have been shown to increase the enhancement of marker compound because of the increased lytic activity associated with them (Laprè et al., 1992). It was also demonstrated that the lytic activity of the mixed micelles increased by the amount of fatty acids as well as increasing hydrophobicity of bile salt. The enhancement in Papp observed following exposure to the mixed micellar systems was PEG 4000 =sCT> mannitol.

Tight junction integrity was unaffected by the absence of Ca^{2+} in the apical medium. This is in agreement with observations made by Meaney (1997) and Anderburg et al., (1993) using the cell culture model. Gumbiner (1987) had already demonstrated that the calcium dependent adhesion molecule uvomorulin of the zonula adherens was on the basolateral side of the tight junction and it was important for sustaining junctional integrity.

The absence of calcium in the apical chamber in the presence of the simple micelle 15mM NaGC and mixed micelle NaGC: LA (15: 1 mM) resulted in a decrease in the transport of sCT and both paracellular markers mannitol and PEG 4000. Many conflicting reports exist in the literature as to the effect of calcium on the action of bile salts. Yamashita et al., (1990) suggested that the enhancement of the bile salts was attributable to the bile salt binding to Ca^{2+} *in-vitro* rat jejunal studies. Similarly, it was suggested in the presence of Ca^{2+} the lytic effect of bile salts was enhanced on human erythrocytes (Child et al., 1986) and isolated rat liver mitochondria (Güldütuna et al. 1999). It has been suggested by numerous authors that the binding of Ca^{2+} to bile salts is a complex dependency on the bile salts' lipophilicity, the pH and ionic strength of the medium, the temperature and the presence of other bile salts (Rajagopalan et al. 1984; Baruch et al. 1991; Hoffmann et al. 1992). It has been demonstrated that Ca^{2+} has a greater binding potential for micelles of dihydroxy bile salts than trihydroxy bile salts suggesting that the more hydrophilic the bile salt the less calcium that was bound. The effect that apical Ca^{2+} had on micellar systems of NaGC was clear because of the statistically significant greater enhancement of sCT, mannitol and PEG 4000 when Ca^{2+} was present. These results suggest that calcium increases the enhancing potential of bile salts (Child et al., 1986; Güldütuna et al., 1999).

The use of bile salt mixed micelles as absorption enhancers has limitations primarily as they are know to cause some toxic effects (Laprè et al., 1992; Meaney, 1997). By monitoring the TEER and the TEM it provided more information on the mechanism of action of these mixed micellar systems.

The formulation of mixed micelles with NaGC and LA caused greater damage to the CaCo-2 cell monolayer as shown by the TEER values and the TEM than their corresponding simple micelles. This is as expected as Laprè et al., (1992) had reported that mixed micelles of fatty acids and bile salts synergistically increased the lytic activity in erythrocytes and CaCo-2 cells as measured by lactate dehydrogenase and alkaline phosphatase activity. The unsaturated fatty acid, linoleic acid (LA) itself did not appear to have any adverse effect on the CaCo-2 cell monolayer (Van Greevenbroek et al 1995). It is apparent from this study that increasing concentration of NaGC in the presence of 1mM LA caused a decrease in the TEER values and corresponding changes in the cell morphology as presented by TEM. Secondly, as the fatty acid loading was increased there was a decrease in TEER and corresponding damage to the CaCo-2 cell monolayer noted. The relationship between TEER and the Papp was demonstrated in this study for the mixed micelles of NaGC and LA but is not as strong as for the simple micellar systems which may indicate that the effect of mixed micelles is not confined solely to an effect on the tight junctions.

CHAPTER 10

The effects of NaGC simple and mixed micelles on the apparent permeabilities of the hydrophilic compounds, mannitol, PEG 4000 and sCT and on the integrity of the CaCo-2: Ht29GlucH co-culture monolayer

10.1 Introduction

The use of the CaCo-2 cell model has been widely accepted as a suitable in-vitro model of the absorptive intestinal epithelium for drug transport and metabolism studies (Hovgaard et al., 1995a). However, one of the major constraints of this model is the absence of a mucus layer, which is present in the in-vivo situation. Meaney and O' Driscoll (1999) developed the mucus producing CaCo-2: Ht29GlucH mucus producing co-culture cell culture model and showed that the permeability of hydrophilic marker compounds, mannitol, PEG 900 and PEG 4000 was greater in the co-culture model compared to the mucus free cell culture model CaCo-2.

Previously we have shown that NaGC simple micelles provided the greatest enhancement with minimal effect to the membrane integrity, Chapter 8. This simple micelle was then combined with various ratio of LA and the results suggested that NaGC: LA (15: 1mM) was a suitable system to investigate, Chapter 9. In this study the modulatory effects of mucus on the transport of sCT was monitored in the presence and absence of these chosen simple and mixed micelles of NaGC using the co-culture model. In addition the paracellular markers, mannitol and PEG 4000, and the integrity of the co-culture model were investigated.

10.2 Demonstrating the presence of mucin

The two methods that were employed to demonstrate that mucin was present in the co-culture were microscopy, including light microscopy (LM), TEM and scanning electron microscopy (SEM) and an enzyme linked lectin assay (ELLA), which detects the carbohydrate epitopes of mucin glycoproteins (Rhodes et al., 1993).

10.2.1 Microscopy

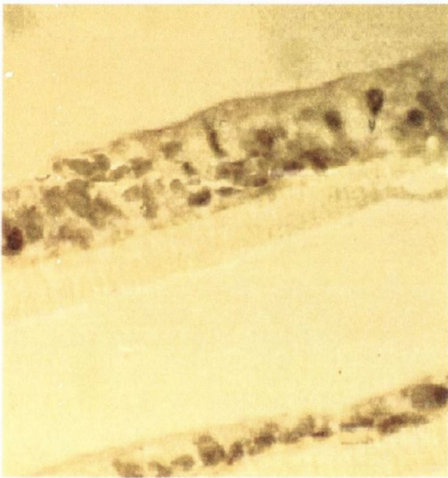
<u>Light microscopy (LM)</u>

LM was used in combination with Alcian Blue and Periodic Acid Schiff's (PAS) reagent, to illustrate the presence of neutral or acidic mucin species stored or secreted by the cell

cultures. The staining of CaCo-2, Ht29GlucH and CaCo-2: Ht29GlucH monolayers were carried out.

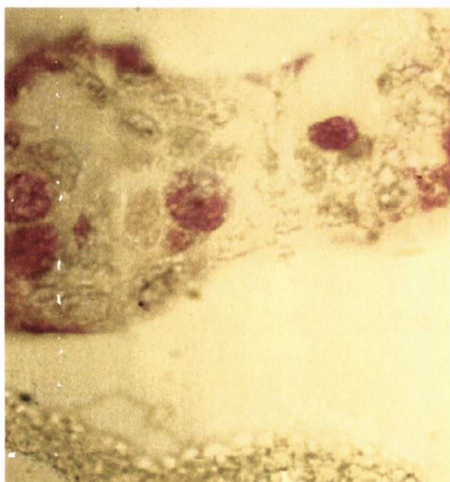
No staining for mucins was observed for CaCo-2 cell culture (Fig. 10.1 (a) and (b)). Ht29GlucH monolayers stained with PAS and alcian blue indicate the presence of intracellular stores of mucins (Fig. 10.1 (c) and (d)). From the co-culture model CaCo-2: Ht29GlucH stained with PAS and alcian blue a number of observations were made. There appeared to be variation in the cell monolayer with distinct areas where the monolayer is stained by either alcian blue or PAS (Fig. 10.1 (e) and (f)). This suggested that both cell types, CaCo-2 and Ht29GlucH, grow together and remain differentiated in the co-culture model.

a)

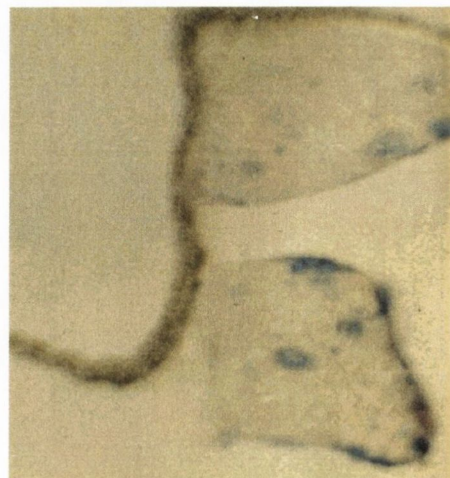


b)

c)



d)



e) f)

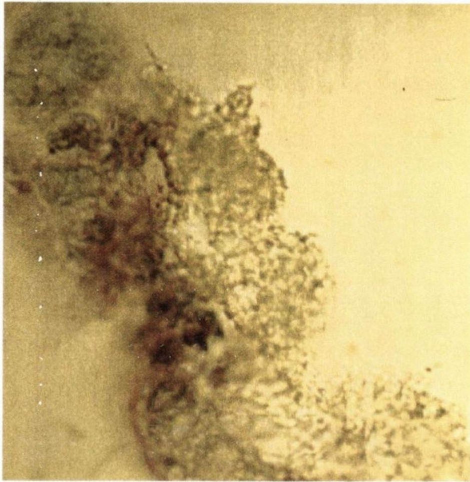


Figure 10.1 LM showing a transverse section of 21-day old

- CaCo-2 monolayers stained with a) PAS or b) Alcian blue
- Ht29GlucH monolayer stained with c) PAS or d) Alcian blue
- CaCo-2: Ht29GlucH co-culture monolayer stained with e) PAS or f) Alcian blue

Electron microscopy (EM)

Scanning electron microscopy (SEM)

The patterns observed for the CaCo-2 cell monolayer are a thick carpet of brush borders (A) and floret formation where the apical ends appeared joined together (B) (Fig. 10.2). This is similar to previously reports that CaCo-2 cell monolayers have two patterns of distribution (Pinto et al., 1983; Meaney 1997).

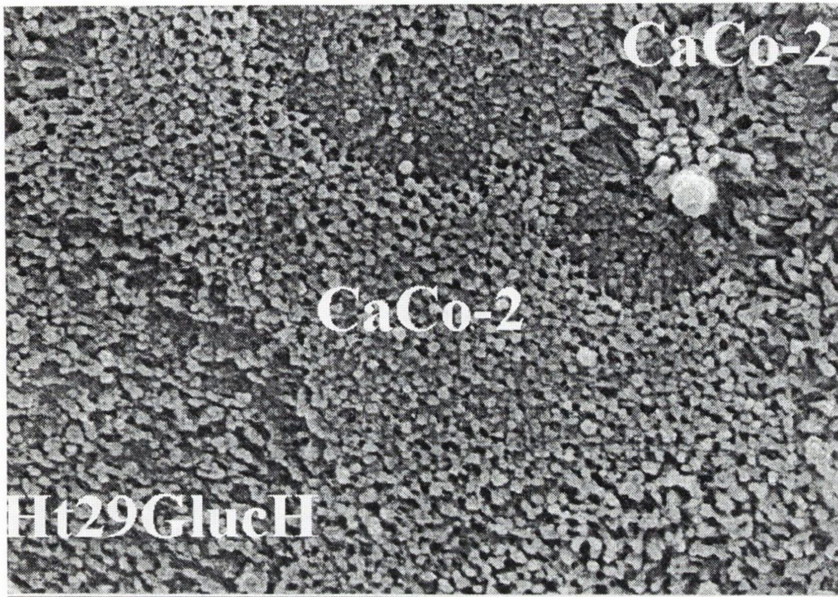


Figure 10.2 Scanning electron micrographs of 21-day old CaCo-2: Ht29GlucH co-culture monolayers illustrating the junctional formation between adjacent CaCo-2 cell and Ht29GlucH.

From Fig. 10.2 it was apparent the cell types of CaCo-2 and Ht29GlucH were interspersed with each other. Each of the cell patterns is visible with the dense carpet and florets of the CaCo-2 and the sparsely distributed areas of the Ht29GlucH. Similarly Meaney (1997) observed that the Ht29GlucH cells in the co-culture system had sparser stub-like shaped villi.

Transmission electron microscopy (TEM)

TEM provided a more intimate view of the CaCo-2: Ht29GlucH cell monolayer. The presence of a junctional complex between the cell types was obvious, Fig. 10.3. The Ht29GlucH cells were distinguished by the presence of the stored mucin granules. The TEM confirmed what had previously been observed using LM and SEM that both cell types display cell polarity and remain differentiated in co-culture.

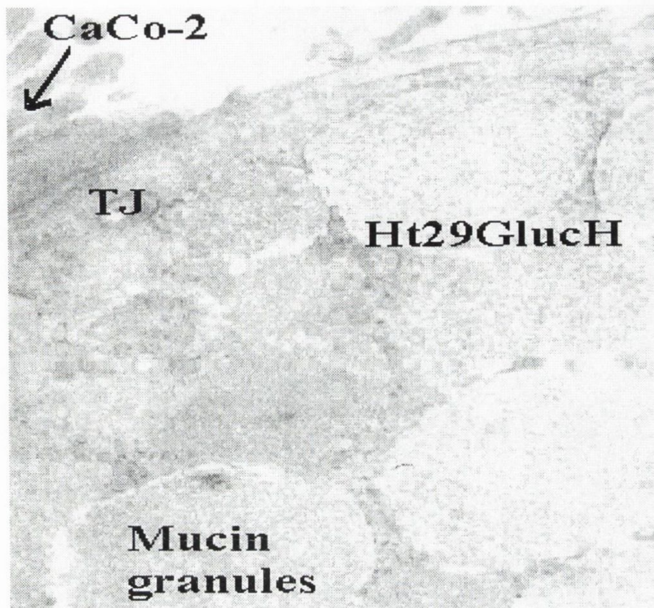


Figure 10.3 Transmission electron micrograph of day 21 co-culture monolayers showing the junctional complex between adjacent CaCo-2 and Ht29GlucH cells (X 24000)

10.2.2 Enzyme lectin linked assay (ELLA)

Rhodes et al. (1993) have demonstrated that peroxidase labelled lectins may be used to quantify mucin glycoprotein. It was shown that these lectins were also suitable for testing intestinal mucus glycoprotein homogenates. The results indicated that only Ht29GlucH and CaCo-2: Ht29GlucH monolayer produce mucin glycoproteins (Table 10.1).

Table 10.1 The concentration of mucin in 21-day old cultured cells of CaCo-2, Ht29GlucH and the co-culture CaCo-2: Ht29GlucH.

Culture type	Mucin Glycoprotein Concentration ± SE (ng/ml) (n)
CaCo-2	0 (6)
Ht29GlucH	17.74 ± 0.32 (9)
CaCo-2: Ht29GlucH	22.32 ± 1.67 (14)

Having established that the CaCo-2: Ht29GlucH co-culture produced a mucus layer the NaGC bile salt micellar systems were investigated to determine their effects on the transport of the peptide sCT and both paracellular markers, mannitol and PEG 4000 and on the integrity of the co-culture monolayer.

10.3 The effects of NaGC simple and mixed micelles on the transport of the hydrophilic compounds across the co-culture model

Salmon calcitonin (sCT)

In the control experiment the transport of sCT across the CaCo-2: Ht29GlucH co-culture monolayers were significantly greater than for CaCo-2 cell monolayers. The Papp for sCT increased from 0.05 ± 0.01 X 10^{-6} cm/sec in the CaCo-2 cell monolayer to 0.45 ± 0.05 X 10^{-6} cm/sec in co-culture monolayers, corresponding to a 9-fold increase relative to the CaCo-2 model, Table 10.2 and Fig. 10.4 (a). Previously it had been reported that the co-culture model was more permeable than the CaCo-2 model (Meaney and O' Driscoll (1999) and Allen (1992)). The presence of the simple micellar systems of NaGC appeared to cause the apparent permeability of sCT to decrease as the concentration of NaGC was increased (table 10.2). In the presence of NaGC 10mM and 15mM the Papp of sCT was reduced 1.55 and 2.5-fold respectively relative to control.

In the presence of the mixed micelle NaGC (15mM): LA (1mM) the Papp increased 6.2-fold from 0.45 ± 0.05 X 10^{-6} cm/sec for sCT alone to 2.78 ± 0.69 X 10^{-6} cm/sec in the presence of the mixed micelle (Table 10.2).

Paracellular markers: Mannitol and PEG 4000

As had been previously observed for the peptide sCT a similar trend was noted for the paracellular marker compound, see Fig. 10.4 (b) and (c). The permeabilities of the paracellular markers, mannitol and PEG 4000 were significantly greater across the co-culture monolayer as compared to the CaCo-2 cell monolayer. The Papp for mannitol and PEG 4000 increased 3.1 and 6.4-fold respectively.

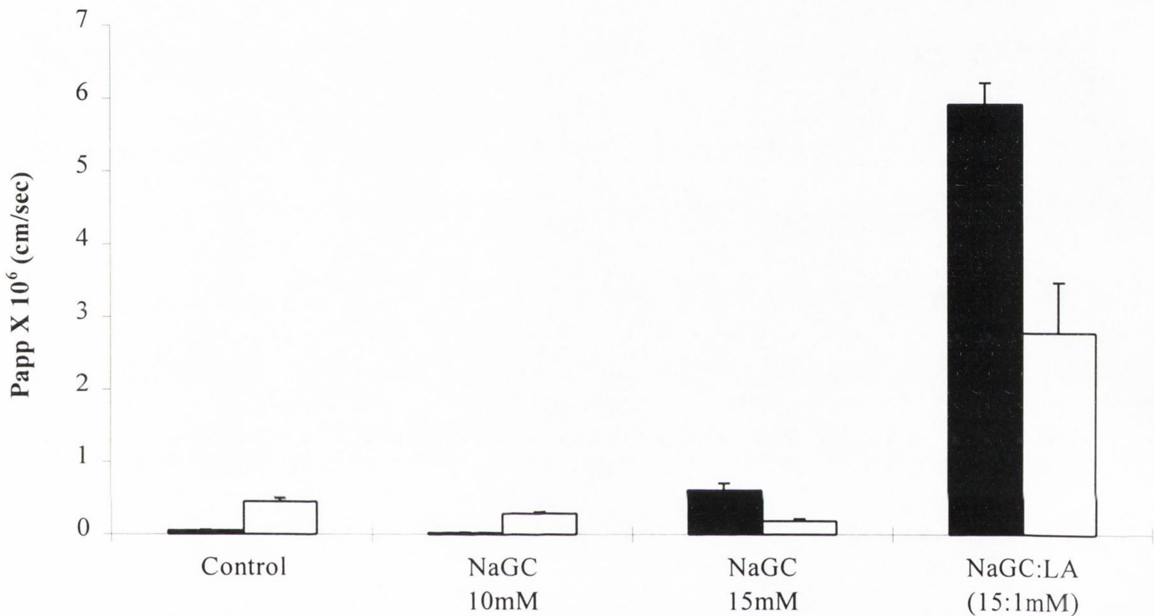
Again as the concentration of NaGC increases there is a decrease in the permeabilities of mannitol and PEG 4000 across the CaCo-2: Ht29GlucH co-culture monolayer, see table 10.2. The Papp for mannitol decreased 1.07 and 1.43-fold in the presence of NaGC 10 mM and 15 mM respectively, relative to control (sCT alone). Similarly, there was a 1.07 and 1.68-fold decrease in the Papp of PEG 4000 in the presence of NaGC 10mM and NaGC 15mM respectively (table 10.2).

In the presence of the mixed micelle system NaGC: LA (15:1 mM) the permeabilities of mannitol and PEG 4000 were enhanced 6.31 and 18.43-fold respectively. However, the degree of enhancement was not as great as was observed in the CaCo-2 cell model.

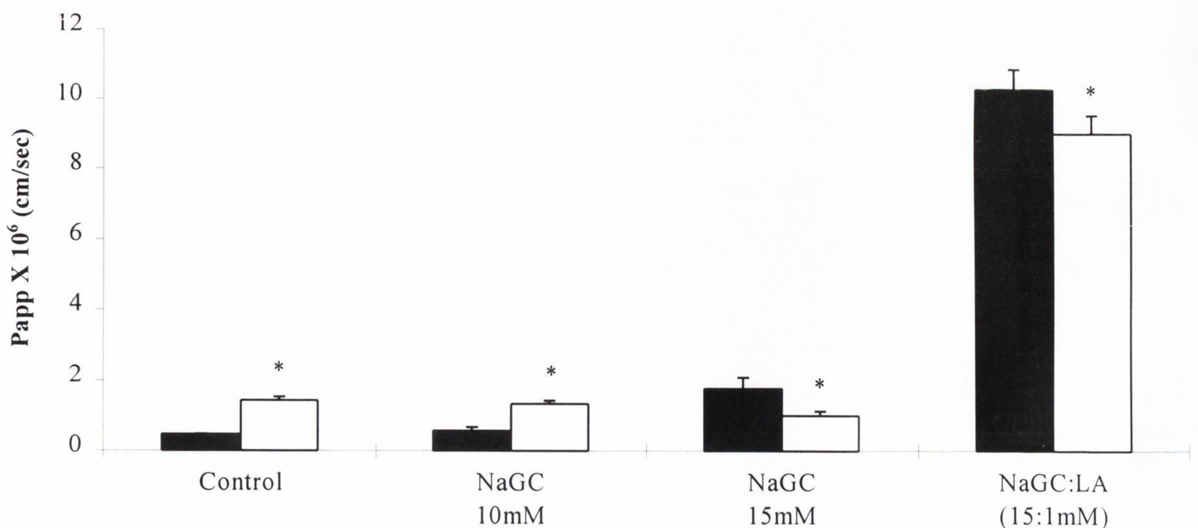
From the results it was clear that the CaCo-2: Ht29GlucH co-culture model was more permeable than the CaCo-2 model, as the Papp for the hydrophilic compounds were greater in the co-culture system. The relative enhancements observed in the transport of the peptide sCT and both paracellular markers due to the simple and mixed micellar systems were less for the co-culture model. This maybe due to the presence of the mucus layer, which acts as a protective mechanism against the micellar systems. It has been reported that bile salts enhance the production of mucin in Ht29 cells, clearly demonstrating that as the hydrophobicity of the bile salt increased there was a

concomitant increase in mucin production (Shekels et al., 1996). Also the diffusivity behaviour of mixed micellar systems has been shown to decrease as the mucin concentration increased *in-vitro* (Li et al., 1996). The results seemed to imply that the mucus layer was a barrier and therefore the effects of these micellar systems were investigated when the mucus layer was removed.

a) sCT



b) Mannitol



c) PEG 4000

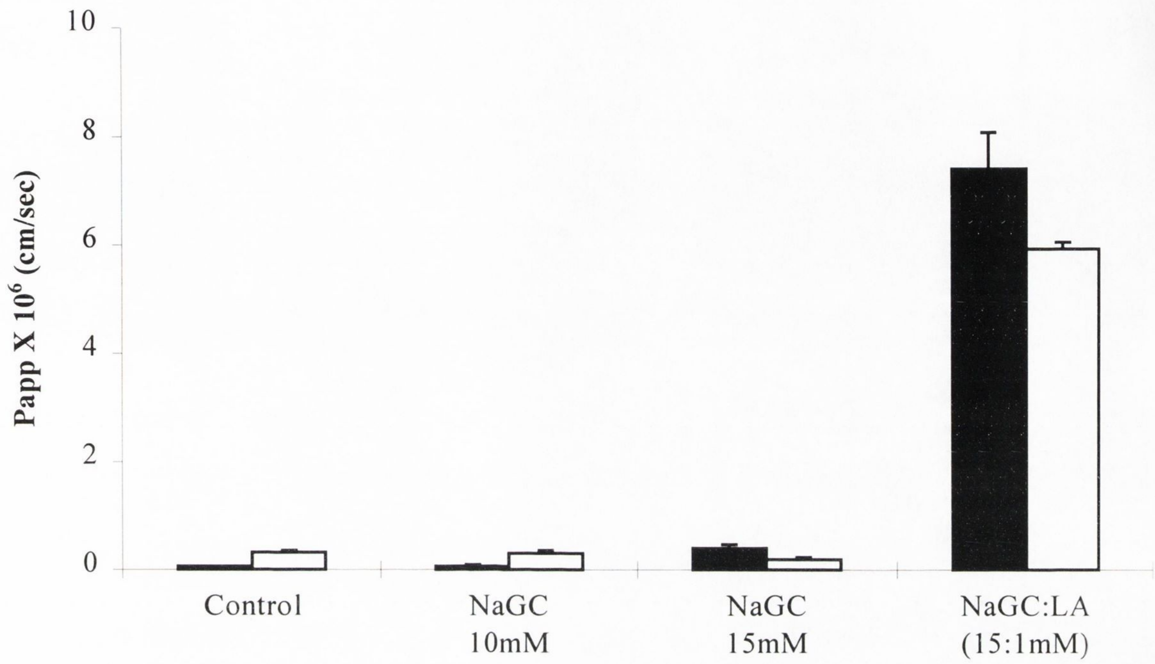


Figure 10.4 Comparison of the Papp of a) sCT, b) mannitol and c) PEG 4000 in CaCo-2 (■) and CaCo-2: Ht29GlucH (□) in the presence of simple and mixed micelles of NaGC and LA.

Table 10.2: The apparent permeability (Papp) across the CaCo-2: Ht29GlucH co-culture monolayer over 4h of the hydrophilic compounds, mannitol, PEG 4000 and sCT for each of the simple and mixed micellar systems of sodium glycocholate (NaGC)

System	Papp ± SD (X10^{6}) (cm/sec) (n ≥ 3) (p<0.05, *) (Total Percentage transported after 4hr)		
NaGC: LA (mM)	Mannitol	PEG 4000	sCT
0: 0	1.43 ± 0.10 (9.64 ± 0.65%)	0.32 ± 0.03 (2.02 ± 1.24%)	0.45 ± 0.05 (2.81 ± 0.28%)
10: 0	1.34 ± 0.09* (9.56 ± 1.89%)	0.30 ± 0.05 (2.09 ± 1.12%)	0.29 ± 0.02 (1.92 ± 0.12%)
15: 0	1.00 ± 0.13* (7.25 ± 3.13%)	0.19 ± 0.04* (1.31 ± 0.92%)	0.18 ± 0.03* (1.22 ± 0.20%)
15: 1	9.03 ± 0.52* (52.52 ± 2.72%)	5.90 ± 0.12* (34.37 ± 0.53%)	2.78 ± 0.69* (16.05 ± 3.92%)

10.4 The effect of the mucolytic agent N-Acetylcysteine (N-AC) has on mucin concentration in the apical chamber of co-culture monolayers

N-AC, a cysteine derivative with a free sulphydryl group, has been used as a mucoregulatory agent in the treatment of chronic obstructive airways disorders. The mechanism of action of N-AC involves breaking the s-s bond of the protein backbone of the mucin molecule, and, in addition the adhesive mucus is made water-soluble by N-AC directly in-vivo in the lumen (Iiboshi et al., 1996; Kelly, 1998).

The mucus was removed from the cells by incubating with N-AC the mucolytic agent for 30min prior to the experiment. Initially studies were performed to select a suitable concentration of N-AC capable of removing mucin when incubated for 30min. Initially a range of concentrations of N-AC (0- 1% w/v) was investigated for their effects on mucin

removal after 30min incubation. In order to see if mucus production had recovered the apical mucin concentration was again measured 4h after the N-AC had been washed off.

For the control i.e. pre-incubated with HBSS, after 30 min exposure no mucus glycoproteins were detected in the apical chamber. In figure 10.5 it was apparent that concentrations of N-AC less than 0.4% w/v had little or no effect on the mucus concentration after 30min exposure indicating that these concentrations of N-AC are not exerting any mucolytic effect. For N-AC 0.4% w/v after 30 min pre-incubation there is a detectable concentration of mucin glycoproteins in the apical chamber, 4.80 ± 2.58 ng/ml. N-AC 0.5% w/v, the concentration of mucolytic used by Meaney and O' Driscoll (1999), was effective in removing the mucus layer with 21.62 ± 4.00 ng/ml of mucin glycoproteins in the apical chamber after 30min incubation. There was a further increase in the concentration of mucin glycoproteins in the apical chamber following 30 min pre-incubation with N-AC 1% w/v. However, from previous work it has been shown that N-AC at this concentration appears to have enhancer-like qualities (Meaney and O' Driscoll, 1999).

After 4 hr post-incubation as the concentration of N-AC increased from 0 - 1% w/v there was a concentration dependant decrease in the amount of mucin present, see figure 10.5. At 0.5% w/v mucins were detected but at a much lower concentration than for control and 1% w/v resulted in no mucins being present. The mucin levels decreased as N-AC concentration increased. This indicated that the recovery to pre-experiment mucin production appeared to be more difficult for cells treated with higher N-AC concentrations.

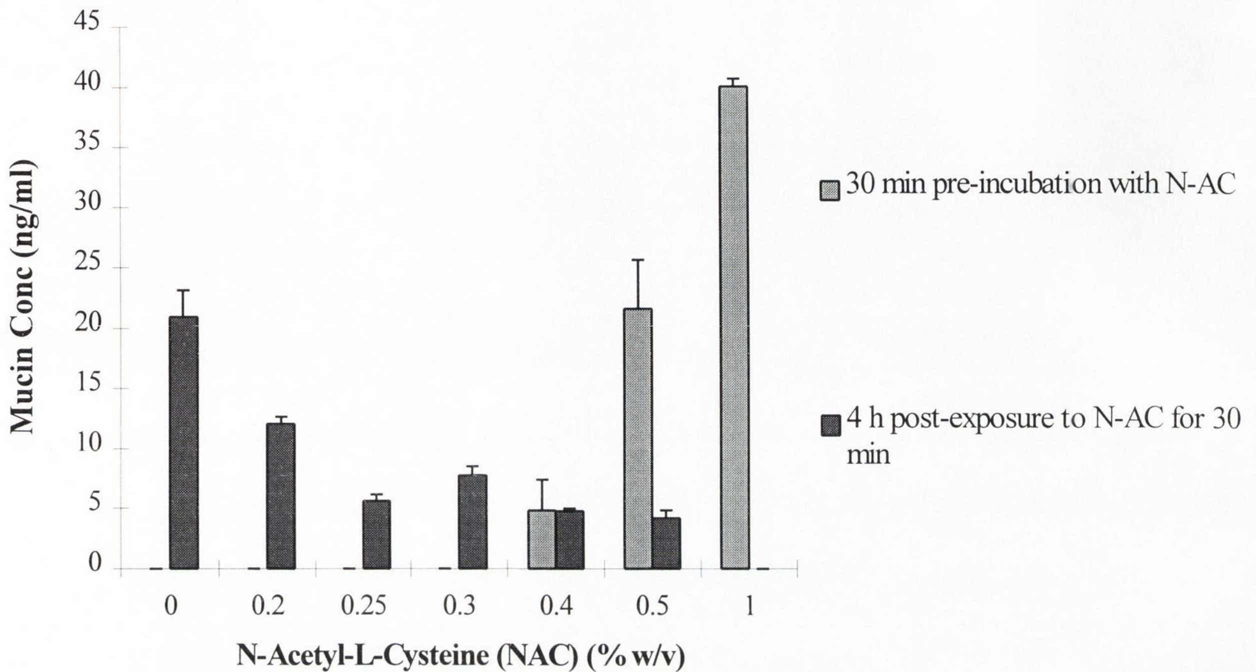


Figure 10.5 Mucin concentration in the apical chamber of CaCo-2: Ht29GlucH co-culture monolayers following incubation with N-Acetyl-L-Cysteine (N-AC) for 30 min, and again after 4hr incubation in HBSS. Values are expressed as mean ± SD ($n \geq 3$).

10.5 Assessment of the mucus layer as a barrier to transport

In order to determine the potential absorption barrier properties of the mucous gel layer the CaCo-2: Ht29GlucH cell monolayers were pre-incubated with N-acetyl-L-cysteine (N-AC). Day 21 CaCo-2: Ht29GlucH co-culture monolayers were incubated with 5mg/ml of N-AC in HBSS for 30min and which was then washed off twice prior to carrying out the transport experiment (Meaney 1997). The effect that this procedure had on the permeability of the paracellular markers, mannitol and PEG 4000 and the peptide, sCT was investigated.

Wikman et al., (1993) had showed that 1%w/v of N-AC reduced the surface coverage of the mucus layer on Ht29GlucH monolayers. However, Meaney and O' Driscoll, (1999)

demonstrated that using a concentration of N-AC greater than 0.5% w/v caused disruption to the integrity of the CaCo-2: Ht29GlucH co-culture monolayer. The results from the ELLA assay implied that 0.5% w/v was the best working concentration of N-AC.

Pre-incubation of the co-culture monolayer to 0.5%w/v N-AC for 30min prior to exposure to buffer alone i.e. no micelles present, caused a significant increase in the permeability of both paracellular markers, mannitol and PEG 4000 and the peptide sCT (Table 10.3). The enhancement in the transport of mannitol, PEG 4000 and sCT on the removal of mucus was 2.15, 1.90 and 2.55-fold respectively.

Table 10.3 The effect of incubation with 0.5% w/v N-Acetyl-L-Cysteine (N-AC) for 30min prior to the transport studies on the apparent permeability coefficient (Papp) 4h of the hydrophilic compounds, mannitol, PEG 4000 and sCT in HBSS across CaCo-2: Ht29GlucH co-culture monolayers, ($p < 0.05$ *).

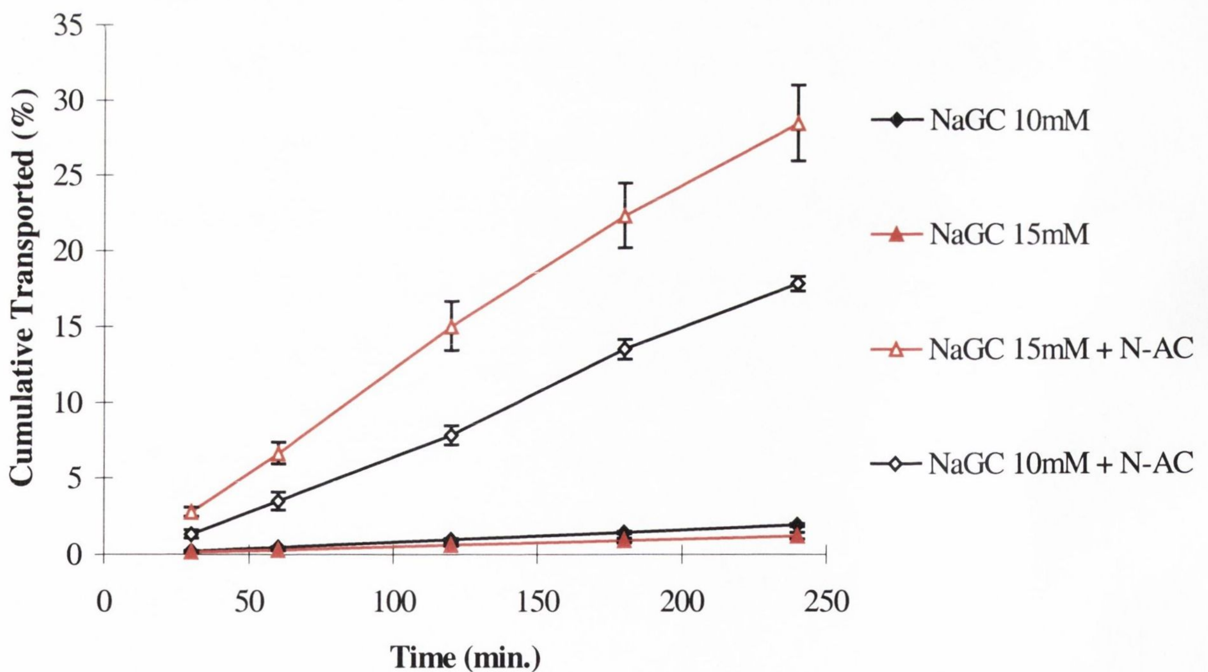
System (n)	**Papp ± SE ($X10^6$) (cm/sec) ($p<0.05$, *) (Total Percentage transported after 4hr)**		
	Mannitol	**PEG 4000**	**sCT**
HBSS (42)	1.43 ± 0.10 (9.64 ± 0.65%)	0.32 ± 0.03 (2.02 ± 1.24%)	0.45 ± 0.05 (2.81 ± 0.28%)
HBSS + N-AC (19)	3.08 ± 0.21* (20.86 ± 1.39%)	0.61 ± 0.05* (3.93 ± 0.31%)	1.15 ± 0.14* (7.10 ± 0.83%)

When the simple micelle NaGC 10mM was added post-incubation with 0.5% w/v N-acetyl-L Cysteine (N-AC) there was a further significant increase in the Papp of both paracellular markers and sCT. The permeability of mannitol, PEG 4000 and sCT increased 3.6, 5.97 and 9.75-fold respectively in the absence of the mucus layer compared to when the layer was present (Fig. 10.6 (a) and table 10.4).

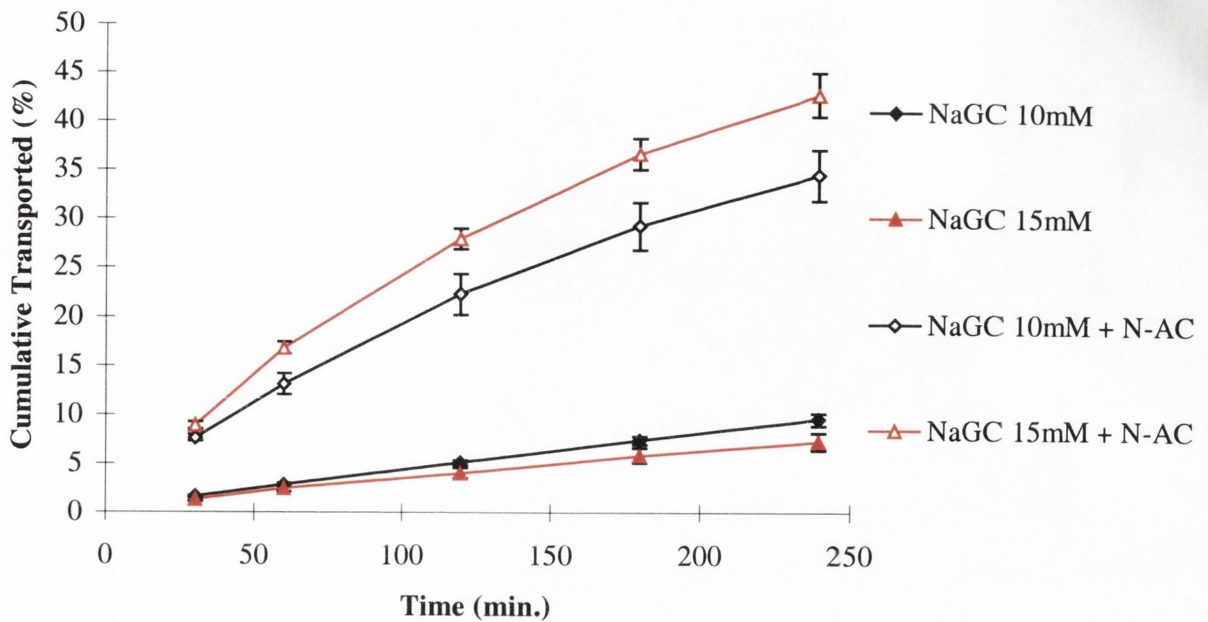
In the presence of NaGC 15mM, on removal of the mucus layer, the apparent permeabilities of mannitol, PEG 4000 and sCT increased significantly. There was a 5.66, 23 and 24.4-fold increase in the Papp of mannitol, PEG 4000 and sCT respectively, compared to CaCo-2: Ht29GlucH co-culture monolayer with mucus present, see Table 10.4 and Fig. 10.6 (b).

As the concentration of bile salt NaGC increased there was an increase in the permeability of the compounds. It also appeared that on removal of the mucus layer and in the presence of NaGC simple micelles the order of relative enhancement on the apparent permeability of the hydrophilic compounds was sCT> PEG 4000> mannitol compared to control pre-incubated monolayers. However, the enhancement caused by these simple micellar systems may be a slight overestimation due to the relatively small enhancement effect caused by N-AC 0.5%w/v alone (table 10.3).

a) sCT



b) Mannitol



c) PEG 4000

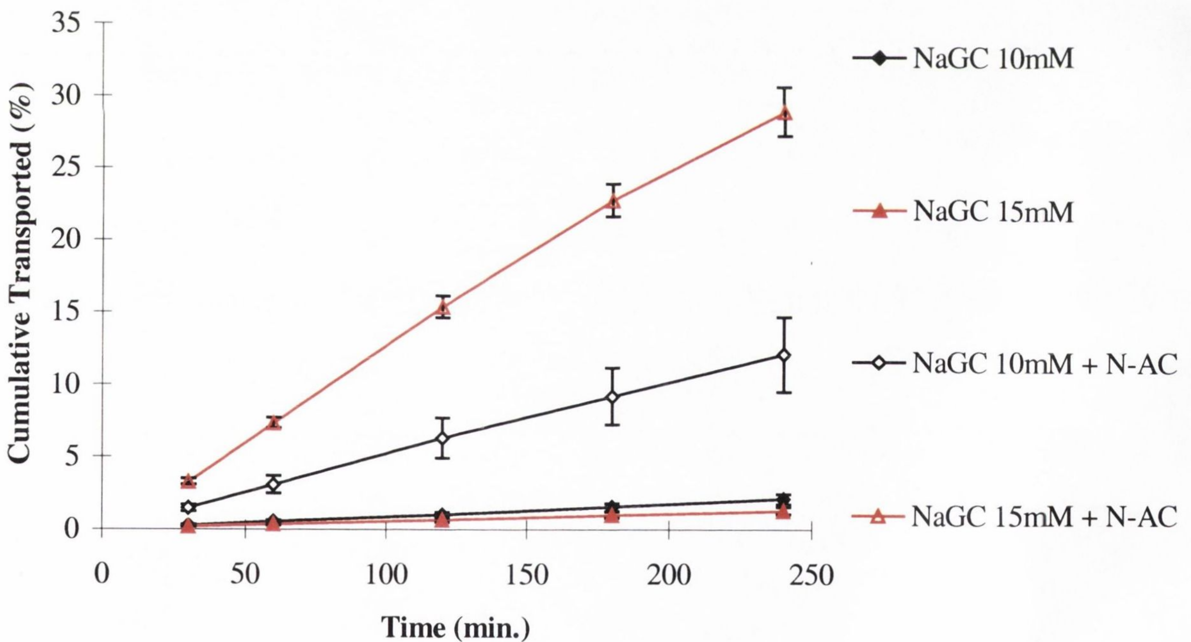


Figure 10.6 The effect of incubation with N-Acetyl-L-Cysteine (N-AC) 0.5% w/v for 30 min on the transport of (a) sCT, (b) mannitol and (c) PEG 4000 across CaCo-2: Ht29GlucH co-culture monolayers in the presence of sodium glycocholate (NaGC) 10mM and NaGC 15mM. Values are expressed, mean ± SE with n ≥ 4 observations.

In the case of the mixed micelle NaGC: LA (15: 1mM) removal of the mucus layer the caused only a slight but not statistically significant increase in the permeability of mannitol and PEG 4000 ($p > 0.05$) (Table 10.4 and Table 10.2). The permeability of sCT was significantly different on removal of the mucus layer with the apparent permeability increasing from $2.78 \pm 0.69 \times 10^{-6}$ cm/sec to $6.55 \pm 0.11 \times 10^{-6}$ cm/sec corresponding to a 2.4-fold enhancement. However the relative enhancement was similar to those seen with monolayer exposed to N-AC 0.5% alone (Table 10.4).

In general the removal of the mucus layer increased the enhancement potential of the NaGC simple micellar systems. The differences that were observed may be in part attributable to their effect on the production and visco-elastic properties of mucus and the increased difficulty for the micelle to travel through the mucus layer.

Table 10.4 The effect of pre-incubation with N-Acetyl-L-Cysteine (N-AC) 0.5% w/v for 30 min on the transport of mannitol, PEG 4000 and sCT across CaCo-2: Ht29GlucH co-culture monolayers in the presence of the simple micelles: sodium glycocholate (NaGC) 10mM, NaGC 15mM and the mixed micellar system: NaGC: LA (15:1 mM). Values are expressed as mean ± SE with at least $n \geq 4$ observations.

System	**Papp ± SE (X10^{6}) (cm/sec) ($n \geq 4$) (Relative enhancement to the mucus layer being present)**		
	Mannitol	**PEG 4000**	**sCT**
HBSS + N-AC	3.08 ± 0.21 (2.15)	0.61 ± 0.05 (1.90)	1.15 ± 0.14 (2.55)
NaGC (10mM)	4.83 ± 0.44 (3.60)	1.79 ± 0.39 (5.97)	2.83 ± 0.06 (9.75)
NaGC (15mM)	5.66 ± 0.38 (5.66)	4.38 ± 0.25 (23.00)	4.39 ± 0.39 (24.40)
NaGC:LA (15:1 mM)	9.68 ± 0.25 (1.07)	6.29 ± 0.40 (1.07)	6.55 ± 0.11 (2.36)

10.6 The effect of NaGC systems on mucin production in the presence and absence of N-AC (0.5% w/v)

An ELLA assay was used to investigate the effect of simple and mixed micelles of sodium glycocholate (NaGC) and linoleic acid (LA) had on the concentration of mucin in the apical chamber of CaCo-2: Ht29GlucH co-culture monolayers in the presence and absence of N-AC 0.5% w/v. This assay as described in Chapter 7 quantifies the concentration of mucin glycoproteins and therefore is an indication of mucin production.

Exposure to NaGC 10mM had no apparent effect on mucin concentrations after 4h relative to the control, sCT alone (Fig. 10.7). This corroborates the permeability results for the paracellular markers, mannitol and PEG 4000 and sCT because the Papp are similar to the control (Table 10.2). It was apparent that in the presence of NaGC 15mM and NaGC: LA (15: 1mM) that there was a significant increase in the presence of mucin in the apical chamber relative to the control ($p < 0.05$). The concentration of mucin increased 13 and 10-fold respectively in the presence of these systems. The increase for the simple micellar system, NaGC 15 mM was significantly greater than for the mixed micellar system NaGC: LA (15:1mM), $p < 0.05$.

Following 30min pre-incubation with N-AC and subsequent exposure of the monolayer to the various test systems there was a decline in the concentration of mucin glycoproteins found in the apical chamber after 4h for all systems, see Fig. 10.7. The lower levels indicate that mucus levels are recovering following pre-treatment with the mucolytic.

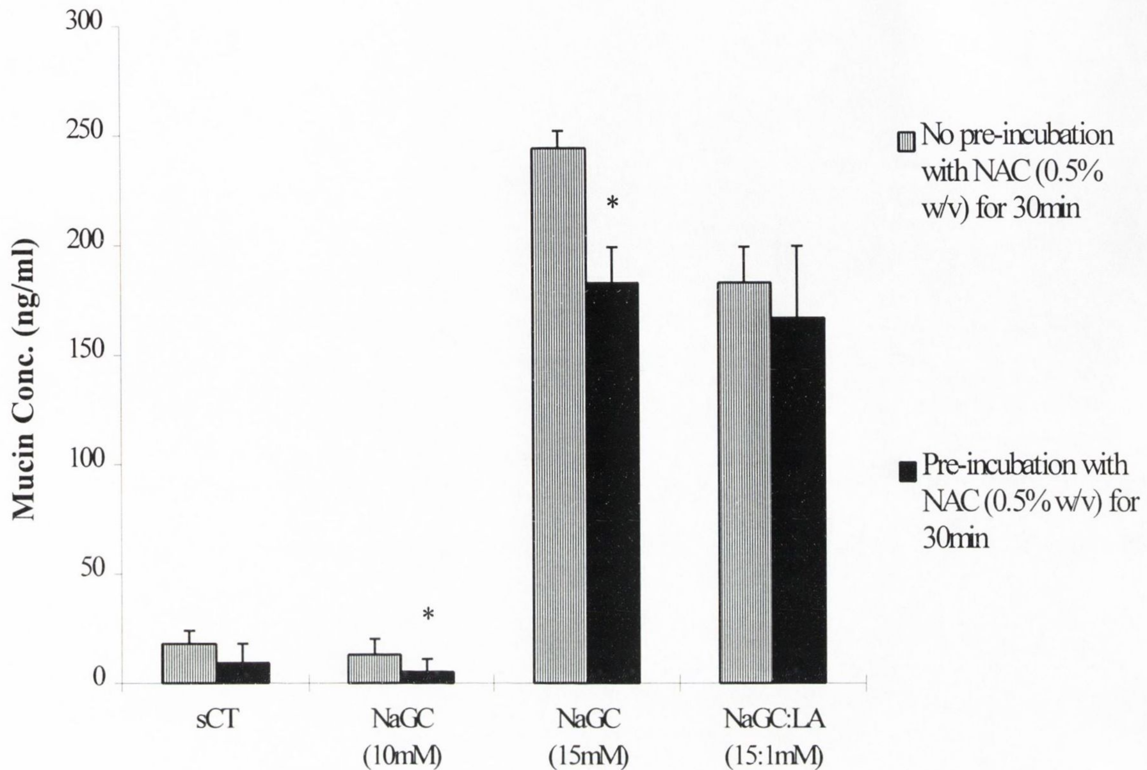


Figure 10.7 The effect of micellar systems on mucin glycoprotein concentration after 4h exposure in the absence and presence of N-Acetyl-L-Cysteine (N-AC) (0.5%w/v) for 30min. Values are expressed as mean ± SD ($n \geq 3$), significance was calculated for the same systems with and without pre-treatment with N-AC ($p > 0.05$, *).

10.7 The effects of micellar systems and N-AC (0.5% w/v) on partially purified porcine mucin (PPPM) viscosity

It has been reported that bile salts are capable of reducing the viscoelastic properties of mucus (Martin et al., 1978). This was further investigated by examining the effect that NaGC had on the rheological properties of partially purified porcine mucus (PPPM). In table 10.5 as the concentration of bile salt was increased there was a significant reduction in the apparent viscosity (η_{app}) of 2.5 and 3% w/v PPPM ($p < 0.05$). The mixed micelle NaGC: LA (15: 1mM) caused a reduction in viscosity similar to NaGC (15mM).

Poelma et al., (1990) previously demonstrated that the molecular cohesion of the freshly scraped pig mucus was reduced after the addition of sodium taurocholate (NaTC) (10mM).

Poelma et al., (1990) also established that L-cysteine (3% w/v), a mucolytic agent, resulted in a reduction of the viscosity to the mucus sample as compared to the control mucus sample. Similarly, when 0.5% w/v N-acetyl-L-cysteine (N-AC) (a chemically modified L-cysteine) was added to 2.5% w/v PPPM; the η_{app} was significantly reduced, ($p < 0.05$), see table 10.5.

Table 10.5 The effect that micellar systems of sodium glycocholate (NaGC) and linoleic acid (LA) (1mM) and N-AC (0.5% w/v) have on the apparent viscosity (η_{app}) of various concentrations of partially purified porcine mucus (PPPM).

	Apparent Viscosity (η_{app}) X 10^{-3} ± SE (Pa. s) p< 0.05, *)		
PPPM Conc. (% w/v) / System (n)	**3.5**	**3**	**2.5**
HBSS (4)	7.63 ± 0.87	6.00 ± 0.05	5.38 ± 0.18
NaGC (15mM) (4)	6.47 ± 0.12*	4.60 ± 0.39*	4.35 ± 0.09*
NaGC (20mM) (4)	ND	3.50 ± 0.08*	3.97 ± 0.18*
NaGC: LA (15:1mM) (5)	ND	ND	4.12 ± 0.18*
N-AC (0.5% w/v) (4)	ND	ND	4.59 ± 0.26*

10.8 The integrity of the CaCo-2: Ht29GlucH co-culture monolayer

The integrity of the co-culture monolayer was monitored by two methods, TEER and TEM. The resistance and morphology of the co-culture monolayer was compared to the CaCo-2 cell monolayer under control conditions and following exposure to NaGC simple and mixed micelle. The effects that pre-incubating the co-culture monolayer to N-AC (0.5% w/v) and resultant exposure to the micellar systems was examined by both TEER and TEM.

10.8.1 Transepithelial electrical resistance (TEER)

CaCo-2: Ht29GlucH co-culture monolayers:

As the permeability of the co-culture monolayer was higher for mannitol, PEG 4000 and sCT than the CaCo-2 cell monolayer it was expected that the corresponding TEER value would be less. The TEER value at Day 21 for the CaCo-2 cell monolayer-2 model was 877 ± 105 $\Omega.cm^2$ however for CaCo-2: Ht29GlucH co-culture model the TEER value was approximately 2.5-fold lower at 308 ± 61 $\Omega.cm^2$. These results were comparable to what has previously been reported, with differences of 1.3 and 2-fold in TEER between both cell models (Larhed et al., 1995; Meaney et al, 1999). The theoretical value for the transepithelial electrical resistance of the co-culture (R_{theor}) was calculated according to the following equation 10.1 (Fuller et al. 1986):

$$R_{theor} = 1/ ((f_H / R_H) + (1 - f_H)/ R_C) \quad \text{Eqn. 10.1}$$

f_H = fraction of the filter covered with Ht29GlucH cells

R_H = resistance of the Ht29GlucH monolayer

R_C = resistance of the CaCo-2 cell monolayer

The TEER values obtained with this predictive model, 323 ± 11 $\Omega.cm^2$, were in good agreement with the values actually measured, 308 ± 61 $\Omega.cm^2$, assuming that the resistance of Ht29GlucH cell monolayer (R_H) was 112 ± 58 $\Omega.cm^2$ (Meaney 1997), the fraction of Ht29GlucH covering the filter (f_H) was 0.25 and the resistance of the CaCo-2 cell monolayer (R_C) was 877 ± 105 $\Omega.cm^2$.

Effect of simple and mixed micelles on CaCo-2: Ht29GlucH co-culture monolayers

Decreases in TEER suggest increased paracellular permeability due to opening of the tight junctions. Therefore the transepithelial electrical resistance (TEER) across the monolayers was monitored before, during and after exposure to the micellar systems. Pre-incubating the co-culture monolayer with N-Acetyl-L-Cysteine (N-AC) (0.5% w/v) showed no effect on the integrity of the monolayer, see table 10.6.

In the presence of NaGC 10mM the TEER remains within control levels which indicated that this concentration of bile salt did not affect the tight junction integrity either when the monolayer had been pre-incubated with N-AC or not. However in the presence of NaGC 15mM on pre-incubation with N-AC (0.5% w/v) there is a statistically significant decrease in the TEER value as compared to when no pre-incubation has occurred ($p < 0.05$). The mixed micelle NaGC: LA (15: 1mM) caused a statistically significant reduction in TEER in the presence and absence of the mucus layer when compared to sCT alone ($p< 0.05$) (table 10.6). However, the TEER value was similar for the mixed micelle system regardless of mucus. This corroborates the permeability data which shows a large flux of mannitol, PEG 4000 and sCT across the co-culture monolayer whether mucus was present or absent suggesting that mucus does not affect the enhancement potential of the mixed micellar system, NaGC: LA (15:1 mM).

Table 10.6 Transepithelial electrical resistance (TEER) values of CaCo-2: Ht29GlucH co-culture monolayers following 4h exposure to sCT (control), simple micellar systems: NaGC 10mM and 15mM and the mixed micellar system: NaGC: LA (15: 1mM) in the presence and absence of N-AC (0.5% w/v). Values are expressed as mean ± SD $(n \geq 3)$, $(p > 0.05, *)$.

System	% TEER at 4h ± SD (n ≥ 6)	
	No N-AC	N-AC for 30 min
sCT	85.8 ± 10.3	85.9 ± 13.1
NaGC (10mM)	89.5 ± 17.3	65.5 ± 13.8
NaGC (15mM)	73.8 ± 10.8	41.2 ± 8.5*
NaGC: LA (15: 1mM)	31.4 ± 7.3	24.8 ± 1.5

A good correlation between the decrease in TEER and Papp of the hydrophilic marker has been previously demonstrated in the CaCo-2 cell monolayer. For the co-culture model it was obvious that in the presence of mucus that the simple micellar systems cause a concentration dependant reduction in the Papp of the hydrophilic compounds with no appreciable decrease in the TEER, see table 10.2 and 10.6), suggesting that the mucus appears to be a barrier to NaGC enhancing potential. Following removal of the mucus layer with N-AC (0.5% w/v) the relationship between the decrease in TEER and increase in Papp was observed (Fig 6.8). This was further evidence to suggest that the mucus layer was a barrier to the simple micellar systems.

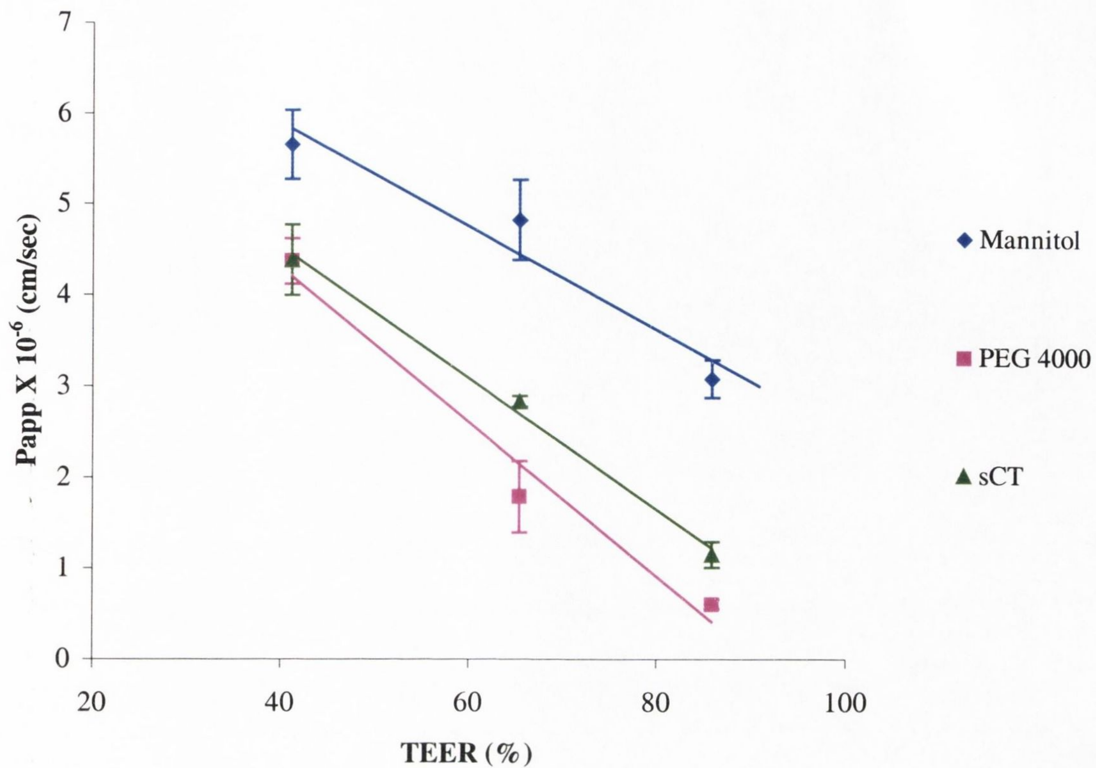
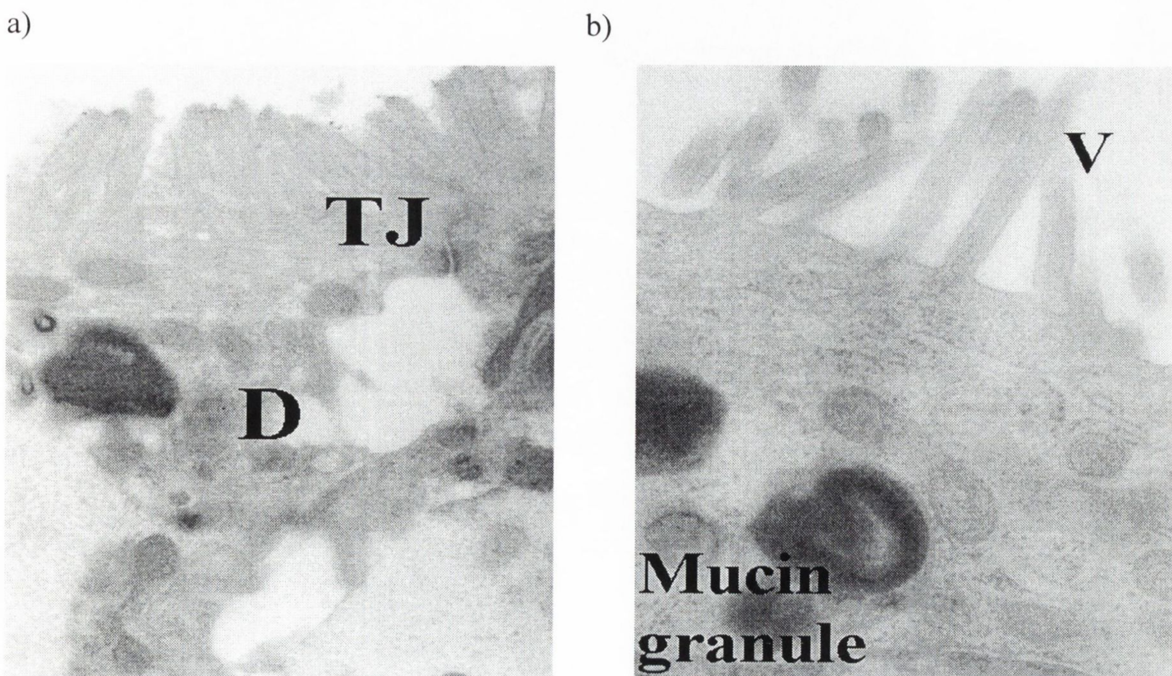


Figure 10.8 The relationship between TEER and apparent permeability (Papp) of mannitol, PEG 4000 and sCT in the presence of simple and mixed micelles of NaGC and LA across CaCo-2: Ht29GlucH co-culture monolayers pre-incubated with N-AC (0.5% w/v). Values are expressed as mean ± SE with n ≥ 4 observations.

10.8.2 Transmission electron microscopy (TEM)

The effect of micellar systems and pre-treatment with N-AC on membrane integrity was also examined by TEM. Figure 10.9a, which is a control co-culture monolayer pre-incubated with N-AC for 30min has slight dilation of the TJ compared to figure 10.3. This corroborates the observed increase in permeability of the hydrophilic compounds, mannitol, PEG 4000 and sCT seen on removal of the mucus layer.

The effect that the simple micelle NaGC had on the co-culture models morphology appears minimal; see figure 10.9b with the villi intact and mucin granules present. On removal of the mucus layer however there appears to be an increase in denudation of the villi (Figure 10.9c) and the junctional complexes appeared dilated. However, for the mixed micellar system NaGC: LA (15; 1mM) there appeared was no difference in the co-culture membranes morphology in the presence or absence of mucus, Figure 10.9d was showing the membrane in the presence of mucus with probable mucin granule labelled (M).



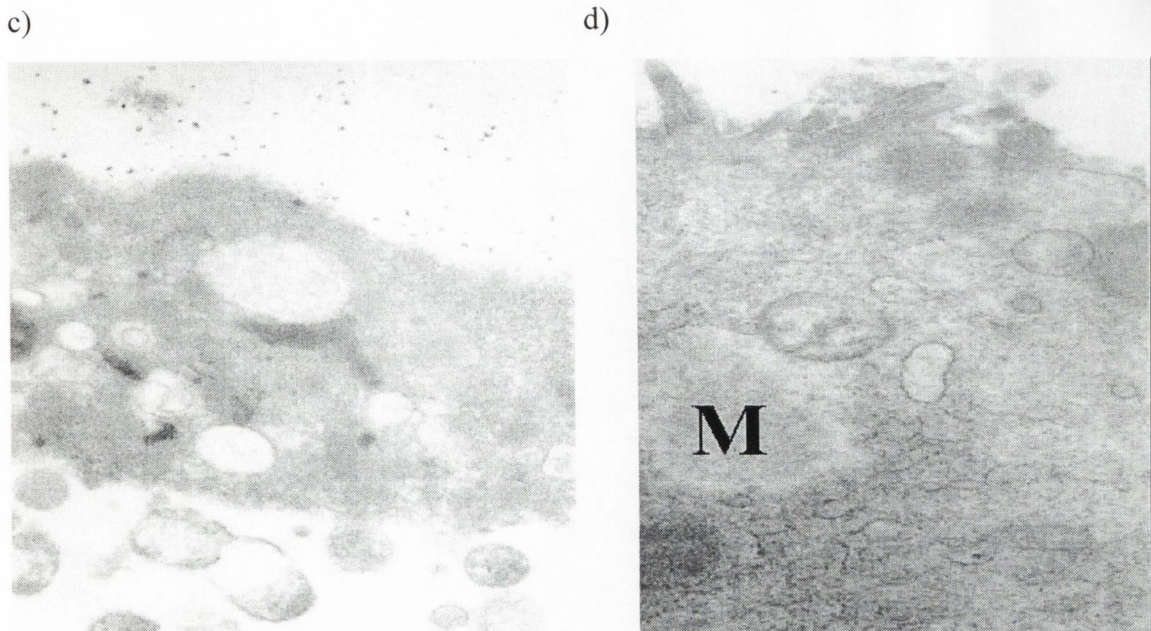


Figure 10.9 Transmission electron micrographs of CaCo-2: Ht29GlucH co-culture monolayers exposed to a) N-AC (0.5% w/v) for 30 min then HBSS for 4 h, b) NaGC (15mM) for 4 h, c) N-AC (0.5% w/v) for 30 min then to NaGC (15mM) for 4h and d) NaGC: LA (15: 1 mM) for 4h in the apical chamber (X 40,000)

10.9 Discussion

The microscopy studies confirmed that the CaCo-2 and Ht29GlucH cell lines co-exist as differentiated cell types in co-culture. The results from the ELLA assay ensured that the co-culture model was producing mucus glycoproteins.

The CaCo-2: Ht29GlucH co-culture monolayer is more permeable than the CaCo-2 cell monolayer; the permeabilities for the peptide sCT and both paracellular markers, mannitol and PEG 4000 were greater in the co-culture model than in the CaCo-2 model. This increase in the permeability of the hydrophilic compounds was confirmed by the 2.5-fold decrease in TEER in the co-culture model. This trend of an increase in the paracellular permeability of markers and decrease in TEER had been previously reported by a number of authors for the CaCo-2: Ht29GlucH co-culture cell system (Meaney and O' Driscoll, 1999; Wikman-Larhed et al., 1995; Allen,

1992).

It has been suggested that bile salts are able to cause an increase in mucin secretions *in-vivo* in dog colon (Lewin et al., 1979) and *in-vitro* in cultured dog gall bladder epithelial cells (Klinkspoor et al., 1995). These increases have been associated in both models with a depletion of intracellular mucin stores. Poelma et al., (1990) noted that there was a reduction in the viscosity of native porcine mucus in the presence of NaTC 10mM. In addition using a chronically isolated segment of the small intestine of the rat *in-vivo* he reported that NaTC caused a concentration dependant increase in the intraluminal release of hexoses (glucose equivalents). In the present study the appearance of mucin glycoproteins, which would correlate with hexoses release in the apical chamber, was determined by an enzyme linked lectin assay (ELLA). It was apparent that in the presence of bile salt NaGC there is an increase in the appearance of mucin glycoproteins in the apical chamber and a reduction in the apparent viscosity of partially purified porcine mucus (PPPM) in a concentration dependant manner.

The mixed micelle of NaGC: LA (15: 1mM) exhibited similar trends namely causing a decrease in viscosity of PPPM and an increase in mucin glycoprotein release. However the extent was not as great as what was observed for the simple micelle of NaGC 15mM. This may be attributable to possible effect that the formation of mixed micelles reduces the mucolytic effect of the bile as was proposed by Newbury et al., (1984); Marriott (1989). They demonstrated that the formation of mixed micelles between the bile salt and phospholipid lysophosphatidylcholine reduced the mucolytic effect of bile salts. In the presence of N-AC the removal of mucin from the surface was effective for concentrations $\geq$ 0.5% w/v. The ELLA indicated that post-incubation to 0.5% N-AC that the co-culture monolayer continued to produce detectable levels of mucin up to 4h.

A slight but statistically significant increase in the transport of the hydrophilic markers, mannitol and PEG 4000 and the peptide sCT across the co-culture monolayer was observed when pre-treated with N-acetyl-L-cysteine (N-AC) 0.5% w/v compared to HBSS alone (Table 10.3). There appears to be conflicting reports in the

literature regarding the effects that the mucolytic has on the permeabilities of hydrophilic markers. Wikman et al., (1992) reported that mannitol permeability was increased by the washing procedure that was used to remove the mucus layer but later, Karlsson et al., (1995), observed no difference in the transport of mannitol in the absence or presence of mucus across Ht29GlucH monolayers. Meaney and O' Driscoll (1999) reported a slight but not statistically significant increase in the permeability of the paracellular markers, mannitol and PEG 4000 in the absence of the mucus layer.

In contrast to the paracellular markers, which are non-ionisable, the increase in transport for sCT on removal of the mucus layer may be attributed to the charge interaction. At pH 7.4 sCT was positively charged (Duncan et al., 1995) and the mucus has a negative charge resulting in an electrostatic attraction which then may reduce sCT apparent permeability. This electrostatic interaction between a positively charged molecule, glucosamine, and native pig mucus negatively charged sialic acid residues have been extensively reviewed (Larhed et al., 1997). It was also reported by Meaney and O' Driscoll, (1999) that at pH 4.5 when dextropropoxyphene was highly ionised that the permeability across the co-culture monolayers was significantly reduced. It also has been reported that a reduction in the diffusion rate of glucouronic acid, a negatively charged molecule was observed for *in-vitro* mucus systems. This may be due to the repulsion between the like charges of the molecule and mucus (Wikman-Larhed et al., 1996).

It appears from the data that NaGC simple micellar systems enhancement potential was reduced in the CaCo-2: Ht29GlucH co-culture model. On removal of the mucus layer the absorption enhancing potential of the simple micellar systems was increased. In addition, the TEER reduced significantly. These results imply that the mucus layer acts as a barrier to the absorption enhancing action of NaGC simple micelles at the concentrations studied. The TEM data supported the previous finding with enhanced effects seen on the morphology of the monolayer for the simple micellar systems following removal of the mucus layer.

The removal of the mucus layer has no significant effect on the ability of the mixed micelle to enhance the transport of the hydrophilic compounds. In addition, no further reduction in TEER was observed in the absence of mucus. These results imply that mucus is not a barrier to the effects of mixed micelles on the cell.

CHAPTER 11

The effects of novel and commercial cyclodextrins (CDs) on the apparent permeabilities of the hydrophilic compounds, mannitol, PEG 4000 and sCT and on the integrity of the CaCo-2 cell monolayer

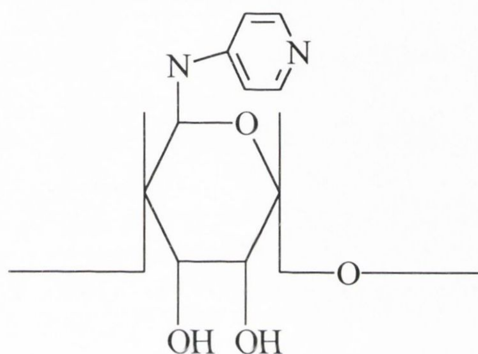
11.1 Introduction

In this chapter a range of commercial and novel CDs are investigated as alternative enhancer systems. The commercially available CDs were modified cyclodextrins and can be classified into three types: hydrophilic, hydrophobic and ionizable derivatives (Uekama et al., 1994). The novel cyclodextrins being investigated were considered to be amphiphilic and therefore similar type enhancer to the previously investigated micellar systems. However it was necessary to compare these novel CDs with the commercially available CDs. The use of cyclodextrins as an enhancer has been previously used to enhance the permeation of peptides and proteins across absorptive mucosae (Sayani and Chien, 1996; Rajewski and Stella, 1996). The exact mechanism of action for these CDs is complex and involves many processes, such as extraction of lipids and proteins from the cell wall (Irie et al., 1997), inhibition of proteolytic enzymes (Irie et al., 1992) and the molecular interaction between the CD and the compound being investigated (Uekama et al., 1994).

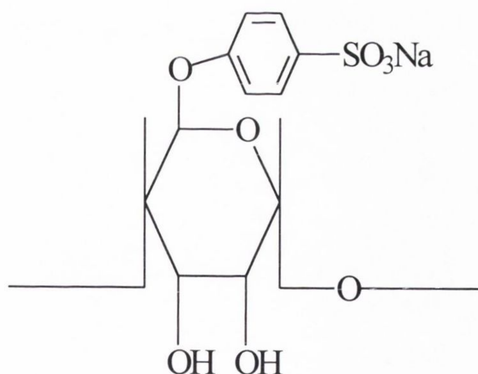
11.2 Commercial and novel cyclodextrins

The commercially available CDs (CCDs) that were investigated were three chemically modified cyclodextrins (CMCD), the hydrophilic derivatives, 2-hydroxylpropyl-β-cyclodextrin (HPCD), 2,3-dimethyl-β-cyclodextrin (DMCD) and the anionic ionizable derivative sulfobutyl ether β-cyclodextrin with 7 degrees of substitution (SBE_7CD). Three novel sulphated amphiphilic cyclodextrins, heptakis (6-deoxy-6-pyridylamino)-β-cyclodextrin sodium salt (ACD), heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin (HCD) and heptakis (6-O-sulphonatophenyl)-β-cyclodextrin sodium salt (PCD) were also studied (figure 11.1). NMR data for the novel cyclodextrins provided information concerning the structure and was used as an in-house quality standard, see Appendix II. These novel CDs were investigated primarily because they are classified as amphiphiles and are capable of forming aggregates (Ramphul, 1998; Holohan, 1999) so therefore are similar to the bile salt systems. HCD was the first NCDs

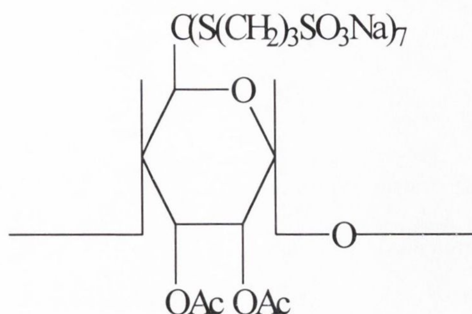
to be investigated, structurally it has a short polar side chain on the primary face and hydrophobic acetyl groups on the secondary face. To ascertain if increasing the side chain length had any effect on the CDs characteristics PCD was synthesised, which incorporates a phenyl group into the side chain on the primary face of the cyclodextrin. An alternative side chain on the primary face of the CDs was the aminopyridyl, which is a long chain hydrophilic moiety. The effect that these CDs had on the transport of the peptide sCT and both paracellular markers, mannitol and PEG 4000 and the integrity of the monolayer were investigated initially in the CaCo-2 cell culture model.



Heptakis (6-deoxy-6-aminopyridyl)-β-cyclodextrin sodium salt (ACD)*



Heptakis (6-O-sulphonylophenyl)-β-cyclodextrin sodium salt (PCD)*



Heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin (HCD)[†]

Figure 11.1 The structure of the novel cyclodextrins synthesised by Ramphul (1998)[†] and Holohan (1999)*.

11.3 Examining the toxicity of cyclodextrins

Prior to transport and stability studies cytotoxicity was examined using the MTT assay and fluorescent microscopy. These assays provided information regarding suitable concentrations of CD to be investigated.

11.3.1 Intracellular Enzyme Activity

Intracellular dehydrogenase activity in the presence of the CCDs was determined by the MTT method (Mossman et al., 1983). The intracellular enzyme activity of CaCo-2 cells decreased after exposure to DMCD at concentrations above 1% w/v with an IC_{50} value of 1.9 ± 0.8 % w/v (Fig. 11.2), which closely resembles previously published values of 1.87 ± 0.27 % by Tötterman et al., (1997) and 3% w/v by Høvgaard et al., (1995b).

The IC_{50} value for HPCD was 14.5 ± 1.1% w/v. Tötterman et al., (1997) found that HPCD increased the intracellular dehydrogenase activity in the concentration range studied 3- 100mM (0.4- 13.3 % w/v), which is below the IC_{50} value calculated. Saarinen-Savolainen et al., (1998) observed that HPCD 13.3 % w/v caused the intracellular

enzyme activity to decrease to 59% of control using human corneal epithelial cells (HCE).

The concentration range 0.2-1% w/v investigated for BCD showed only a slight decrease in enzyme activity (67.6- 87.8% cells viability), and examining higher concentrations was not possible due to the poor aqueous solubility of BCD (1.86% w/v).

SBE_7CD exhibited little effect on intracellular enzyme activity over 1 –10% w/v with the enzyme activity decreasing to 75 ± 3.1 % at the maximum concentration of 10% w/v which correlated with Tötterman et al., (1997).

As the MTT was based on a colourimetric analysis it was unsuitable for testing the novel CDs since they produced a colour in solution, which interfered with the assay.

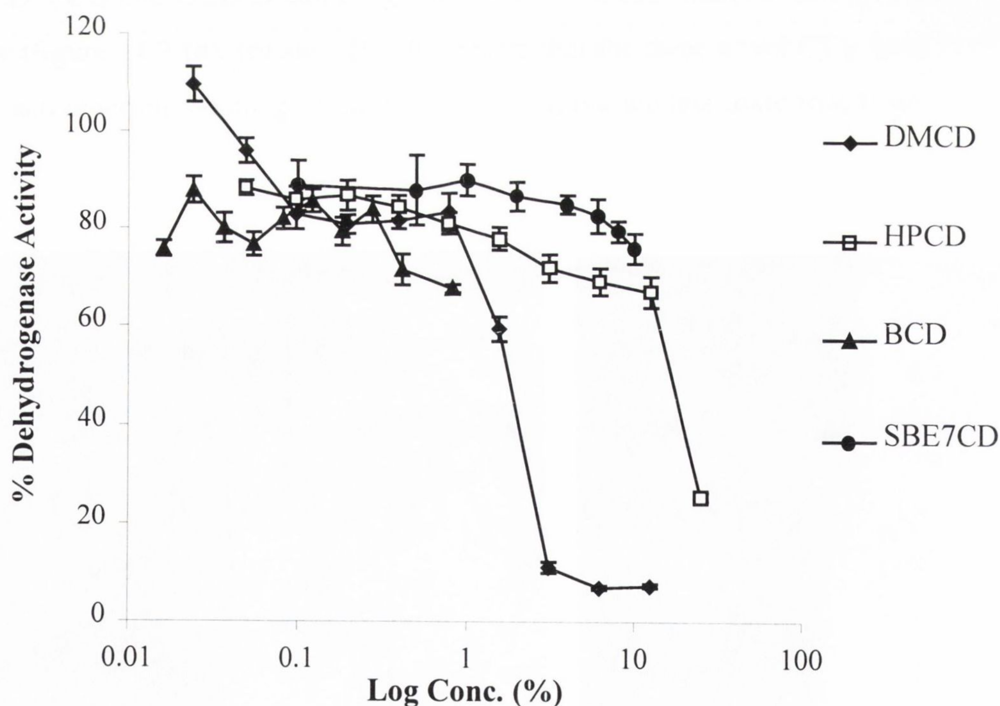


Figure 11.2 The effects of 2,3-dimethyl-β-cyclodextrin (DMCD), 2-hydroxylpropyl-β-cyclodextrin (HPCD), β-cyclodextrin (BCD) and sulfobutyl-β-cyclodextrin (SBE7CD)

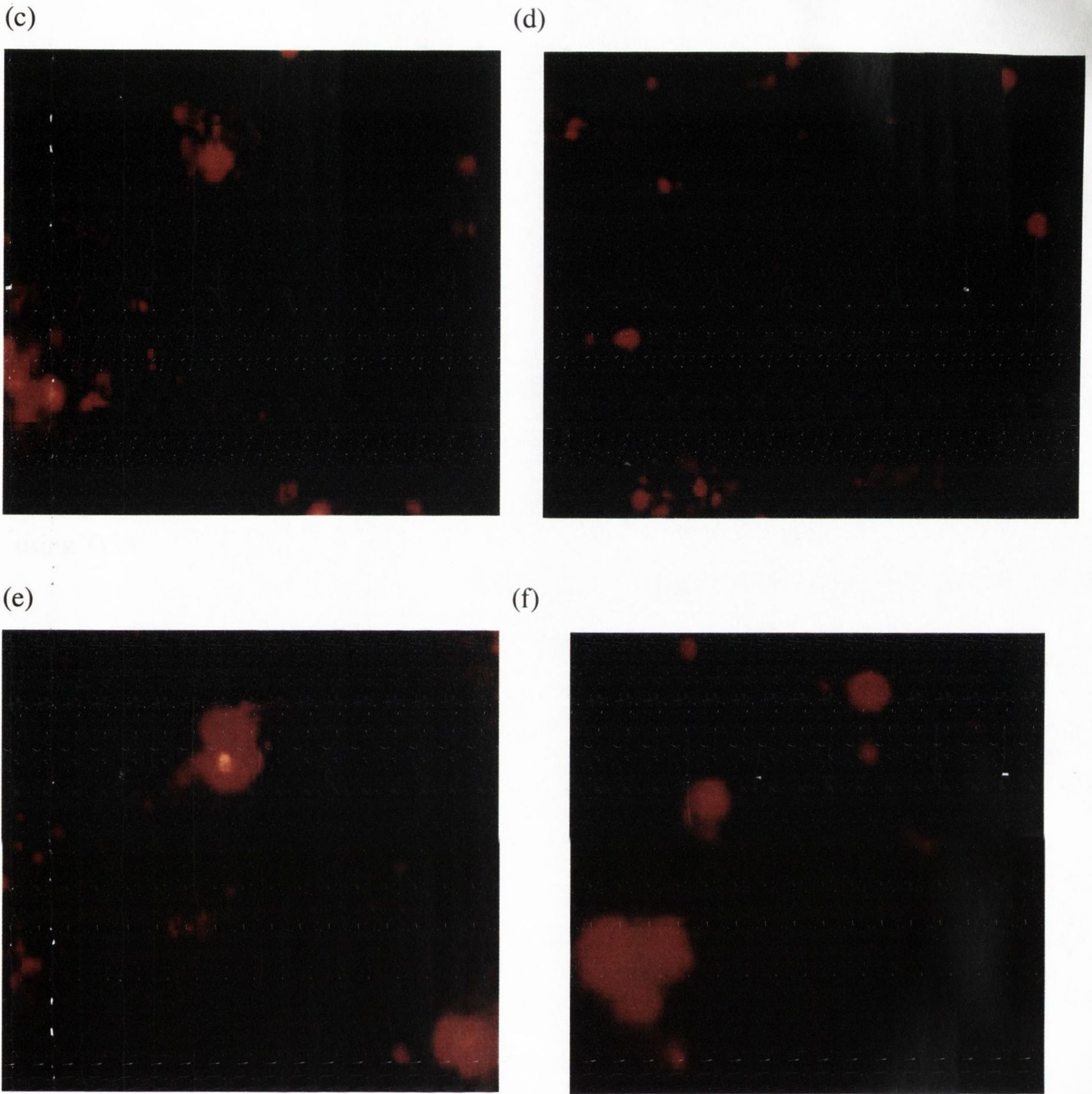


Fig. 11.3 Photographs of CaCo-2 cells incubated with

(a) Control HBSS (b) DMCD (3% w/v)

(c) HPCD (10%w/v) (d) ACD (10%w/v)

(e) PCD (10% w/v) (f) HCD (10% w/v)

for 1 hr respectively and then stained with propidium iodide.

11.4 The effects of commercial and novel cyclodextrins on the stability of sCT

Due to the labile nature of peptides the stability of salmon calcitonin (sCT) was investigated in the presence of the cyclodextrins. It has been reported that CDs have enhanced the stability of dry powder peptide formulations and also have enzyme inhibitory effects (Brewster et al., 1991; Haeberlin et al., 1996). The concentration range studied was based on the IC_{50} values (see figure 11.2) and the propidium iodide staining (see figure 11.3). To detect intact sCT an assay involving the precipitation of the peptide using TCA as outlined in materials and methods see Chapter 7, was used.

Commercial cyclodextrins (CCDs)

2,3-dimethyl-β-cyclodextrin (DMCD)

The stability of the peptide calcitonin (sCT) and CDs sample in the apical chamber was investigated immediately after preparation and warming to 37^0C, time = 0min. The concentration range of DMCD investigated was 1%- 3 %w/v, which incorporates the IC_{50} value as estimated by the cytotoxicity experiments. The percentage of sCT degraded at time 0 min was similar to what was seen for the drug alone (Table 11.1). However, after 4h exposure there was a significant increase in the degradation of sCT in the presence 3 % w/v (Appendix 1).

2-hydroxylpropyl-β-cyclodextrin (HPCD)

In the presence of the HPCD concentration range studied the effect on sCT stability was similar to sCT alone (Table 11.1). However, there it appeared that the lower concentration of CDs offered greater protection to the peptide. There was no destabilisation of the peptide, sCT in the presence of HPCD over the time course of the experiment (4h).

Sulfobutyl-β-cyclodextrin (SBE_7CD)

At the beginning of the experiment in the presence of SBE_7CD 10% w/v and 20% w/v the concentration of sCT degraded was similar to the control (Table 11.1). in the presenc of SBE_7CD the stability of sCT was not affected with the amount degraded at 4h similar to control (Appendix 1)

The results from the stability experiment indicated that DMCD 3% w/v affected the stability of the peptide salmon calcitonin (sCT) at 4h whereas this was not seen in the presence of other commercial CDs at the concentrations studied.

Table 11.1 The effect of commercially available CDs on the stability of sCT in the apical chamber of the CaCo-2 cell monolayer at the onset of a 4h experiment.

System	% sCT Degraded ± SE of the Initial Starting Conc. at onset of experiment (n ≥ 3)
sCT	7.92± 4.09
DMCD 1% w/v	5.44 ± 2.04
DMCD 3% w/v	8.08 ± 2.27
HPCD 1% w/v	1.26 ± 1.21
HPCD 5% w/v	5.74 ± 3.35
HPCD 10% w/v	10.10 ± 5.55
SBE_7CD 10% w/v	11.09 ± 1.04
SBE_7CD 20% w/v	16.84 ± 3.67

Novel cyclodextrins (NCDs)

Heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin (HCD)

At the beginning of the experiment in the presence of the higher concentration of HCD 10 % w/v and 20% w/v the stability of the peptide sCT decreased when compared to the control, see Table 11.2.

Heptakis (6-deoxy-6-pyridylamino)-β-cyclodextrin sodium salt (ACD)

At the beginning of the experiment there appears to be a concentration dependant increase in the percentage of degraded sCT, see Table 11.2.

Heptakis (6-O-sulphonatophenyl)-β-cyclodextrin sodium salt (PCD)

A similar trend was seen for PCD as the concentration increased the percentage sCT degraded increased at the initial onset of the experiment.

Generally at the onset of the experiment as the concentration of NCDs increases the percentage of degraded sCT increased.

Table 11.2 The effect of novel CDs on the stability of sCT in the apical chamber of the CaCo-2 cell monolayer at the onset of a 4h experiment.

System	% sCT Degraded ± SE of the Initial Starting Conc. at onset of experiment (n ≥ 3)
sCT	7.92± 4.09
HCD 5 % w/v	6.34 ± 0.60
HCD 10 % w/v	13.57 ± 6.55
HCD 20 % w/v	45.21 ± 11.53
ACD 5 % w/v	12.86 ± 1.91
ACD 10 % w/v	16.98 ± 8.89
ACD 20 % w/v	23.40 ± 6.35
PCD 5 % w/v	8.98 ± 2.12
PCD 10 % w/v	14.61 ± 2.89
PCD 20 % w/v	15.12 ± 3.15

11.5 Transport of hydrophilic compounds in the presence of CCDs and NCDs

Following these initial studies, the apparent permeability coefficients (P_{app}) of the hydrophilic compounds, mannitol (MW 182), sCT (MW 3413) and PEG 4000 across the CaCo-2 cell monolayer were estimated in the presence of the cyclodextrins.

Commercially available cyclodextrins (CCDs)

2,3-dimethyl-β-cyclodextrin (DMCD)

In the presence of DMCD there appeared to be a concentration dependant increase in the transport of sCT across the CaCo-2 cell monolayer. The Papp increased 2.6 and 7.6- fold

for DMCD 1% w/v and DMCD 3% w/v respectively. A similar trend was seen for the paracellular markers, mannitol and PEG 4000, as the concentration of DMCD increased the transport of these compounds increased. The Papp for mannitol increased 1.3 and 3.8-fold and for PEG 4000 increased 3.6 and 7.6-fold for DMCD 1% w/v and 3% w/v respectively, see table 11.3. Similar increases in the relative enhancement of mannitol permeability in the presence of DMCD have previously been reported (Tötterman et al., 1997). In that study the absorption of mannitol in the presence of DMCD 2% w/v and 3% w/v increased 2.12 and 3.7-fold respectively. Hovgaard et al., (1995) indicated that DMCD 2.5% w/v caused a slight increase in the transport of PEG 4000 compared to control, with approximately 1.8% of the marker in the basolateral chamber after 225min exposure. In this study after 240min exposure to DMCD 1% w/v and 3% w/v, 1.22 ± 0.30 % and 2.33 ± 0.35 % PEG 4000 was in the basolateral chamber respectively.

<u>2-hydroxylpropyl-β-cyclodextrin (HPCD)</u>

In the case of HPCD as the concentration increased from 1% w/v to 5% w/v to 10% w/v the Papp of sCT increased. However the enhancement was not significantly different from the sCT alone i.e. control for 1%w/v and 5%w/v HPCD. However, the Papp of the paracellular markers, mannitol and PEG 4000 in the presence of HPCD 10% w/v were significantly enhanced compared to the control, see table 11.3. This corresponds to what has previously been shown (Tötterman et al., 1997 and Hovgaard et al., 1995). In these studies it was apparent that concentrations of HPCD 5% w/v and less caused no increase in the transport of mannitol and PEG 4000 compared to the control.

<u>Sulfobutyl-β-cyclodextrin (SBE_7CD)</u>

In the presence of SBE_7CD 20% w/v the transport of sCT increased 11.6-fold. No significant enhancement in the transport of sCT was seen with SBE_7CD 10% w/v, see table 11.3. A similar trend was observed for the paracellular markers, mannitol and PEG 4000 in the presence of SBE_7CD 10% w/v and 20% w/v. Tötterman et al., (1997) noted

no enhancement effects on mannitol across the CaCo-2 cell monolayer using concentrations of SBE_7CD 3, 4.5 and 6% w/v.

Table 11.3 The apparent permeability (Papp) and the percentage transported across the CaCo-2 cell monolayer over 4 hours of the hydrophilic compounds, mannitol, PEG 4000 and sCT for each of the commercially available cyclodextrins (CCDs): 2-hydroxylpropyl-β-cyclodextrin (HPCD), 2,3-dimethyl-β-cyclodextrin (DMCD) and sulfobutyl ether β-cyclodextrin (SBE_7CD).

System	Papp ± SD ($X10^6$) (cm/sec) ($n \geq 3$) ($p<0.05$, *) (Total Percentage transported after 4hr)		
	Mannitol	**PEG 4000**	**sCT**
sCT	0.46 ± 0.02 (2.9 ± 0.17%)	0.05 ± 0.01 (0.34 ± 0.04%)	0.05 ± 0.01 (0.33 ± 0.05%)
DMCD 1% w/v	0.60 ± 0.03 (4.19 ± 0.25%)	0.18 ± 0.04 (1.22 ± 0.30%)	0.13 ± 0.05 (0.97 ± 0.09%)
DMCD 3% w/v	1.74 ± 0.38* (10.68 ± 2.41%)	0.38 ± 0.05* (2.33 ± 0.35%)	0.38 ± 0.07* (2.34 ± 0.41%)
HPCD 1% w/v	0.47 ± 0.03 (0.18 ± 0.04%)	0.03 ± 0.01 (0.18 ± 0.04%)	0.03 ± 0.005 (0.18 ± 0.05%)
HPCD 5% w/v	0.41 ± 0.04 (3.71 ± 0.81%)	0.03 ± 0.01 (0.54 ± 0.37%)	0.05 ± 0.02 (0.35 ± 0.10%)
HPCD 10% w/v	0.76 ± 0.13* (5.16 ± 0.98%)	0.21 ± 0.09* (1.36 ± 0.64%)	0.15 ± 0.07 (1.06 ± 0.49%)
SBE_7CD 10% w/v	0.32 ± 0.01 (2.15 ± 0.07%)	0.05 ± 0.01 (0.30 ± 0.07%)	0.05 ± 0.02 (0.29 ± 0.07%)
SBE_7CD 20% w/v	4.10 ± 0.64* (25.66 ± 3.79%)	0.63 ± 0.08* (4.06 ± 0.57%)	0.58 ± 0.07* (3.79 ± 0.47%)

Novel cyclodextrins (NCDs)

Heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin (HCD)

As the concentration of HCD increase there was no statistically significant increase in the transport of sCT. The Papp for 5% w/v, 10% w/v and 20% w/v being similar, 0.17 ± 0.03 X 10^{-6} cm/sec, 0.19 ± 0.03 X 10^{-6} cm/sec and 0.14 ± 0.01 X 10^{-6} cm/sec respectively. There was a statistically significant increase in the permeability of the marker PEG 4000 with all concentrations of HCD, however for mannitol the Papp was significantly increased in the presence of HCD 10% w/v and HCD 20% w/v, see table 11.4.

Heptakis (6-deoxy-6-pyridylamino)-β-cyclodextrin sodium salt (ACD)

There appeared to be a concentration dependant increase in the transport of sCT and the paracellular markers across the CaCo-2 cell monolayer as the concentration of ACD increased, see table 11.4. ACD 5% w/v caused no enhancement in the transport of the hydrophilic compounds compared to the control. ACD 10% w/v had a minor effect on the transport of sCT, mannitol and PEG 4000 with the Papp increasing 4.0, 1.63 and 2.6-fold respectively. However for ACD 20% w/w the increase in the apparent permeability of the compounds was statistically greater with an 29.6, 17.1 and 15.8-fold increase for sCT, mannitol and PEG 4000 respectively, $p < 0.01$.

Heptakis (6-O-sulphonatophenyl)-β-cyclodextrin sodium salt (PCD)

For this cyclodextrin concentrations of 10% w/v and 20% w/v caused an increase in the permeability of the hydrophilic markers, see table 11.4. PCD 10% w/v caused a similar fold enhancement to ACD 10% w/v with a 2.6, 1.6 and 3.2-fold increase in the Papp of sCT, mannitol and PEG 4000. When the concentration was increased to 20% w/v there was a statistically significant increase in the transport of sCT, mannitol and PEG 4000 was 12.2, 2.13 and 13.6-fold respectively, $p< 0.05$.

Table 11.4 The apparent permeability (Papp) and the percentage transported across the CaCo-2 cell monolayer over 4 hours of the hydrophilic compounds, mannitol, PEG 4000 and sCT for each of the novel cyclodextrins (NCDs): Heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin (HCD), Heptakis (6-deoxy-6-pyridylamino)-β-cyclodextrin sodium salt (ACD) and Heptakis (6-O-sulphonatophenyl)-β-cyclodextrin sodium salt (PCD).

System	Papp ± SD (X10^{6}) (cm/sec) (n ≥ 3) (p<0.05, *) (Total Percentage transported after 4hr)		
	Mannitol	PEG 4000	sCT
sCT	0.46 ± 0.02 (2.9 ± 0.17%)	0.05 ± 0.01 (0.34 ± 0.04%)	0.05 ± 0.01 (0.33 ± 0.05%)
HCD 5 % w/v	0.38 ± 0.09 (2.64 ± 0.17%)	0.19 ± 0.04* (1.32 ± 0.25%)	0.17 ± 0.03 (1.16 ± 0.21%)
HCD 10 % w/v	0.77 ± 0.20* (5.09 ± 1.38%)	0.46 ± 0.10* (2.99 ± 0.70%)	0.19 ± 0.03 (1.23 ± 0.13%)
HCD 20 % w/v	0.85 ± 0.05* (4.94 ± 0.25%)	0.19 ± 0.03* (1.12 ± 0.12%)	0.14 ± 0.01 (0.81 ± 0.06%)
ACD 5 % w/v	0.16 ± 0.009 (1.22 ± 0.07%)	0.02 ± 0.0003 (0.11 ± 0.01%)	0.01 ± 0.0004 (0.07 ± 0.01%)
ACD 10 % w/v	0.75 ± 0.04* (5.22 ± 0.40%)	0.13 ± 0.01 (0.88 ± 0.13%)	0.20 ± 0.03 (1.30 ± 0.17%)
ACD 20 % w/v	7.88 ± 0.05* (46.45 ± 0.29%)	0.79 ± 0.04* (4.82 ± 0.23%)	1.48 ± 0.04* (8.74 ± 0.47%)
PCD 5 % w/v	0.36 ± 0.06 (2.29 ± 0.34%)	0.05 ± 0.01 (0.30 ± 0.07%)	0.04 ± 0.01 (0.25 ± 0.07%)
PCD 10 % w/v	0.74 ± 0.17* (4.62 ± 1.15%)	0.16 ± 0.05 (1.03 ± 0.36%)	0.13 ± 0.03 (0.82 ± 0.20%)
PCD 20 % w/v	0.98 ± 0.05* (6.18 ± 0.29%)	0.68 ± 0.06* (4.18 ± 0.33%)	0.61 ± 0.05* (3.69 ± 0.24%)

From the data it appears that ACD 20% w/v and PCD 20% w/v are the most suitable of the novel cyclodextrins as candidates for increasing the transport of the hydrophilic compounds.

Because these cyclodextrins were produced as sodium salts with high degrees of substitution of 2-5, high osmolarity has been associated with such formulations and could have been responsible for the enhanced transport. It has previously been reported that SBE_7CD at concentrations greater than 5% w/v were hypertonic (Saarinen-Savolainen et al., 1998; Croyle et al., 1998) and this may be contributing to the enhancement seen in the hydrophilic compounds. Therefore it was important to investigate that the effects that hypertonic solutions had on the transport of sCT, mannitol and PEG 4000 across the CaCo-2 cell monolayer.

11.6 Effect of osmolarity on the transport of sCT, mannitol and PEG 4000 across the CaCo-2 cell monolayer.

Osmolarities of all the cyclodextrins were measured in triplicate using a freezing point depression osmometer (table 11.5). To factor out the potential effects of hypertonicity Mannitol solutions (5% w/v, 10% w/v and 15% w/v) were prepared with similar osmolarity to the cyclodextrin formulations and their effects on the transport of sCT, mannitol and PEG 4000 across the CaCo-2 cell monolayer were examined. Mannitol was chosen as a suitable candidate to prepare hypertonic solutions based on previous reports (Jørgensen et al., 1993).

In this study the term isotonic is taken to be 290-310 mOs kg^{-1} as isotonic buffers were within this range and corresponded to previously published data (Croyle et al., 1998). The osmolarity of the cyclodextrin formulations varied from 346.3 ± 3.2 mOs kg^{-1} to 1004.3 ± 2.5 mOs kg^{-1} depending on concentration (Table 11.5). It has previously been shown that osmolarities up to 350 mOs kg^{-1} do not affect the integrity of the cell monolayer (Tötterman et al., 1997; Anderberg et al., 1993).). In table 11.6 a hypertonic mannitol solution (5% w/v) did not increase the transport of the hydrophilic compounds

when compared to an isosmotic control. However, Mannitol 10% w/v (925.7 ± 2.1 mOs kg $^{-1}$) caused a 3.2, 1.6 and 2-fold enhancement in the transport of sCT, mannitol and PEG 4000 respectively. A further increase in concentration of mannitol to 15% w/v having an osmolarity of 1199.7 ± 2.1 mOs kg $^{-1}$ produced an 8.2, 2.5 and 9.8-fold increase in the transport of the hydrophilic compounds, sCT, mannitol and PEG 4000.

Table 11.5 The osmolarity of the commercially available and novel cyclodextrin formulations.

Cyclodextrin (% w/v) in HBSS	**Osmolarity (mOs kg $^{-1}$)**
0	310.0 ± 4.7
CCDs	
HPCD (10)	442.7 ± 0.6
DMCD (3)	346.3 ± 3.2
SBE_7CD (10)	615 ± 3.5
SBE_7CD (20)	1004.3 ± 2.5
NCDs	
ACD (10)	463 ± 1.7
ACD (20)	579 ± 1
PCD (10)	572.3 ± 0.6
PCD (20)	844 ± 2

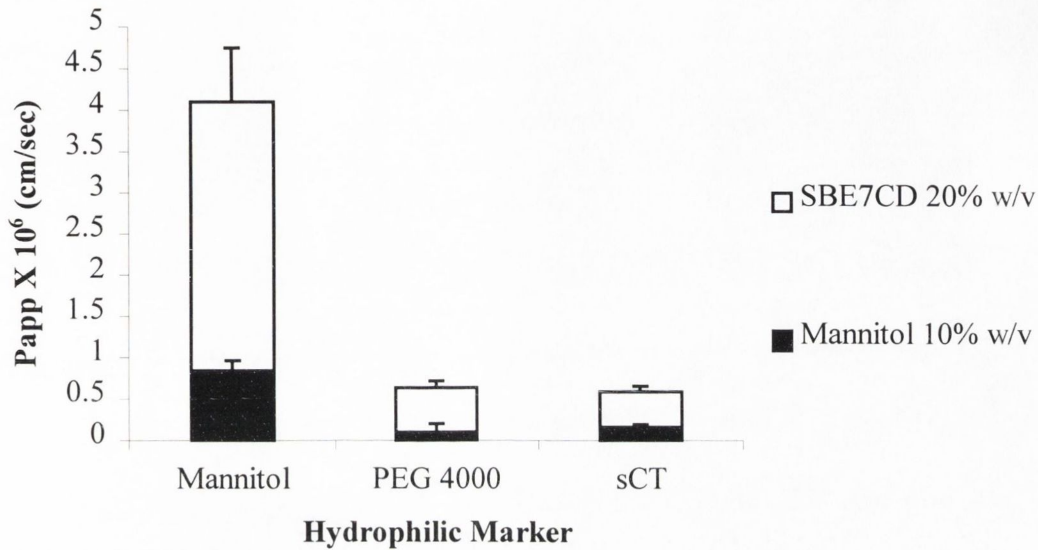
Table 11.6 The effect that hypertonic solutions of mannitol had on the apparent permeabilities of sCT, mannitol and PEG 4000 across the CaCo-2 cell monolayer.

Mannitol (% w/v)	Osmolarity ($mOs\ kg^{-1}$)	Papp ± SD ($X10^{-6}$) (cm/sec) ($n \geq 3$) ($p < 0.05$, *; $p < 0.01$, **)		
		Mannitol	PEG 4000	sCT
0	310.0 ± 4.7	0.46 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
5	634.3 ± 2.1	0.34 ± 0.02	0.05 ± 0.02	0.06 ± 0.004
10	925.7 ± 2.1	0.84 ± 0.12*	0.10 ± 0.10	0.16 ± 0.03
15	1199.7 ± 2.1	1.16 ± 0.10**	0.49 ± 0.06**	0.41 ± 0.02**

It is clear that most of the CDs formulations have osmolarity value less than or equivalent to mannitol 5% w/v and consequently tonicity will not be a significant contributor to the absorption enhancement of theses excipients at the concentrations studied. Only PCD 20% w/v (844 ± 2 $mOs\ kg^{-1}$) and SBE_7CD 20% w/v (1004.3 ± 2.5) have higher osmolarity values, similar to Mannitol 10% w/v, which may contribute to the absorption enhancement.

Figure 11.4 (a) and (b) compares the Papp achieved by a mannitol solution (10%w/v) of similar hypertonicity to the total Papp achieved with SBE_7CD (20% w/v) and PCD (20% w/v). In the case of SBE_7CD the hypertonicity does not appear to be the major influence on the overall Papp. For PCD the same trend was observed for larger molecular weight compounds, PEG 4000 and sCT.

a) SBE_7CD (20% w/v)



b) PCD (20% w/v)

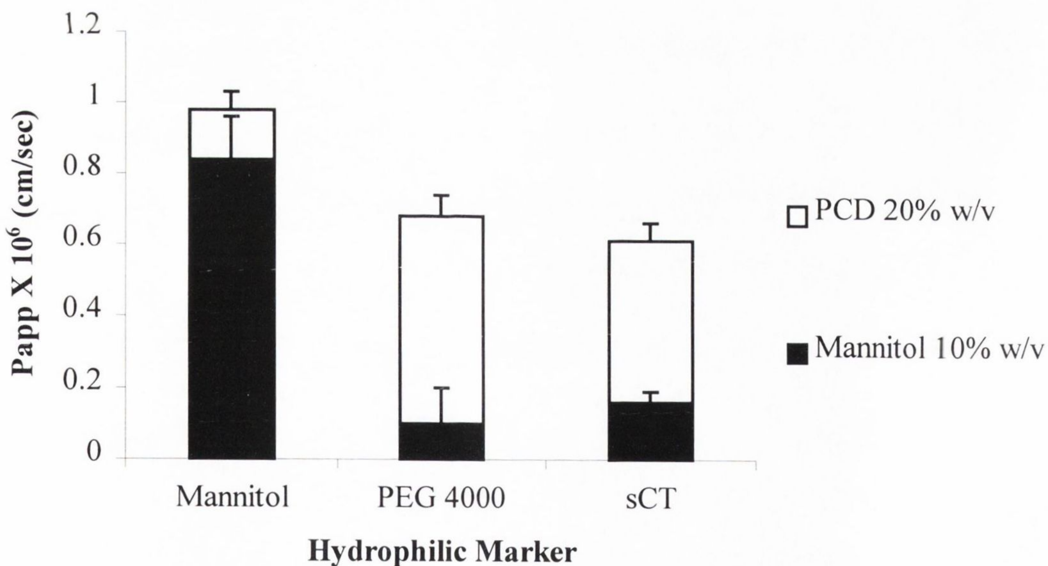


Figure 11.4 The Papp of the hydrophilic compounds, sCT, mannitol and PEG 4000 across the CaCo-2 cell monolayer in the presence of (a) SBE_7CD (20% w/v) and (b) PCD (20% w/v) compared to Mannitol (10% w/v) with similar tonicity. Values are expressed as mean ± SD for at least 3 observations.

11.7 The Effect of CCDs and NCDs on CaCo-2 Cell Monolayer Integrity

In preceding sections commercially available and novel cyclodextrins have enhanced the transport of the peptide, salmon calcitonin (sCT) and both paracellular markers mannitol and PEG 4000. It has been previously reported that this absorption enhancement may be associated with the extraction of membrane components of the monolayer and also an alteration of the tight junction permeability (Hovgaard et al., 1995; Haeberlin et al., 1996). The methods employed to determine the effect that these cyclodextrin systems had on the CaCo-2 cell monolayer were transepithelial electrical resistance (TEER) and transmission electron microscopy (TEM).

11.7.1 Transepithelial Electrical Resistance (TEER)

The TEER was monitored throughout the transport experiment as a measure of monolayer integrity. The initial TEER value was determined following 30 min equilibration in HBSS prior to beginning of the transport experiments at room temperature (25^0C). In all cases TEER values were expressed as a percentage of this initial value i.e. 100%.

Commercially available cyclodextrins (CCDs)

There was a concentration dependent decrease in TEER values for 2,3-dimethyl-β-cyclodextrin (DMCD) as the concentration increased (Table 11.7). . Following 1hr exposure of the CaCo-2 cell monolayer to DMCD 1% w/v the TEER was similar to original value at $101.26 \pm 1.14\%$ of the. At the end of the transport experiment 4hr later the TEER value had dropped to 71.72 ± 5.11 %. However, after exposure of the monolayer to DMCD 3% w/v a greater decrease was seen between 1 hr and 4 hr compared to DMCD 1% w/v. The changes in the TEER correlate with the increase in the transport of the hydrophilic compounds, sCT and the paracellular markers, mannitol and PEG 4000, see section 11.5. The reductions in TEER values for the other commercially available cyclodextrins (CCDs) 2-hydroxylpropyl-β-cyclodextrin (HPCD) and

sulfobutyl-β-cyclodextrin (SBE_7CD) were similar to those previously been reported (Tötterman et al., 1997). It is clear that HPCD had less of an effect on the monolayer at the concentrations studied compared to DMCD. From the TEER data it was noted that DMCD 1% w/v and HPCD 10% w/v caused similar reductions with TEER dropping to 71.72 ± 5.11% and 73.67 ± 4.69% respectively after 4h. In the case of SBE_7CD there was a concentration dependant decrease in the TEER. This was also reflected in the transport data for the peptide sCT, and the two paracellular markers, mannitol and PEG 4000.

Table 11.7 The effect of commercially available cyclodextrins, DMCD, HPCD and SBE_7CD on TEER of CaCo-2 cell monolayers after 1hr and 4 hr exposure.

System (n)	% TEER ± SD ($p < 0.05$,*) 1 hr	4 hr
sCT (33)	94.56 ± 10.6	83.59 ± 15.62
DMCD 1 %w/v (4)	101.26 ± 1.14	71.72 ± 5.11
DMCD 3 %w/v (4)	67.76 ± 2.85	36.69 ± 2.01*
HPCD 1% w/v (3)	101.77 ± 0.67	88.50 ± 0.59
HPCD 5% w/v (6)	105.32 ± 15.75	93.23 ± 1.70
HPCD 10% w/v (3)	102.74 ± 2.86	73.67 ± 4.69
SBE_7CD 10% w/v (3)	98.32 ± 1.21	70.29 ± 3.24
SBE_7CD 20% w/v (6)	74.12 ± 17.92	39.92 ± 1.37*

The relationship between the increase in the Papp of sCT, mannitol and PEG 4000 and the decrease in TEER after 4h in the presence of the CDs was not good. An R^2 correlation of less than 0.9 was calculated for all cases with R^2 equal to 0.684, 0.782 and 0.888 for mannitol, PEG 4000 and sCT respectively. This may suggests that the CCDs are operating as permeation enhancers by not only affecting the integrity of the tight

junction but also as the literature suggests affecting the lipid structure of the cell membrane (Ohtani et al., 1989; Shao et al., 1992; Shiotani et al., 1995).

Novel cyclodextrins (NCDs)

In the case of the NCDs it was apparent from table 11.8 that there was a concentration dependant effect on the TEER values. HCD at the lower concentration 5% w/v had little or no effect on the TEER. The TEER however decreased drastically in the presence of 10% w/v and 20% w/v HCD, and after 1h exposure, was less than 50% of the original value in both cases, 44.90 ± 5.85 % and 25.09 ± 0.96% respectively. Exposure of the membrane to HCD 10% and 20% for 4h resulted in a further decrease in the TEER. For PCD and ACD similar trends were observed, see table 11.8. In the case of the novel cyclodextrins no direct correlation exits between decrease in TEER and the increase in Papp of the hydrophilic compounds, mannitol, PEG 4000 and sCT.

Table 11.8 The effect of novel cyclodextrins, HCD, PCD and ACD on the TEER of the CaCo-2 cell monolayers after 1hr and 4 hr exposure.

System (n)	% TEER ± SD ($p < 0.05$, *) 1 hr	4 hr
sCT (33)	94.56 ± 10.6	83.59 ± 15.62
HCD 5 %w/v (3)	88.63 ± 6.12	78.32 ± 1.02
HCD 10 %w/v (3)	44.90 ± 5.85*	30.04 ± 0.76*
HCD 20 %w/v (3)	25.09 ± 0.96*	14.55 ± 0.55*
ACD 5 % w/v (3)	92.17 ± 1.60	110.93 ± 14.38
ACD 10 % w/v (3)	50.11 ± 3.72*	35.42 ± 2.10*
ACD 20 % w/v (3)	38.62 ± 4.20*	32.62 ± 3.23*
PCD 5 % w/v (3)	75.05 ± 9.87	76.15 ± 6.25
PCD 10 % w/v (3)	41.09 ± 1.21*	33.52 ± 0.55*
PCD 20 % w/v (3)	34.15 ± 1.22*	24.08 ± 0.95*

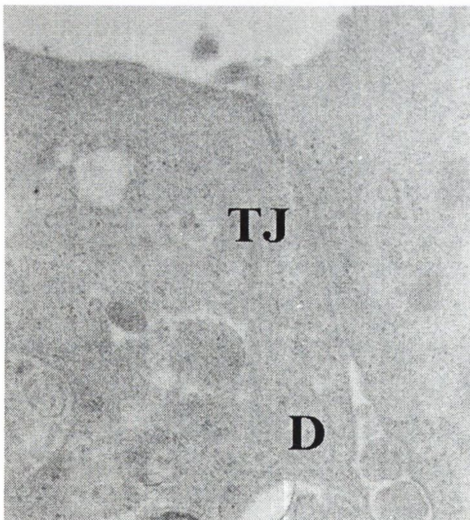
11.7.2 Transmission electron microscopy (TEM)

The TEER values indicated that there was some disruption to the integrity of the CaCo-2 cell monolayer due partly to alteration of the tight junctional complex. Transmission electron microscopy (TEM) was used to complement and extend the TEER studies. This technique enables one to view the effect that the commercially available and novel cyclodextrins have on the structure of the CaCo-2 cell membrane and tight junction.

Commercially available cyclodextrins (CCDs)

Exposing the CaCo-2 cell monolayer to the commercial cyclodextrins result in some morphological changes to the membrane, see Fig 11.5 (a-d). DMCD 3%w/v demonstrated that it had a greater effect on the membrane compared to the other commercial CDs (Fig 11.5a) with the cells at the apical surface parted, no villi present and dilation of the junction. It appears that HPCD 10% w/v (Fig 11.5b) caused little effect, which was reflected in this compound overall lower toxicity than DMCD 3% (Loftsson and Brewster, 1996). For SBE_7CD 10% and 20% both of which are hypertonic, typical morphological changes are apparent with the appearance of vacuoles (Va) and disordered villi (DV) but the membrane remained intact (Fig 11.5c and d respectively). These finding have reflect previous findings when CaCo-2 cell monolayer were exposed to hypertonic glucose solutions (Jorgenson et al., 1993)

a)



b)

d) e)

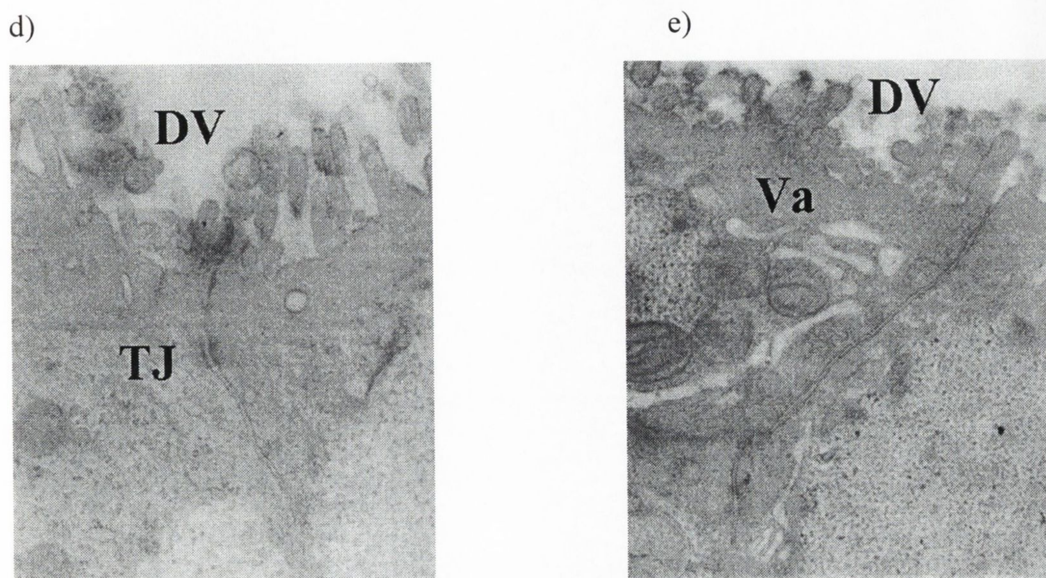


Figure 11.5 The effect that the commercial cyclodextrins (a) DMCD 3% w/v, (b) HPCD 10% w/v, (c) SBE_7CD 10% w/v and SBE_7CD 20% w/v have on the morphology of the CaCo-2 cell monolayer

Novel cyclodextrins (NCDs)

The TEM data reflects previous finding with minor changes observed for the lower NCDs concentration with significant effect for ACD and PCD 20% w/v. Figure 11.6 (a) is an example of the lower concentration of 10% w/v PCD effect on the membrane, eliciting only very minor changes. All other concentrations of novel CDs at 10% w/v caused similar effects on the CaCo-2 cell monolayer. From figures 11.6 (b-d) the effect that hypertonicity was clear with vacuolisation and disorganisation of the villi in most cases. It would also appear that the cells are being detached from each other, which may be attributable to the decrease in TEER and increase in Papp seen in the presence of these NCDs particularly PCD and ACD.

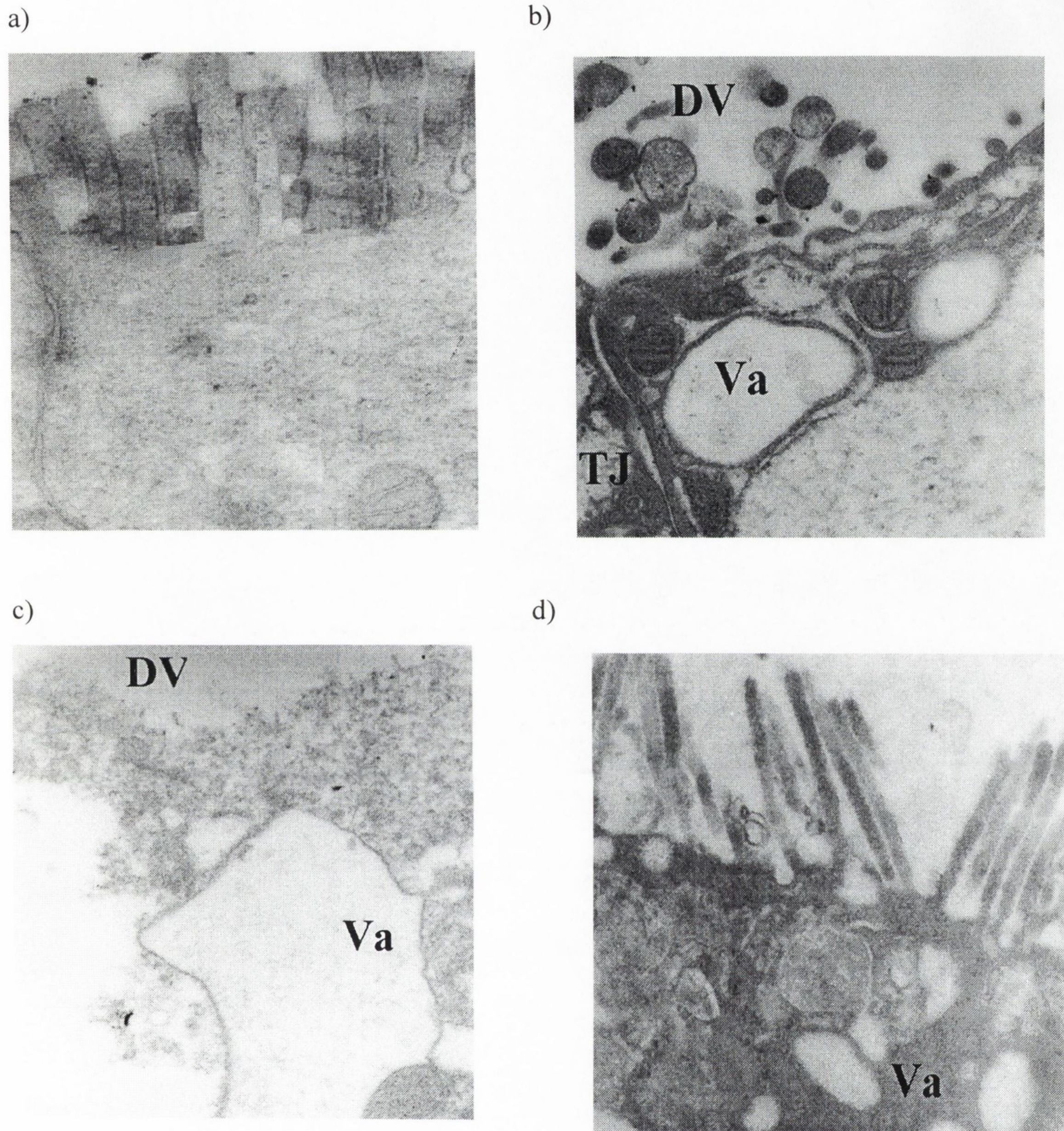


Figure 11.6 The effect that the novel cyclodextrins a) PCD 10% w/v, b) HCD 20% w/v, c) PCD 10% w/v and d) ACD 20% w/v have on the morphology of the CaCo-2 cell monolayer. (X 40,000).

11.8 Discussion

CDs have been used to stabilise various biomedically important peptides and proteins such as growth hormone and interlukin-2 (Brewster et al., 1991; Charman et al., 1993). The use of cyclodextrins as absorption enhancers has been well documented for a wide range of pharmaceuticals (Szeijtli (1988); Uekama et al., 1994)

In this present study, the aim was to assess the effect of commercially available cyclodextrins on the stability and transport of sCT and compare the results to a group of novel amphiphilic cyclodextrins bearing a common sulphur group.

The toxicity of cyclodextrins has been previously delineated by their ability to affect erythrocytes (Shiotani et al., 1995; Irie and Uekama, 1997). The haemolytic activity of the four types of BCD derivatives towards rabbit erythrocytes have been shown to be DMCD> BCD> HPCD> SBE_7CD. From the results of the MTT assay it appears that a similar trend was observed with the IC_{50} for DMCD> HPCD> SBE_7CD. These results are further supported by previous findings that have reported similar IC_{50} values (Tötterman et al., 1997; Hovgaard et al., 1995; Saarinen-Savolainen et al., 1998).

The results from the propidium iodide (PI) staining indicate that 10%w/v of the three novel CDs have similar amounts of fluorescence and was comparative to HPCD 10%. However, the commercial cyclodextrins DMCD (3% w/v) has a more widespread and greater intensity of staining compared to the other CDs investigated.

The stability of sCT was established at the beginning and the end of the 4h experiment. The data suggested that initially the commercial cyclodextrins do not cause an acceleration of sCT degradation and there was no indication that they protected sCT from degradation. For HPCD as the concentration increased there was an increase in the amount of sCT degraded at the beginning and end of the experiment. Loftsson and Brewster (1996) have already reported this interesting

occurrence where maximum benefit was usually obtained at low cyclodextrin concentrations in the presence of large drug molecules such as peptides and proteins.

The novel CDs similarly did not demonstrate any protection from degradation of the peptide. However, it would appear that only HCD 20% w/v and ACD 20% w/v have a detrimental effect on the stability of the peptide causing a substantial increase in the amount of degraded peptide, 44.83 ± 11.53 % and 23.40 ± 6.35 % respectively at the beginning of the experiment.

As the stability was being monitored the transport of sCT and the paracellular markers, mannitol and PEG 4000 were also assessed. It is clear from the results that there is a concentration dependant increase in the transport of sCT, mannitol and PEG 4000 across the CaCo-2 cell monolayer in the presence of increasing concentrations of cyclodextrins (Tötterman et al., 1997). The commercially available cyclodextrins (CCDs) investigated which caused most enhancement to the peptide, sCT and both paracellular markers was SBE_7CD 20% w/v> DMCD 3% w/v> DMCD 1% w/v= HPCD 10% w/v> HPCD 5% w/v= HPCD 1% w/v= SBE_7CD 10% w/v= sCT. Usually the enhancement in Papp observed following exposure to the CCDs was PEG 4000= sCT> mannitol.

In the presence of the novel cyclodextrins (NCDs) ACD and PCD there appears to be a concentration dependant increase in the permeabilities of the hydrophilic compounds. However, for HCD this relationship does not exist. The enhancement of sCT, mannitol and PEG 4000 was as follows ACD 20% w/v> PCD 20% w/v> ACD 10% w/v= PCD 10% w/v=HCD 20% w/v= HCD 10% w/v> ACD 5% w/v= PCD 5% w/v= HCD 5% w/v= sCT. Similarly the enhancement was usually greater for the larger molecular weight hydrophilic compounds, sCT and PEG 4000 than for mannitol. Overall the ranking for the cyclodextrins investigated was ACD 20% w/v> PCD 20% w/v> SBE_7CD 20% w/v> DMCD 3% w/v> DMCD 1% w/v= HPCD 10% w/v > sCT and the other CDs concentrations studied.

It has previously been shown that SBE_7CD at concentrations greater than 5% w/v are hypertonic primarily due to the presence of sodium salts and the high degree of substitution, 7 (Croyle et al., 1998; Saarinen-Savolainen et al., 1998). When the cyclodextrin solutions were measured at the concentrations investigated in the transport studies the NCDs, PCD was more hypertonic than ACD because of the presence of the sodium salts, but not as great as SBE_7CD. To ensure that the transport of the hydrophilic compounds across the CaCo-2 cell monolayer was not entirely attributable to solvent drag, similar hypertonic solutions of mannitol were prepared. The enhancement achieved with the hypertonic mannitol solutions were lower that those observed with the CDs. This implied that the hypertonicity of the CDs solutions was not the only factor driving the enhancement in the Papp of the hydrophilic compounds.

The use of cyclodextrins as absorption enhancers has limitations primarily as they are known to cause some toxic effects. By monitoring TEER and the TEM it provided more information on the mode of action of the commercially available and novel cyclodextrins studied.

There was a concentration dependant decrease in TEER as the cyclodextrin concentration was increased. It would appear that the more lipophilic cyclodextrin, DMCD caused the greatest reduction in TEER at lower concentration compared to other commercial and novel cyclodextrins. Tötterman et al., (1997) and Høvgaard et al., (1995) both demonstrated that DMCD caused a greater decrease in TEER compared to the more hydrophilic derivative, HPCD. It appeared that high concentrations of cyclodextrins (20% w/v) caused a huge decrease in TEER after 4h exposure; this was seen for all the sulphur containing commercial and novel CDs. The TEM corroborated the permeability and electrical resistance data.

The exposure of the monolayer to increasing concentrations of CCDs and NCDs did enhance the permeabilities of the paracellular markers, mannitol and PEG 4000 and sCT and a concomitant reduction in TEER. However, a direct linear correlation between the Papp and the reduction in TEER for both the CCDs and NCDs was not

observed. This may be attributable to the mechanism of enhancement of the cyclodextrin, which has been shown not only to affect the tight junctional complex but also extract membrane components, which may cause an increase in membrane permeability (Shiotani et al., 1995; Shao et al., 1992; Haeberlin et al., 1996).

CHAPTER 12

The effects of novel and commercial cyclodextrins (CDs) on the apparent permeabilities of the hydrophilic compounds, mannitol, PEG 4000 and sCT and on the integrity of the CaCo-2: Ht29GlucH co-culture monolayer

12.1 Introduction

In Chapter 11 the effect that commercially available and novel cyclodextrins had on the transport of hydrophilic compounds, the stability of sCT and the effect on the integrity of the monolayer was investigated. Since the CaCo-2: Ht29GlucH co-culture monolayer is considered to be more physiological the effects of these systems were investigated using this model.

Meaney and O' Driscoll (1999) developed the mucus producing CaCo-2: Ht29GlucH co-culture model and showed that the permeabilities of hydrophilic marker compounds, mannitol, PEG 900 and PEG 4000 were greater in the co-culture model compared to the mucus free cell culture model CaCo-2.

In this study the modulatory effects of mucus on the transport and stability of sCT was monitored in the presence and absence of commercially available (CCDs) and novel cyclodextrins (NCDs) using the co-culture model. In addition the transport of the paracellular markers, mannitol and PEG 4000, and the integrity of the co-culture model was investigated. The effects of removing the mucus layer on the absorption potential of the commercially available (CCDs) and novel cyclodextrins (NCDs) were also studied.

12.2 The effects of commercial and novel cyclodextrins on the stability of sCT in the presence of the co-culture monolayer

The stability of salmon calcitonin (sCT) was investigated following exposure to the co-culture model in the presence of the cyclodextrins. It has been reported that CDs have enzyme inhibitory effects (Brewster et al., 1991; Haeberlin et al., 1996).

Commercial cyclodextrins (CCDs)

The stability of the peptide calcitonin (sCT) with the CDs sample in the apical chamber was investigated immediately after preparation and warming to 37^{0}C, time = 0min.

At the beginning of the experiment in the presence and following removal of the mucus layer with N-AC (0.5% w/v) there appears to be no statistically significant difference in the concentration of sCT degraded, see Table 12.1. Appendix I demonstrates that the instability of sCT increases with time over 4h. For all the commercially available cyclodextrins (CCDs) it appears that the stability of sCT is similar whether the mucus layer is present or not when compared to sCT alone (Table 12.1 and appendix I).

Table 12.1 The effect of commercially available cyclodextrins (CCDs) on the stability of sCT in the apical chamber of the CaCo-2: Ht29GlucH cell monolayer at the onset of a 4h experiment in the presence and absence of mucus.

	% sCT Degraded ± SE of the Initial Starting Conc. (n ≥ 3)	
System	**Mucus**	**No mucus**
sCT	11.31 ± 2.34	6.89 ± 0.70
DMCD 3% w/v	16.49 ± 1.47	11.44 ± 3.51
HPCD 10% w/v	11.81 ± 1.47	9.64 ± 1.67
SBE_7CD 20% w/v	5.50 ± 0.38	5.50 ± 0.38

Novel cyclodextrins (NCDs)

Table 12.2 demonstrates the effect that the novel cyclodextrins, ACD and PCD have on the stability of sCT in the apical chamber of co-cultures when mucus was present or not. There appears to be a trend toward an increase in degradation as the concentration of NCDs was increased from 10% w/v to 20% w/v in the presence of the mucus layer. However, significant increase in degradation was only observed in the presence of ACD 20% w/v irrespective to the presence of a mucus layer. Following removal of the mucus layer in the presence of PCD 20% w/v the degradation of sCT is similar to the control.

Table 12.2 The effect of novel cyclodextrins (NCDs) on the stability of sCT in the apical chamber of the CaCo-2: Ht29GlucH cell monolayer at the onset of a 4h experiment in the presence and absence of mucus. ($p < 0.05$, *)

	% sCT Degraded ± SD of the Initial Starting Conc. (n ≥ 3)	
System	**Mucus**	**No mucus**
sCT	11.31 ± 2.34	6.89 ± 0.70
ACD 10 % w/v	18.83 ± 6.52	ND
ACD 20 % w/v	32.16 ± 4.52*	41.46 ± 3.09*
PCD 10 % w/v	16.29 ± 0.71	ND
PCD 20 % w/v	17.76 ± 2.45	11.20 ± 5.30

The presence or absence of mucus appeared not to affect the stability of the peptide sCT. Generally at the onset of the experiment as the concentration of ACD increased the percentage of degraded sCT increased. The degradation of sCT in the presence of ACD 20% following the removal of the mucus layer was similar to when mucus was present.

12.3 Transport of hydrophilic compounds in the presence of CCDs and NCDs across the CaCo-2: Ht29GlucH co-culture monolayer

Having previously established that the CaCo-2: Ht29GlucH co-culture produced a mucus layer (Chapter 10, Section 10.2), this model was used to study the effects of the commercially available and novel cyclodextrins (CCDs and NCDs) on the transport of the peptide sCT and both paracellular markers, mannitol and PEG 4000.

We previously demonstrated that the presence of mucus influenced the effect of the bile salt, NaGC on the transport of the hydrophilic compounds, sCT, mannitol and PEG 4000

(Chapter 10, Section 10.3). Therefore the effect that the mucus layer had on the enhancing potential of the cyclodextrins was also investigated.

Salmon calcitonin (sCT)

In the presence of mucus it appeared that the apparent permeability of sCT increased 8.44 and 2.60-fold in the presence of the commercially available cyclodextrins, DMCD 3% w/v and HPCD 10% w/v respectively. In contrast to the enhancement observed in the CaCo-2 cell model SBE_7CD 20% w/v did not significantly enhance the Papp of sCT in the co-culture model when mucus was present (Table 12.3).
In the presence of the novel amphiphilic cyclodextrin, PCD the Papp increased 5.4 and 7.9-fold for PCD 10% w/v and 20% w/v respectively, compared to sCT alone. The relative enhancement for sCT in the presence of ACD 10% w/v and 20% w/v was less than observed for PCD, with a 4.1 and 5.9-fold increase respectively.

Paracellular markers: Mannitol and PEG 4000

Similar trends to those observed for the peptide sCT were noted for the paracellular marker compound, Table 12.3. Increases in the Papp of mannitol and PEG 4000 were seen with cyclodextrins, which previously caused an increase in the Papp of sCT.
In the presence of the commercially available cyclodextrins, the Papp for mannitol increased 6.78 and 3.45-fold and for PEG 4000 increased 3.28 and 19.1-fold for HPCD 10% w/v and DMCD 3% w/v respectively. In the presence of SBE_7CD, which carries a negative charge (Irie and Uekama, 1997), the Papp of both marker compounds was not enhanced to any great extent for 20% w/v compared to the control, sCT. This may be attributable to the possible repulsion by the negatively charged mucus layer and the negatively charged CDs (Table 12.3).

Table 12.3 The apparent permeability (Papp) across the CaCo-2: Ht29GlucH co-culture monolayer over 4h of the hydrophilic compounds, mannitol, PEG 4000 and sCT for each of the commercially available and novel cyclodextrins. Values are expressed as mean ± SD of at least 3 observations and statistical significance, $p < 0.05$,* as compared to sCT.

System (% w/v)	Papp ± SD (X10^{6}) (cm/sec) (n ≥ 3) (p<0.05, *) (Total Percentage transported after 4hr)		
	Mannitol	**PEG 4000**	**sCT**
sCT	1.43 ± 0.10 (9.64 ± 0.65%)	0.32 ± 0.03 (2.02 ± 1.24%)	0.45 ± 0.05 (2.81 ± 0.28%)
Commercially Available Cyclodextrins (CCDs)			
HPCD (10)	3.45 ± 0.38* (20.20 ± 6.71%)	1.05 ± 0.11* (6.15 ± 2.13%)	1.17 ± 0.11* (7.39 ± 2.09%)
DMCD (3)	9.70 ± 0.19* (55.98 ± 2.98%)	6.11 ± 0.31* (35.82 ± 4.83%)	3.80 ± 0.20* (21.32 ± 2.34%)
SBE_7CD (20)	1.71 ± 0.07 (10.75 ± 0.51%)	0.50 ± 0.06 (3.18 ± 0.40%)	0.55 ± 0.05 (3.44 ± 0.33%)
Novel Cyclodextrins (NCDs)			
PCD (10)	1.50 ± 0.01 (8.66 ± 0.11%)	2.93 ± 0.27* (15.45 ± 1.44%)	2.44 ± 0.31* (12.51 ± 2.33%)
PCD (20)	7.65 ± 0.35* (49.56 ± 3.29%)	2.06 ± 0.48* (12.97 ± 3.08%)	3.58 ± 0.06* (22.98 ± 2.28%)
ACD (10)	8.34 ± 1.45* (55.13 ± 13.56%)	4.97 ± 2.1* (29.39 ± 13.17%)	1.86 ± 0.20* (11.41 ± 0.89%)
ACD (20)	8.12 ± 0.48* (49.38 ± 2.93%)	1.61 ± 0.17* (9.70 ± 1.00%)	2.64 ± 0.29* (15.60 ± 1.62%)

In the presence of the novel cyclodextrins, PCD and ACD the permeabilities of mannitol and PEG 4000 were enhanced. For PCD the Papp for mannitol was enhanced 1.08 and 5.34-fold and for PEG 4000 was enhanced 9.15 and 6.44-fold in the presence of PCD 10% w/v and 20% w/v respectively. Similarly for ACD the Papp of both paracellular markers were similar whether 10% w/v or 20% w/v was used.

It appears from the transport of the peptide, sCT and both paracellular markers, mannitol and PEG 4000 that the cyclodextrins, with the exception of the anionic SBE_7CD (20% w/v), are capable of enhancing permeability in the presence of the mucus layer indicating that mucus may not be a significant barrier. This is in contrast to previous results with the bile salt simple micellar systems (Chapter 10). In order to clarify the further the role of mucus on the effects of commercially available and novel cyclodextrins transport studies were performed following removal of mucus with N-AC (0.5% w/v).

12.4 Assessment of the mucus layer as a barrier to transport

To remove the mucus the Ht29GlucH co-culture monolayers were incubated with 5mg/ml of N-AC in HBSS for 30min and which was then washed off twice prior to carrying out the transport experiment (Chapter 10, section 10.4).

Previously it was shown that N-AC 0.5% w/v caused a statistically significant increase in the permeability of the hydrophilic compounds. The increase observed for the peptide, sCT on removal of the mucus layer might be due to the electrostatic attraction that existed between the positively charged sCT and the negative charged mucus layer (Duncan et al., 1995; Baudyš et al., 1996).

Following the removal of the mucus layer there was no further enhancement relative to when the mucus layer was present in the transport of the peptide, sCT and the paracellular markers mannitol and PEG 400 in the presence of commercially available and novel cyclodextrins with the exception of SBE_7CD (Table 12.4). In the case of SBE_7CD (20% w/v), an anionic cyclodextrin derivative there was a statistically

significant increase in the transport of mannitol and PEG 4000 following removal of the mucus layer ($p < 0.05$) when compared to the enhancement caused by SBE_7CD (20% w/v) when mucus was present. This may be due to electrostatic repulsion that may have occurred between the negatively charged mucus layer and the negatively charged CDs when the mucus layer was present. No statistically significant enhancement was observed for sCT following removal of the mucus layer with the Papp being similar to sCT alone (Table 12.4).

Table 8.4 The effect of pre-incubation with N-Acetyl-L-Cysteine (N-AC) 0.5% w/v for 30 min on the transport of mannitol, PEG 4000 and sCT across CaCo-2: Ht29GlucH co-culture monolayers in the presence of the commercially available and novel cyclodextrins. Statistical significance, $p<0.05$ compares to sCT following removal of mucus.

System (% w/v)	Papp ± SD ($X10^6$) (cm/sec) ($n \geq 3$) ($p<0.05$, *)		
	Mannitol	**PEG 4000**	**sCT**
sCT	3.08 ± 0.21	0.61 ± 0.05	1.15 ± 0.14
Commercially Available Cyclodextrins (CCDs)			
HPCD (10)	5.86 ± 0.26	2.32 ± 0.21	2.35 ± 0.15
DMCD (3)	9.68 ± 0.14	6.50 ± 0.22	5.25 ± 0.24
SBE_7CD (20)	5.22 ± 1.27*	1.22 ± 0.21*	1.13 ± 0.12
Novel Cyclodextrins (NCDs)			
PCD (20)	6.61 ± 0.37	2.64 ± 0.21	3.43 ± 0.32
ACD (20)	8.93 ± 0.18	1.83 ± 0.17	2.97 ± 0.49

12.5 The integrity of the CaCo-2: Ht29GlucH co-culture monolayer in the presence of CCDs and NCDs

The integrity of the co-culture monolayer was monitored by two methods, TEER and TEM. The resistance and morphology of the co-culture monolayer was compared under control conditions and following exposure to the commercially available and novel cyclodextrins. The effects that pre-incubating the co-culture monolayer to N-AC (0.5% w/v) and resultant exposure to the cyclodextrins was examined by both TEER and TEM.

12.5.1 Transepithelial electrical resistance (TEER)

Decreases in TEER suggest that changes in the integrity of the co-culture monolayer were occurring. Therefore the transepithelial electrical resistance (TEER) across the monolayers was monitored before, during and after exposure to the cyclodextrins. Pre-incubating the co-culture monolayer with N-Acetyl-L-Cysteine (N-AC) (0.5% w/v) showed no effect on the integrity of the monolayer, see Chapter 10, table 10.6.

In the presence of HPCD 10% w/v the TEER remains within control levels which indicated that this concentration of cyclodextrin did not significantly affect the tight junction integrity when the mucus layer was present ($p > 0.05$). However on pre-incubation with N-AC (0.5% w/v) there is a statistically significant decrease in the TEER value as compared to sCT alone ($p < 0.05$) for HPCD 10% w/v. The decrease in TEER observed for this system correlated with an increase, which was not statistically significant in the permeabilities of the hydrophilic compounds ($p > 0.05$). Whether in the presence or absence of mucus DMCD cause a statistically significant decrease in TEER with a concomitant increase in the permeabilities of the hydrophilic compounds. In the presence of SBE_7CD 20% w/v there was a statistically significant decrease in TEER following pre-incubation with N-AC 0.5% w/v ($p < 0.05$) as compared to when mucus was present. This was supported by a statistically significant increase in the transport of mannitol and PEG 4000 (Table 12.4 and 12.5).

The novel cyclodextrins, ACD and PCD 10% w/v were not significantly different to the control, sCT alone ($p > 0.05$). For both ACD 20% w/v and PCD 20% w/v in the presence and absence of mucus the significant decrease in TEER correlates to a significant increase in the Papp of the hydrophilic compounds.

Table 12.5 Transepithelial electrical resistance (TEER) values of CaCo-2: Ht29GlucH co-culture monolayers following 4h exposure to sCT (control), novel and commercially available cyclodextrins in the presence and absence of N-AC (0.5% w/v). Values are expressed as mean ± SD ($n \geq 3$), ($p > 0.05$, *).

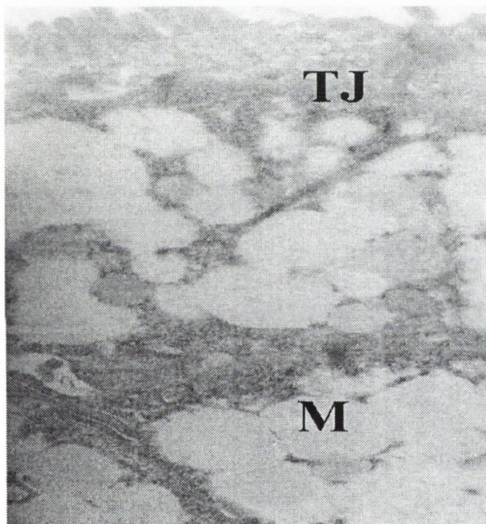
System	% TEER at 4h ± SD (n ≥ 3)	
	No N-AC	N-AC for 30 min
sCT	85.8 ± 10.3	85.9 ± 13.1
Commercially Available Cyclodextrins		
HPCD 10% w/v	72.8 ± 2.3	65.4 ± 2.2*
DMCD 3% w/v	47.3 ± 5.7*	53.8 ± 0.9*
SBE_7CD 20% w/v	62.1 ± 0.5	45.2 ± 1.1*
Novel Cyclodextrins		
ACD 10% w/v	81.7 ± 2.1	ND
ACD 20% w/v	49.1 ± 1.0*	42.4 ± 1.8*
PCD 10% w/v	71.7 ± 7.5	ND
PCD 20% w/v	48.2 ± 0.6*	43.7 ± 0.6*

8.5.2 Transmission electron microscopy (TEM)

The effect of commercially available and novel cyclodextrins and pre-treatment with N-AC on membrane integrity was also examined by TEM. Previously in Chapter 10 it was shown that N-AC (0.5% w/v) appeared to cause an increase in the dilation of the

junctional complexes and that the novel CDs: ACD, PCD and HCD at concentrations of 20%w/v caused vacuolisation and denudation of the villi (Chapter 11, section 11.7.2.) and the commercially CDs: DMCD 3% and SBE_7CD 20% w/v appeared to affect the morphology of the CaCo-2 cell monolayer to the greatest extent (Chapter 10, section 10.8.2.). The commercial CDs were initially investigated and it appeared that the morphology of the cell was unaltered in the presence and absence of the mucus layer. It appeared that in both cases the mucus vesicles were empty which may be attributed to the physiological protective mechanism of mucus (Figure 12.1 (a) and (b)).

a) DMCD 3% w/v with mucus (X10K)



DMCD 3% w/v without mucus (X20K)

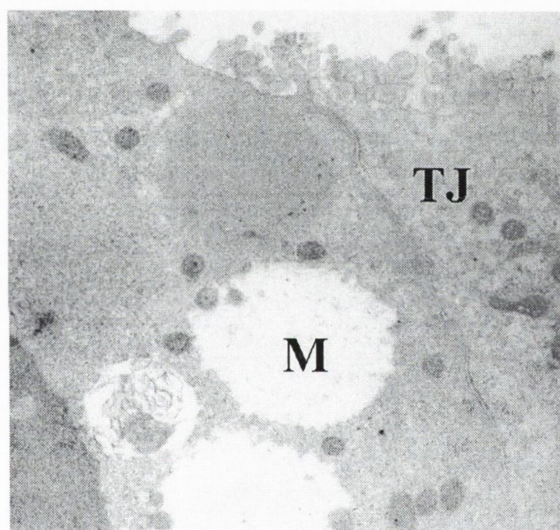
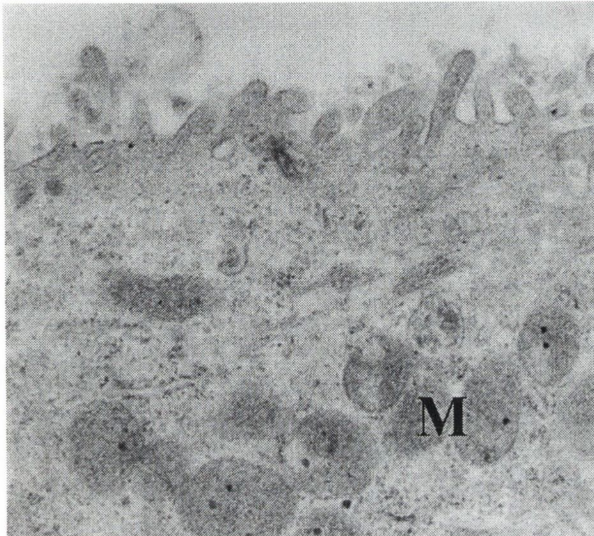


Figure 12.1 Transmission electron micrograph showing the effect that mucus removal has on the enhancement potential of DMCD 3% w/v. M = mucin granules which appear to be empty whether treated with a mucolytic or not.

The hypertonic SBE_7CD 20% w/v, which appeared to have an electrostatic repulsion to when the mucus layer was present, demonstrated to have a greater effect following removal of the mucus, see Figure 12.2 (a and b). It appears following removal of the mucus layer that there is an increase in the disorganisation of the villi with some budding and increased vacuolisation.

a) SBE_7CD 20%w/v with mucus



b) SBE_7CD 20% w/v without mucus

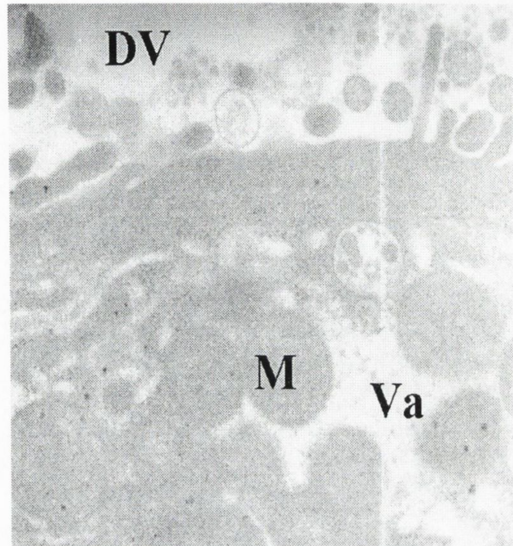
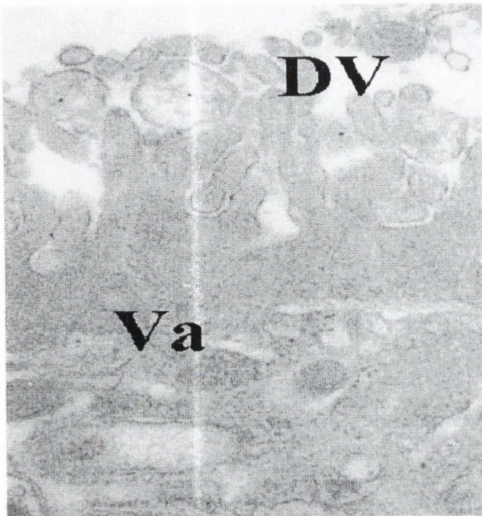


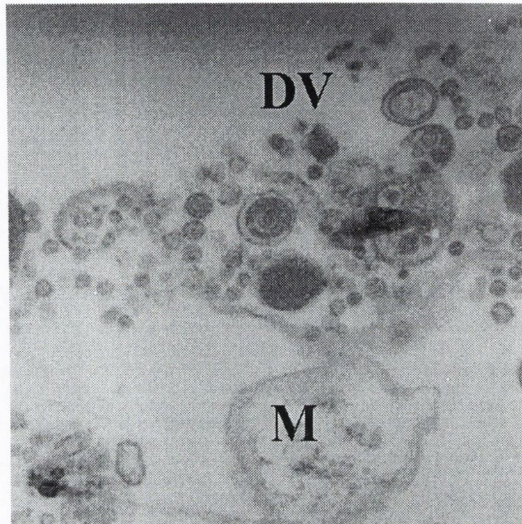
Figure 12.2 Transmission electron micrograph demonstrating the effect that mucus has on the enhancement potential of SBE_7CD 20% w/v.

The novel cyclodextrins that were investigated using the co-culture model, PCD and ACD 20% w/v had previously demonstrated that their enhancement potential was unaffected by mucus. The TEM demonstrated that while mucus was present the cell morphology was altered, with the villi budding and sloughing off (DV) and an increased number of vacuoles (Va). Following the removal of the mucus it appeared that the cell walls were targeted to a greater extent with cell lysis being observed.

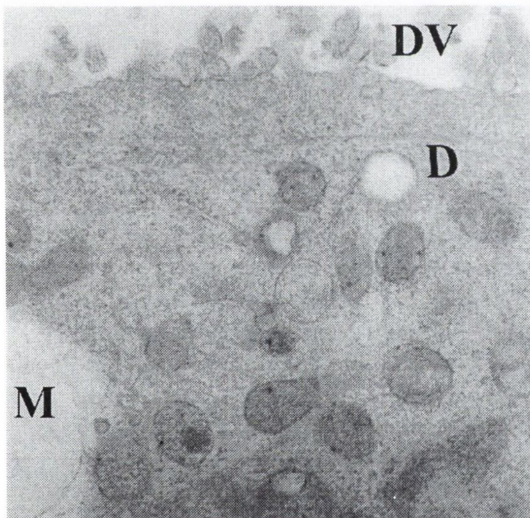
a) PCD 20%w/v with mucus



b) PCD 20%w/v without mucus



c) ACD 20% w/v with mucus



d) ACD 20% w/v without mucus

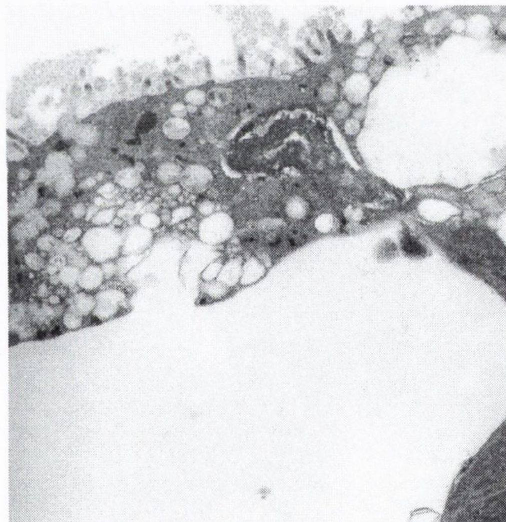


Figure 12.3 Transmission electron micrograph demonstrating the effects that PCD 20% w/v and ACD 20% w/v have on the co-culture membrane in the presence and absence of the mucus layer.

12.6 Discussion

The stability of sCT in the presence of the CDs was not affected except for ACD 20% w/v. This NCDs caused a statistically significant increase in the sCT degraded from the onset of the experiment.

In the presence of the CDs there was a significant increase in the permeabilities of the hydrophilic compounds in the presence of mucus. However, SBE_7CD demonstrated poor enhancing abilities in the co-culture model compared to the CaCo-2 model. This may be attributable to the negative charge associated with this compound (Irie and Uekama, 1997) and therefore the electrostatic repulsion that may exist between the negatively charged mucus layer (Hoogdalem et al., 1989) and the enhancer. This electrostatic repulsion has been previously demonstrated by numerous authors (Hoogdalem et al., 1989; Wikman-Larhed et al., 1997). It appears that DMCD 3% w/v, HPCD 10% w/v, PCD 20% w/v and ACD 20% w/v enhancement potentials were unaffected by the presence of the mucus layer. This was demonstrated by the similar permeabilities of the hydrophilic compounds and TEER values in the presence and absence of mucus.

Similar Papp values were obtained for 20% w/v Heptakis (6-O-sulphonatophenyl)-β-cyclodextrin sodium salt (PCD) and Heptakis (6-deoxy-6-aminopyridyl)-β-cyclodextrin sodium salt (ACD) and as 2,3-dimethyl-β-cyclodextrin (DMCD) (3% w/v) indicating that DMCD is more potent. It has been widely reported *in-vitro* (Høvgaard et al., 1995:Tötterman et al., 1996) and *in-situ* (Merkus et al., 1991; Shao et al., 1992, 1994; Schipper et al., 1994) that DMCD was the one of the most potent enhancers in the commercially available cyclodextrin family. It has also been suggested by using confocal scanning microscopy that it primarily affects the paracellular route to enhance permeability (Irie and Uekama, 1997; Junginger et al., 1998).

The integrity of the co-culture monolayer was monitored using TEER. Pre-incubation with N-AC (0.5% w/v) had no effect on the TEER value suggesting that the integrity was maintained. On removal of mucus the TEER values did not significantly change in the

presence of novel and commercially available CDs this was consistent with the transport data for the hydrophilic markers which suggests that mucus is not a major barrier to the enhancement potential of CDs. The exception to this was sulfobutyl-β-cyclodextrin (SBE_7CD) 20% w/v following removal of the mucus layer a significant reduction in TEER was seen. This appears to reflect the transport data for the hydrophilic compound with the reduction in TEER corresponding to an increase in their apparent permeabilities.

The morphology of the CaCo-2: Ht29GlucH co-culture monolayer when exposed to the commercially available and novel cyclodextrins in the presence of a mucus layer clearly showed that the mucus although not a barrier to the absorption enhancement potential of these systems appeared to offer some protection to the membrane.

CHAPTER 13

General Discussion

Initial studies were performed to assess the degree and mechanism of membrane transport of the peptide, salmon calcitonin (sCT). The apparent permeability of sCT was 0.05 ± 0.01 (SE) X 10^{-6} cm/sec although the permeability is low, it does indicate that sCT is transported across the CaCo-2 cell monolayer.

The transport pathway of sCT was investigated to determine if any active, passive or carrier mediated process was involved. In order to determine the involvement of any carrier mediated process equal concentrations of sCT (10µg/ml) were placed in the apical (AP) and basolateral (BA) chamber. The concentration of sCT was determined in both chambers at different time intervals over 4h. No significant changes in sCT concentration were seen with the ratio of AP: BA remaining at 0.99: 1.00. This suggests that the involvement of a carrier mediated process in one direction for the transport of sCT is unlikely.

Active transport mechanisms tend to be polarised on the apical or basolateral side of the membrane e.g. P-GP. Therefore to ensure that the transport of sCT was not active, sCT (10µg/ml) flux was measured from AP → BA and from BA → AP and compared. No difference was observed between the apparent permeability coefficients (Papp) of sCT, 0.048 ± 0.009 (SD) X 10^{-6} cm/sec in the apical to basolateral direction and 0.044 ± 0.009 (SD) X 10^{-6} cm/sec in the basolateral to apical direction. This further suggests that an active transport system is not involved in the transport of sCT across the CaCo-2 cell monolayer. The Papp of sCT was measured at 4^0C because at this temperature metabolic processes would be slowed which would reduce any active process. Again the results indicated that no active mechanism was involved in the transport of this peptide across the CaCo-2 cell monolayer, as the Papp was not significantly different at either temperature. The Papp at 37^0C was 0.05 ± 0.01 X 10^{-6} cm/sec and at 4^0C was 0.04 ± 0.01 X 10^{-6} cm/sec.

The passive process involved in the transport of sCT was investigated by using a series of increasing concentrations of sCT placed in the apical chamber and then amount quantified in the basolateral chamber over 4 h. This data again suggests that sCT is

passively transported across the cell membrane in the concentration range studied (0.1-50 μg). Passive transport across the membrane can be either paracellular or transcellular. The potential membrane permeability of sCT, a large hydrophilic compound, is affected by a number of barriers e.g. physical –chemical properties associated with sCT and its stability characteristics. sCT is a large peptide compound with a MW of 3414, carries a positive charge at physiological pH and is inherently enzymatically unstable. From molecular modelling studies, sCT was constructed and its estimated molecular cross-sectional diameter to be 11.5 ± 1.2Å, see figure 13.1. Previous work has calculated that the pore size radius in the CaCo-2 cell monolayer 12.0 ± 1.9Å (Adson et al., 1994). Based on theses dimensions it is potentially possible for sCT to traverse the membrane via the paracellular route, which was consistent with the CFSLM results (Figure 8.15).

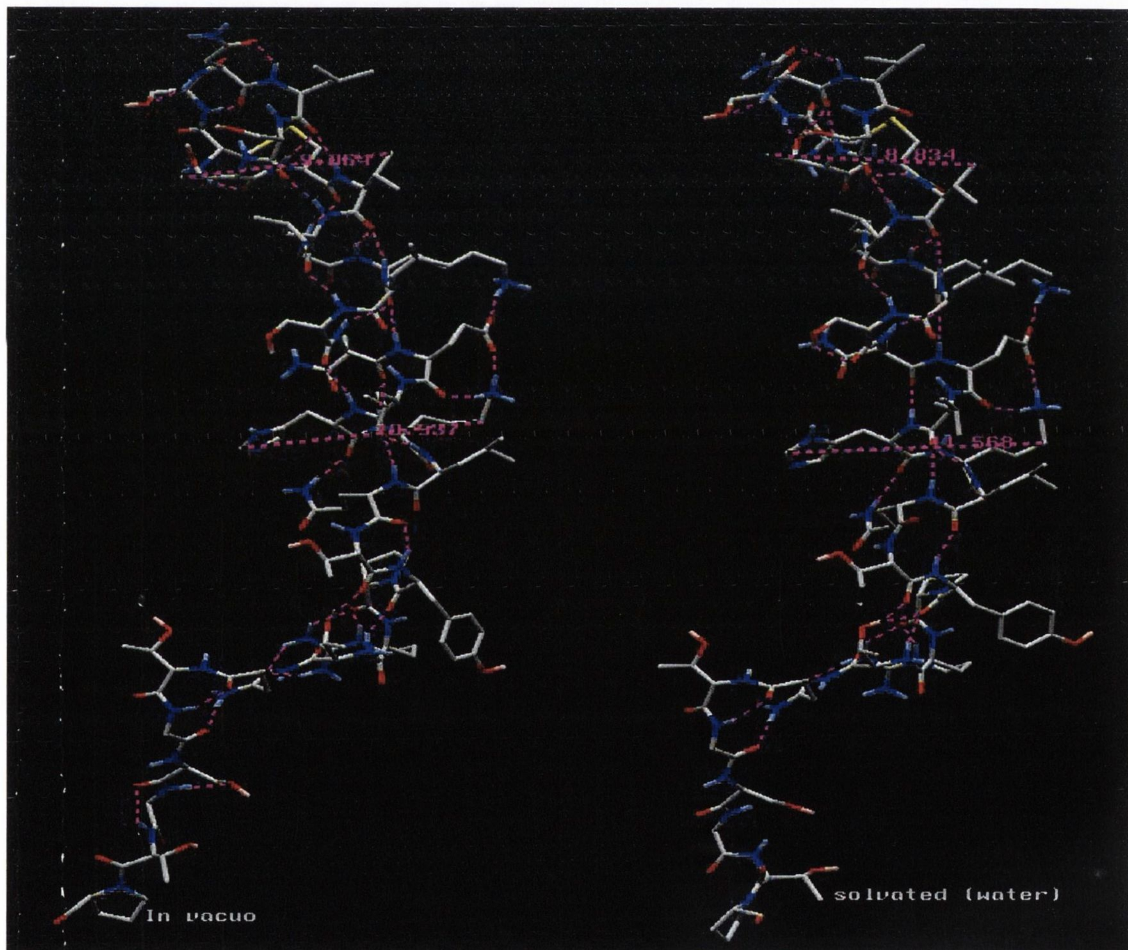


Figure 13.1 Computer generated model of salmon calcitonin (sCT) *in-vacuo* and solvated in water. The pink-hatched lines indicate distances; carbon is represented by blue, nitrogen by red and hydrogen by white/grey.

The stability of sCT was monitored at the beginning of an experiment and again after 4h. The stability of the peptide decreased from 0 to 4h, with 7.92 ± 4.09 % and 22.79 ± 2.14 % degraded in the apical chamber of the CaCo-2 cell monolayer respectively. Because of the decrease in stability of sCT and its poor apparent permeability the use of enhancers with potential enzyme inhibition was investigated.

Initial screening of a variety of bile salts, NaC, NaTC, NaGC and Deoxy was undertaken to assess their abilities to enhance the permeability and stability of sCT without having detrimental effects on the integrity of the cell monolayer. Both the tri-hydroxy conjugated and unconjugated bile salts, NaC and NaTC caused an increase in the degraded sCT, with the greatest effect seen in the presence of NaTC. It has been shown in our laboratory that *in-vitro* stability of sCT in rat intestinal homogenates was compromised in the presence of NaTC (20mM) with the degradation rate being approximately 2-fold greater than for the control (O' Donnell, 2000). The enhanced degradation caused by bile salts has previously been observed for insulin (Lane, 1997). In this study NaC (40mM) caused rapid degradation of insulin in rat intestinal perfusions and was attributed to the deaggregation of the insulin dimer thereby exposing it to further enzymatic degradation. Because sCT is known not to aggregate this suggested mechanism does not explain the effect of NaTC and NaC on the stability of sCT. The enhanced degradation seen in the presence of NaTC may be attributable to unfolding of the protein thereby exposing the hydrophobic residues to subsequent enzymatic degradation. A further consideration in the enhanced degradation of sCT maybe due to taurine residue interacting with the peptide, as this rapid degradation is only seen for the taurine conjugate of the trihydroxy bile salt NaC. In contrast NaGC did not enhance the degradation of sCT, with the percentage degraded generally being less than for sCT alone.

In the presence of the bile salt simple micelles, NaGC (15mM and 20mM), NaTC (20mM) and NaC (5mM) there was an increase in the permeability of sCT, mannitol and PEG 4000. It has been shown that trihydroxy conjugated bile salts, including NaGC act on the tight junctions (Werner et al., 1996; Tengamnuay and Mitra, 1990). Werner et al., (1996) have shown by staining the actin with FITC-labelled phallodin that an increase in

the permeability of the CaCo-2 cell monolayer in the presence of NaTC (< 20mM) was due to dilation of the tight junction. The apparent permabilities of sCT, mannitol and PEG 4000 increased with increasing concentrations of NaGC. This concentration dependant increase had been previously reported by a number of authors (Donovan et al., 1990; Muranishi, 1990; Jørgensen et al., 1993).

From CFSLM it appeared that in the presence of NaGC (15mM) sCT was being transported via the paracellular route. To further support that the mechanism of action of the simple micelles of NaGC was on the tight junctions the permeability of sCT was compared to PEG 4000 in the presence of increasing concentrations of this bile salt. PEG 4000 is a hydrophilic unionised compound, which has been extensively used as a paracellular marker (Meaney, 1997). In addition it has similar molecular weight and similar molecular cross-sectional diameter to sCT, 12Å, (Lane et al., 1996). From figure 13.2 it is clear that there is a strong correlation, $R^2 = 0.94$, in the permeabilities of sCT and PEG 4000. This suggests that sCT is being transport across the CaCo-2 cell monolayer in the presence of simple micelles of NaGC via the paracellular route.

In addition as previously demonstrated (Chapter 8, section 6.1.2) there was a good correlation between the decrease in TEER and the increase in the Papp of the hydrophilic compounds as the concentration of NaGC increased. The TEM suggested that there appeared to be dilation of the junctional complex. All of this data together strongly indicates that the mechanism of enhancement of NaGC at the concentrations studied is via the paracellular route.

From the screening of the simple micelles of the tri-hydroxy bile salt, NaC and its glycine and taurine conjugates and the di-hydroxy bile salt, Deoxy, it was clear that the most suitable candidate for mixed micelle studies was the tri-hydroxy glycine conjugate, NaGC. Mixed micelles with linoleic acid (LA) have previously been reported to enhance permeability to a greater extent than the simple micelle (Tengamnuay and Mitra, 1990b; O'Reilly et al., 1994a). Mixed micelles of NaGC: LA (15: 5mM) increased the nasal absorption of [D-Arg^2]Kyotorphin almost 2-fold compared to NaGC (15mM) alone (Tengamnuay and Mitra, 1990a).

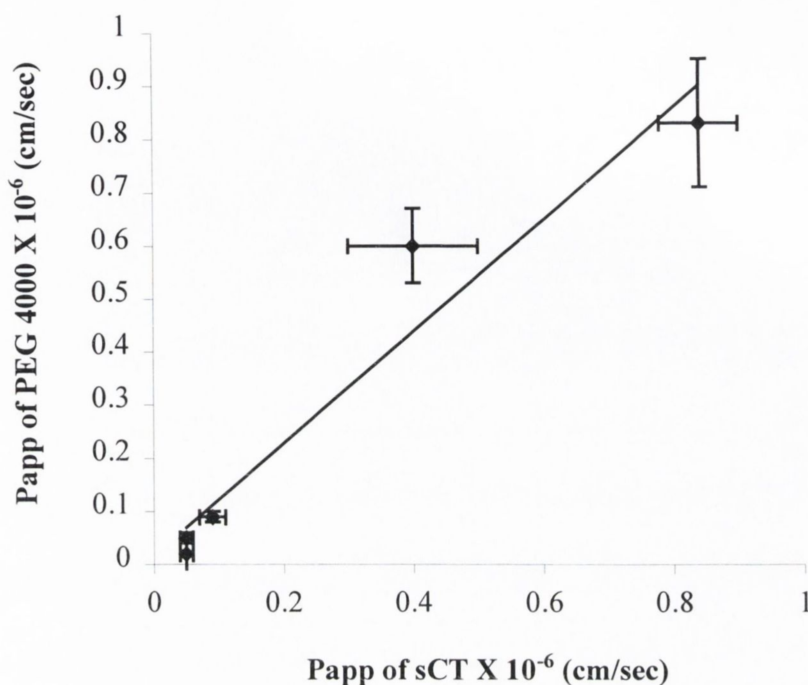


Figure 13.2 The apparent permeability of PEG 4000 expressed versus the apparent permeability of salmon calcitonin (sCT) across the CaCo-2 cell monolayer in the presence and absence of NaGC (10, 12.5, 15 and 20mM) simple micelles. Values are mean ± SE of at least n≥ 6 observations.

In general mixed micelles incorporating the long-chain unsaturated fatty acid, linoleic acid (LA), caused a greater enhancement in the Papp of the hydrophilic compounds compared to the corresponding simple micelles and this effect appeared to depend on fatty acid loading. In the presence of the mixed micelles of NaGC: LA with the concentration of bile salt remaining constant i.e. 15mM and the fatty acid loading varied from 0.5- 1mM the Papp of the hydrophilic compounds increased significantly only when the fatty acid loading was >0.85mM. Below this concentration the permeabilities of mannitol, PEG 4000 and sCT were lower than for NaGC (15mM), which may be due to the membrane protective effect of the fatty acid (Feldman and Gibaldi, 1969). O'Reilly et al., (1994b) observed that the permeability of PEG 4000 across isolated rat intestine was decreased in the presence of fatty acid loading of 10-20mM mixed micelles with

NaC (40mM) compared to NaC 40mM alone. It was suggested that this might be attributed to the protective effect of the fatty acid in the mixed micelle. However, when the ratio of bile salt: fatty acid was increased to 1:1 this membrane protective effect was substantially reduced and the Papp of PEG 4000 increased significantly compared to bile salt alone for the rat intestinal model.

In addition to fatty acid loading the concentration of bile salt incorporated into the micelles also affects the enhancement potential (Figure 13.3). The Papp of the hydrophilic compounds are increased as the bile salt is increased when the fatty acid loading is kept constant at 1mM. This suggests that there is a concentration dependant increase in the permeability of sCT, mannitol and PEG 4000 in the presence of simple and mixed micelle. The absorption enhancing effects of bile salt mixed micelles has been reported previously in the CaCo-2 cell model (Meaney, 1997).

The formation of mixed micelles increased the lytic activity of the bile salt simple micelle as shown by MTT. NaGC (10mM) simple micelle was shown not to affect the cells viability however the incorporation of 1mM LA to form mixed micelles reduced the cell viability by 43%. The MTT further demonstrated that as the fatty acid loading increased the toxicity of the mixed micelles increased.

The integrity of the monolayer was monitored using TEER and TEM. There was a reduction in TEER as the fatty acid loading was increased, and, also as the concentration of bile salt increased with constant fatty acid loading (1mM). The TEM results imply in the case of NaGC simple micelles as the concentration increased there appeared to be an increase in denudation of the villi, and increased dilation of the junctional complex. On formation of the mixed micelle the TEM data reflects greater villi loss, increased dilation of the junctions and in some areas cell lysis, which implies that mixed micelles cause greater membrane damage as compared to simple micellar systems. Previously in our laboratory, the effects that mixed micelles of NaTC and NaC with 1mm LA were investigated and the TEER and TEM data reflected the enhanced transport of the markers, PEG 4000 and mannitol (Meaney, 1997). The correlation between the reduction in TEER and the increase in the Papp of the hydrophilic compounds (Chapter 8) was poor

markers, PEG 4000 and mannitol (Meaney, 1997). The correlation between the reduction in TEER and the increase in the Papp of the hydrophilic compounds (Chapter 8) was poor which indicates that the paracellular route may not be the only route that this system affects.

To investigate if the increased permeability, the reduction in TEER and the changes in cell morphology seen with the micellar systems were transient, reversibility studies were undertaken. After 4h exposure to the simple micelle NaGC (15mM) complete recovery was seen with the permeability of the paracellular markers and TEER data returning to pre-exposure levels after 24h. However, in the presence of the mixed micelles NaGC: LA (15: 1mM) recovery was only seen for monolayers that had been exposed to the mixed micelle for a maximum of 1h.

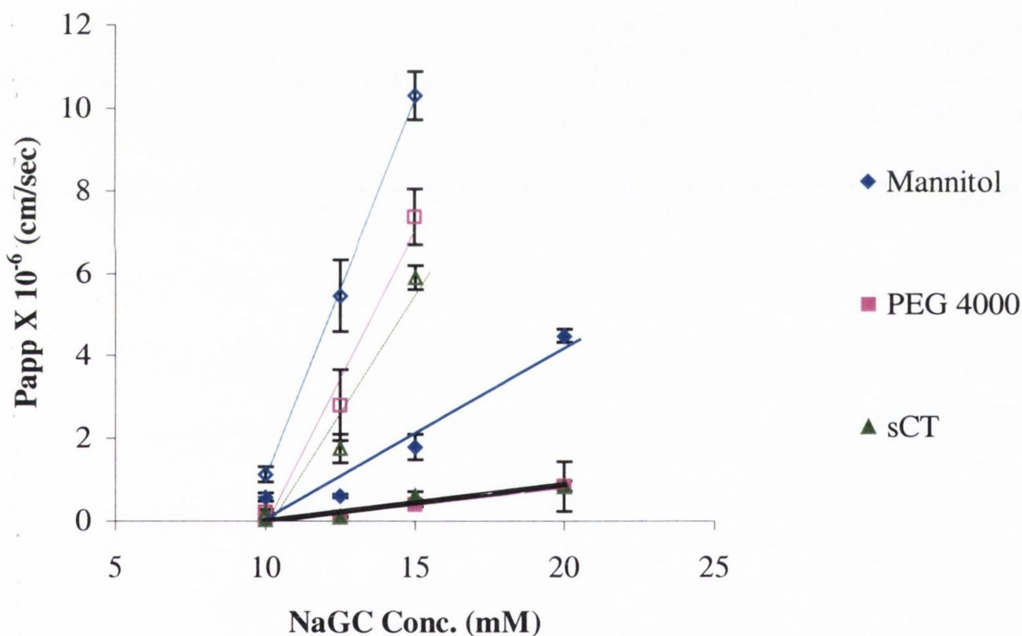


Figure 13.3 The effect of NaGC: LA mixed micelles (open symbol) with the fatty acid loading constant at 1mM relative to NaGC simple micelles (closed symbols) on the permeability of the mannitol, PEG 4000 and sCT across the CaCo-2 cell monolayer. Each value is the mean ± SE (n ≥ 4).

systems is not so clear and may involve a combination effect on both the paracellular and transcellular routes.

The second group of enhancer type investigated was cyclodextrins. In this work the ability of commercially available cyclodextrins (CDs) and custom synthesised CDs obtained from Dr. Raphael Darcy, Dept of Chemistry, U.C.D., to enhance the hydrophilic compounds, mannitol, PEG 4000 and sCT were compared. It was shown that 2,3-dimethyl-β-cyclodextrin (DMCD) 3%w/v and sulfobutyl-β-cyclodextrin (SBE_7CD) 20% w/v were the most potent of the commercially available CDs. However, some of the enhancement potential of the SBE_7CD was associated with its hypertonicity. The novel CDs Heptakis (6-deoxy-6-aminopyridyl)-β-cyclodextrin sodium salt (ACD), Heptakis (6-O-sulphonatophenyl)-β-cyclodextrin sodium salt (PCD) and Heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin (HCD) caused enhancement of the hydrophilic compounds, but only at concentrations greater than 10%w/v. It appears that the enhancement potential of PCD 20% w/v, ACD 20% w/v and DMCD 3% w/v are similar but that DMCD is more potent an enhancer because of the lower concentration that is required.

When the Papp of sCT and PEG 4000 were compared a good correlation was achieved for each of the NCDs and CCDs with $R^2 > 0.95$ except for HCD which has a poor correlation of 0.66. This suggests that CDs are affecting the tight junctions which is consistent with Haeberlin et al., (1996) who reported that β-cyclodextrin, γ-cyclodextrin, DMCD and HPCD at 1% w/v enhanced the transport of octreotide across the CaCo-2 cell monolayer via the paracellular route.

TEER has been previously been shown to be a measure of tight junctional integrity and therefore paracellular permeability (Junginger et al., 1997). For the CDs there was a poor correlation ($R^2 \leq 0.88$) between the reduction in TEER and increase in Papp of the hydrophilic compounds. These results imply an effect on tight junctions may not be the only effect that CDs are having on the cell membrane. It has been shown that CDs have the ability to extract membrane proteins and lipids, which results in changes to the

membrane integrity (Shiotani et al., 1995). Høvgaard et al., (1995) postulated that the primary effect of DMCD was extraction of membrane components in addition to tight junction dilation. Shao et al. (1992) had earlier suggested this proposed mechanism, because of the ability of DMCD to remove the membrane bound 5'-nucleotidase protein in the rat nasal model. The exact mechanism of the CDs is unclear but there is evidence to suggest that they are acting on the paracellular route as well as the transcellular route. Further work with these enhancers is necessary to completely elucidate their enhancement mechanism, which could be done with CFLSM and using model marker compounds, which are transcellular transported e.g. propranolol.

In the CaCo-2 cell monolayer the relative enhancement of the hydrophilic compounds in the presence the enhancers which have been investigated suggested that NaGC: LA (15:1 mM) > ACD 20% w/v> NaGC (20mM)> NaGC (15mM) ≈ SBE_7CD 20%w/v ≈ PCD 20%w/v> DMCD 3% w/v > sCT.

From preliminary permeability studies a synergism in the enhancement potential was observed when HPCD and NaGC were combined, see Table 13.1. There was a significant enhancement in the Papp of the peptide sCT and the paracellular markers mannitol and PEG 4000 for this combination compared to the individual excipients ($p < 0.05$).

Table 13.1 The apparent permeability (Papp) across the CaCo-2 cell monolayer over 4h of the hydrophilic compounds, mannitol, PEG 4000 and sCT for the simple micelle, sodium glycocholate (NaGC) 10mM, the commercially available cyclodextrin, 2-hydroxylpropyl-β-cyclodextrin (HPCD) (1% w/v) and a combination of both excipients.

System	Papp ± SD ($X10^6$) (cm/sec) ($n \geq 3$) ($p<0.05$, *)		
	Mannitol	PEG 4000	sCT
Control	0.46 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
NaGC (10mM)	0.57 ± 0.01	0.05 ± 0.04	0.02 ± 0.01
HPCD 1% w/v	0.47 ± 0.03	0.03 ± 0.01	0.03 ± 0.005
NaGC (10mM) & HPCD (1% w/v)	1.29 ± 0.65*	1.10 ± 0.62*	1.18 ± 0.30*

Complexation of bile acids and cyclodextrins has previously been reported as a 1:1 complex between β-cyclodextrin and sodium glycocholate (Tan and Lindenbaum, 1991). This study reported that the nature of complexation varied with the bile salt structure. Abe et al., (1995) reported that the combination of HPCD with a lipophilic absorption enhancer, 1-[2-9decylthio)ethyl]azacyclopentane-2-one (HPE-101) resulted in a significant increase in rat nasal permeability of FD-4, compared to when the enhancer was used on its own. The formation of a complex between NaGC (10mM) and HPCD (1% w/v) may partly explain the synergistic effect.

One of the major criticisms of the CaCo-2 cell monolayer as a model of intestinal permeability is the lack of a mucus layer, which is present in the *in-vivo* situation. The mucus layer may have a protective function and thereby limit the effectiveness of the absorption enhancer, in addition an electrostatic effect with drugs or excipients may occur. This co-culture model has the ability to produce and secrete mucus into the apical chamber, as was determined by an ELLA assay and staining techniques. It has been suggested that this co-culture model is a better representative of the small intestine because of the similarity in electrical resistance, permeability characteristics and the presence of the mucus layer (Meaney and O'Driscoll, 1999). In order to ascertain if

mucus was a barrier to the absorption enhancers, studies were performed using the CaCo-2: Ht29GlucH co-culture model.

In the case of the NaGC simple micelles when mucus was present there was a concentration dependant decrease in the permeabilities of sCT, mannitol and PEG 4000 relative to the control. The effect that the micelle system had on mucin production was investigated using the ELLA and it was evident that NaGC 15mM stimulated the release of mucin into the apical chamber. The stimulation of mucin release from differentiated Ht29 cells in the presence of bile salts has previously been shown (Shekels et al., 1996). The *in-situ* rat intestinal model has shown that as the concentration of NaTC increased there was an increase in the release of hexoses (mucin components) (Poelma et al., 1990). This mucin may impede the diffusion of the bile salt micelles and therefore decrease access of the enhancer to the membrane. Following removal of the mucus layer and subsequent exposure to the simple micelles of NaGC there was a significant increase in the permeability of the hydrophilic markers, which was greater than the apparent enhancement calculated for the control monolayers. Therefore from this study it appears that mucus act as a barrier to the enhancement potential of NaGC simple micelles.

The possibility of electrostatic interaction between sCT and the mucus layer exists. As sCT at pH 7.4 is positively charged (Duncan et al., 1994) it may be bind to the mucus layer because of an electrostatic attraction. In addition, in the case of the enhancer system, SBE_7CD 20% w/v there may be an electrostatic repulsion because this CDs is negatively charged (Okimoto et al., 1996). This electrostatic interaction has been demonstrated by numerous authors using *in-vitro* techniques and co-culture cell model (Wikman-Larhed et al., 1996, 1998; Meaney and O'Driscoll et al., 1999). On removal of the mucus layer there was a statistically significant increase in the permeability of sCT across the co-culture monolayer, however this increase may partly be associated with the enhancing effect of the mucolytic agent. For SBE_7CD 20% w/v in the absence of a mucus layer the relative enhancement seen for this system was greater than the control monolayer which may be attributable to removal of the repulsion caused by the mucus layer.

The mixed micelle NaGC: LA (15: 1mM) and the cyclodextrins, both novel and commercial except SBE_7CD 20% w/v, all caused statistically significant increases in the permeabilities of the hydrophilic compounds in the presence of mucus. No further enhancement was seen on the removal of the mucus layer. This would indicate that the mucus layer was not a barrier to the absorption potential of NaGC: LA (15: 1mM), ACD 20% w/v, PCD 20% w/v, DMCD 3% w/v and HPCD 10% w/v. The effects of the cyclodextrins on mucin production and viscosity have been investigated, and it would appear that they have no mucoregulatory effect at the concentrations investigated (Appendix 3).

The major findings or conclusions of this body of work are as follows:

- Salmon calcitonin (sCT) was transported across the CaCo-2 and CaCo-2: Ht29GlucH cell culture models. The apparent permeability being 9-fold greater for the co-culture model as compared to the monoculture.
- The permeability mechanism of sCT did not involve an active localised transporter but appeared to be a passive mechanism. From confocal laser microscopy it appeared that the X–Y scanning micrographs that were taken down through the monolayer suggested that sCT was localised in the cell wall area.
- The stability of sCT was compromised in the presence of sodium taurocholate (NaTC) (20mM) and sodium cholate (NaC) (5mM), but not in the presence of sodium glycocholate (NaGC) (10-20mM).
- The simple bile salt micelles of NaGC caused a concentration dependant increase in the permeability of sCT and the paracellular markers, PEG 4000 and mannitol.
- The mixed micelles of NaGC and linoleic acid (LA) enhancing effects were shown to depend both on the fatty acid loading and also the concentration of bile salt that was incorporated.
- There appeared to be a linear correlation between apparent permeability and TEER for NaGC simple micellar systems, which suggested that these systems act primarily on the tight junctional complex.
- The effect of the simple micellar system NaGC (15mM) was reversible, however the mixed micelle system NaGC: LA (15: 1mM) was not reversible.

- The CaCo-2: Ht29GlucH co-culture model produced a detectable mucus layer.
- The removal of the mucus layer was achieved using 0.5% w/v N-Acetyl-Cysteine (N-AC), however this resulted in an increase in the permeability of sCT and the paracellular markers, mannitol and PEG 4000.
- The mucus layer appeared to be a barrier to the enhancing potential of the simple micellar systems of NaGC. However, for the mixed micelle NaGC: LA (15: 1mM) the presence of mucus had no effect on its' enhancing potential.
- On removal of the mucus layer there was a concentration dependant increase in the transport of the hydrophilic compounds in the presence of the simple micelles of NaGC.
- The cyclodextrin dimethyl-β-cyclodextrin (DMCD) 3% w/v had the greatest enhancing effect on the transport of the hydrophilic compounds, sCT, mannitol and PEG 4000.
- The novel synthesised cyclodextrins, Heptakis (6-deoxy-6-aminopyridyl)-β-cyclodextrin sodium salt (ACD) (20% w/v) and Heptakis (6-O-sulphonatophenyl)-β-cyclodextrin sodium salt (PCD) (20% w/v) caused a statistically significant increase in the apparent permeabilities of sCT, mannitol and PEG 4000.
- Mucus appeared not to affect the enhancing potential of the cyclodextrins with the exception of sulfobutyl-β-cyclodextrin (SBE_7CD) (20% w/v), which may be associated with a charge effect.
- The molecular modelling of sCT resulted in a similar molecular radius to PEG 4000 and from that a linear correlation of Papp for sCT and PEG 4000 was seen in the presence of simple micelles of NaGC. This relationship was not as strong for the cyclodextrin systems, which further suggested that these enhancers affected not only the tight junctional complex but may be implicated in disruption of the cell matrix.

Appendix I

Table I: The % of salmon calcitonin (sCT) degraded in the apical chamber of the CaCo-2 cell monolayer at the end of a 4 hour transport experiment for each of the bile salt micelles, commercially available and novel cyclodextrins, ($p < 0.05$ * compared to sCT alone)

System	% sCT Degraded ± SE of the Initial Starting Conc. at the end of 4h experiment ($n \geq 3$)
sCT	22.79 ± 2.14
Bile salt simple micelles	
NaGC (10mM)	17.98 ± 1.60
NaGC (12.5mM)	17.72 ± 0.81
NaGC (15mM)	18.79 ± 1.40
NaGC (20mM)	21.13 ± 1.21
NaTC (20mM)	64.65 ± 3.14*
NaC (5mM)	35.41 ± 8.82*
Deoxy (1mM)	21.76 ± 0.32
NaGC: LA Mixed micelles	
10: 0	17.98 ± 1.60
10: 1	10.42 ± 1.26
10: 1.25	16.36 ± 0.71
12.5: 0	17.72 ± 0.81
12.5: 1	17.43 ± 2.96
15: 0	18.79 ± 1.40
15: 0.5	5.84 ± 1.44
15: 0.75	10.81 ± 9.52
15: 0.85	9.24 ± 2.10
15: 0.9	9.42 ± 0.57
15: 1	14.47 ± 1.19
Commercially available cyclodextrins	
DMCD 1% w/v	24.51 ± 10.90
DMCD 3% w/v	42.03 ± 6.67
HPCD 1% w/v	9.16 ± 1.91
HPCD 5% w/v	15.14 ± 1.98
HPCD 10% w/v	14.95 ± 1.41
SBE_7CD 10% w/v	10.45 ± 2.25
SBE_7CD 20% w/v	14.33 ± 3.38
Novel cyclodextrins	
HCD 5 % w/v	16.20 ± 1.63
HCD 10 % w/v	19.69 ± 3.14
HCD 20 % w/v	13.19 ± 4.10
ACD 5 % w/v	10.25 ± 2.93
ACD 10 % w/v	4.82 ± 0.30
ACD 20 % w/v	5.17 ± 4.04
PCD 5 % w/v	18.11 ± 1.88
PCD 10 % w/v	23.24 ± 1.24
PCD 20 % w/v	8.30 ± 4.12

Table II The percentage sCT degraded in the apical chamber of CaCo-2: Ht29GlucH co-culture monolayer at the end of a 4 hr transport experiment in the presence of mucus. ($p < 0.05$, *)

System	% sCT Degraded ± SE of the Initial Starting Conc. at the end of 4h experiment(n ≥ 3)
sCT	18.07 ± 3.52
NaGC Simple and Mixed Micelles (NaGC: LA)	
10: 0	11.06 ± 4.13
15: 0	15.71 ± 8.88
15: 1	14.93 ± 0.97
Commercially Available Cyclodextrins (CCDs)	
DMCD 3% w/v	32.98 ± 1.22*
HPCD 10% w/v	14.42 ± 2.89
SBE_7CD 10% w/v	5.63 ± 0.50*
SBE_7CD 20% w/v	6.05 ± 0.02*
Novel Cyclodextrins (NCDs)	
ACD 10 % w/v	2.65 ± 0.73
ACD 20 % w/v	9.25 ± 1.31
PCD 10 % w/v	16.03 ± 2.10
PCD 20 % w/v	7.62 ± 1.40

Table III The effect of commercially available and novel cyclodextrins (CCDs and NCDs) on the stability of sCT in the apical chamber of the CaCo-2: Ht29GlucH cell monolayer at the onset and end of a 4h experiment in the absence of mucus. ($p < 0.05$, *)

System	% sCT Degraded ± SE of the Initial Starting Conc. at the end of 4h experiment(n ≥ 3)
sCT	9.04 ± 0.76
NaGC Simple and Mixed Micelles (NaGC: LA)	
10: 0	5.60 ± 0.50
15: 0	12.12 ± 8.79
15: 1	8.56 ± 2.11
Commercially Available Cyclodextrins (CCDs)	
DMCD 3% w/v	26.76 ± 0.73*
HPCD 10% w/v	9.15 ± 1.24
SBE_7CD 10% w/v	6.41 ± 0.61
SBE_7CD 20% w/v	5.70 ± 0.56
Novel Cyclodextrins (NCDs)	
ACD 20 % w/v	7.72 ± 1.50
PCD 20 % w/v	7.71 ± 0.72

Appendix II

NMR Results for Novel cyclodextrins obtained from the Dept. of Chemistry, UCD:

Heptakis (6-(1-sulphonatopropyl-3-thiol)-2,3-di-O-acetyl)-β-cyclodextrin (HCD)

^{1}H NMR (270 MHz, D_2O)	5.25 (2H, H-3(t); H-1(d); 4.94 (dd, 2H, H-2); 4.15 (m, 1H, H-5); 3.98 (t, 1H, H-4); 3.03-3.17 (m, 2H, H-6a, H-6b); 2.97 (m, 4H, S$\underline{CH_2}\underline{CH_2}$-); 2.85 (t, 2H, $J_{1,2}$ = 7.2 Hz, $J_{2,3}$ = 7 Hz, -$\underline{CH_2}$); 2.09 (s, 6H, OAC, $\underline{CH_3}$)
^{1}H NMR (270 MHz, DMSO)	5.18 (t, 1H, H-3; 5.08 (d, 1H, H-1);4.69 (dd, 1H, H-2) 4.10 (m, 1H, H-5); 3.79 (t, 1H, H-4); 2.88-3.03 (m, 2H, H-6a, H-6b); 2.75 (t, 2H, $J_{1,2}$ = 7.1Hz, $J_{2,3}$= 7.3Hz, -$\underline{CH_2}$); 1.98 (s, 6H, OAC, $\underline{CH_3}$); 1.86- 1.94 (m, 4H, $J_{1,2}$ = 7.3Hz, $J_{2,3}$= 7.5Hz, S$\underline{CH_2}\underline{CH_2}$)
^{13}C NMR (125 MHz, DMSO)	173.34 (CO); 172.58 (CO); 99.94 (C-1); 82.32 (C-4); 74.85 (C-5); 74.07 (C-3); 73.90 (C-2); 54.35, 53.67 (SCH_2CH_2); 37.35 (C-6); 29.34 (CH_2); 23.91 (OAC)
$C_{91}H_{133}S_{14}O_{63}Na_7$	Theory: C 34.42%, H 4.71%, S 15.78%, Na 5.66% Found : C 33.56%, H 3.97%, S 15.21%, Na 5.12%

Heptakis (6-deoxy-6-aminopyridyl)-β-cyclodextrin sodium salt (ACD)

^{1}H NMR (270 MHz, D_2O) 7.88 (d, J= 7.15 Hz, 2H, H-2 of Pyr), 6.83 (d, J=7.15 Hz, 2H, H-3 of Pyr), 5.15 (d, J=3.2 Hz, 1H, H-1), 4.36 (m, 1H, H-5), 3.99 (m, 3H, $H6/H6^{1}$, H-3), 3.62, (dd, 1H, H-2), 3.46 (t, 1H, H-4).

^{13}C NMR (68 MHz, D_2O) 158.847 (C-4 of aminopyr), 148.267 (C-3 of aminopyr), 143.795 (C-2 of aminopyr), 101.904 (C-1), 82.724 (C-4), 72.22 (C-3), 71.87 (C-2), 69.99 (C-5), 58.23 (C-6).

$C_{77}H_{98}N_{14}O_{28}$	Theory	C 54.46%, H 5.88%, N 11.76%
	Found	C 55.18%, H 5.91%, N 12.23%

Heptakis (6-O-sulphonatophenyl)-β-cyclodextrin sodium salt (PCD)

^{1}H NMR (270 MHz, D_2O) 7.69 (d, 2H, J= 7.87 Hz phenyl H's beside SO_3Na), 6.99 (d, 2H, J =7.87 Hz phenyl H's beside O-), 4.00 (d, 1H, J= 3.4 Hz, H-1), 4.14 (m, 2H, H-3, H-5), 4.00 (t, 1H, H-4), 3.83 (m, 1H, H-2), 3.59 (m, 2H, H6/H6').

^{13}C NMR (67 MHz,DMSO) 162.609 (C-SO_3Na), 143.738 (C-O), 131.369 (C of phenyl ring), 117.386 (C of phenyl ring), 106.44 (C-1), 86.145 (C-4), 76.987 (C-3), 76.291 (C-2), 73.87 (C-5), 71.007 (C-6).

$C_{84}H_{91}Na_7O_{56}S_{23}$	Theory	C 42.35%, H 3.82%, Na 6.76%, S 9.41%
	Found	C 40.52%, H 3.78%, Na 6.98%, S 10.12%

Appendix III

The effect of commercially available cyclodextrins on the viscosity of partially purified porcine mucin (PPPM). No statistical difference was observed between Mucin control and Mucin and cyclodextrins. Mean number of observations ≥4.

Cyclodextrin & Mucus	Viscosity Coefficient	se
2.5% Mucus	0.005382	0.000179622
Mucus 2.5% + DMBCD 3%	0.005735	0.000469867
Mucus 2.5% + HPBCD 10%	0.0058	0.000246881
Mucus 2.5% + SBE7 20%	0.005312	0.000345563

To ascertain if commercial CDs had to ability to promote the release or production of mucus an ELLA assay was carried out the results suggested that there was no difference to control levels i.e. sCT alone. n= 3 observations.

System	sCT	HPBCD (10%w/v)	DMBCD (3%w/v)
Mucin Conc. (ng/ml)	7.017361	4.378472222	9.638888889
SD	1.924501	1.16500688	2.512087388

References

Abe, K., T. Irie, H. Adachi, and K. Uekama. "Combined use of 2-hydroxypropyl-beta-cyclodextrin and a lipophilic absorption enhancer in nasal delivery of the LHRH agonist, buserelin acetate, in rats." *International Journal of Pharmaceutics* 123, no. 1 (1995): 103-12.

Adachi, H., H. Arima, T. Irie, F. Hirayama, K. Uekama, T. Yano, M. Saita, and K. Noda. "Combined use of cyclodextrin derivatives and penetration enhancer HPE-101 in transdermal delivery of Prostaglandin-E." *Journal of Pharmacobio-Dynamics* 15, no. 2 (1992): S35.

Adson, A., P. S. Burton, T. J. Raub, C. L. Barsuhn, K. L. Audus, and N. F. H. Ho. "Passive diffusion of weak organic electrolytes across CaCo-2 cell monolayers - uncoupling the contributions of hydrodynamic, transcellular, and paracellular barriers." *Journal of Pharmaceutical Sciences* 84, no. 10 (1995): 1197-204.

Adson, A., T. J. Raub, P. S. Burton, C. L. Barsuhn, A R. Hilgers, K. L. Audus, and N. F. H. Ho. "Quantitative approaches to delineate paracellular diffusion in cultured epithelial-cell monolayers." *Journal of Pharmaceutical Sciences* 83, no. 11 (1994): 1529-36.

Agerholm, C., Bastholm L, Johansen PB, Nielsen MH, and Elling F. "Epithelial transport and bioavailability of intranasally administered human growth hormone formulated with the absorption enhancers didecanoyl-l-alpha-phosphatidylcholine and alpha-cyclodextrin in rabbits." *Journal of Pharmaceutical Sciences* 83 (1994): 1706-11.

Allen, J.D., G.P. Martin, C. Marriott, I. Hassan, and I. Williamson. "Drug transport across a novel mucin secreting cell model, comparison with the CaCo-2 cell system." *Journal of Pharmaceutical Pharmacology* 43 60P (1991).

Allen, J.D. "A mucus secreting cell co-culture model for drug absorption studies." *Ph.D. Thesis, Brighton Polytechnic* (1992).

Anderberg, E. K., and P. Artursson. "Epithelial transport of drugs in cell culture. VIII: effects of sodium dodecyl sulfate on cell membrane and tight junction permeability in human intestinal epithelial (Caco-2) cells." *Journal of Pharmaceutical Science* 82, no. 4. (1993a): 392-8.

Anderberg, E. K., T. Lindmark, and P. Artursson. "Sodium caprate elicits dilatations in human intestinal tight junctions and enhances drug absorption by the paracellular route." *Pharmaceutical Research* 10, no. 6 (1993b): 857-64.

Anderberg, E. K., C. Nyström, and P. Artursson. "Epithelial Transport of drugs in cell culture. VII: Effects of pharmaceutical surfactant excipients and bile acids on transepithelial permeability in monolayers of human intestinal epithelial (Caco-2) cells." *Journal of Pharmaceutical Science* 81, no. 9 (1992): 879-87.

Anderle, P., E. Niederer, W. Rubas, C. Hilgendorf, H. Spahn-Langguth, H. Wunderli-Allenspach, H. P. Merkle, and P. Langguth. "P-Glycoprotein (P-GP) mediated efflux

in CaCo-2 cell monolayers: the influence of culturing conditions and drug exposure on P-Gp expression levels." *Journal of Pharmaceutical Sciences* 87, no. 6 (1998): 757-62.

Anderson, B. W., A. S. Levine, D. G. Levitt, J. M. Kneip, and M. D. Levitt. "Physiological measurement of luminal stirring in perfused rat jejunum." *American Journal of Physiology* 254, no. 6 Pt 1 (1988): G843-8.

Arima, H., K. Wakamatsu, T. Aritomi, I. Irie and K. Uekama "Improvement in nasal delivery of insulin with simultaneous use of chemically modified cyclodextrins in rats. In "*Proceedings of the 5th Symposium on Cyclodextrins*". (Eds. Duchêne, D.) March (1991): 487-90. Published Edition Santé, Paris.

Artursson, P. "Cell cultures as models for drug absorption across the intestinal mucosae." *Critical Reviews Therapeutic Drug Carrier Systems* 8, no. 4 (1991): 305-30.

Artursson, P. "Epithelial Transport of Drugs in Cell Culture. I: a model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells." *Journal of Pharmaceutical Science* 79, no. 6 (1990a): 476-82.

Artursson, P., and J. Karlsson. "Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells." *Biochemical & Biophysical Research Communications* 175, no. 3 (1991): 880-5.

Artursson, P., A.L. Ungell, and J.E., Lofroth. "Selective paracellular permeability in two models of intestinal absorption: cultured monolayers of human intestinal epithelial cells and rat intestinal segments." *Pharmaceutical Research* 10, no. 8 (1993): 1123-29

Artursson, P., and C. Magnusson. "Epithelial transport of drugs in cell culture. II: Effect of extracellular calcium concentration on the paracellular transport of drugs of different lipophilicities across monolayers of intestinal epithelial (Caco-2) cells." *Journal of Pharmaceutical Sciences* 79, no. 7 (1990b): 595-600.

Artursson, P., K. Palm, and K. Luthman. "Caco-2 monolayers in experimental and theoretical predictions of drug transport." *Advanced Drug Delivery Reviews* 15, No. 22(1-2), (1996) : 67-84.

Asada, H., T. Douen, Y. Mizokoshi, T. Fujita, M. Murakami, a. Yamamoto, and S. Muranishi. "Stability of acyl derivatives of insulin in the small-intestine- relative importance of insulin association characteristics in aqueous-solution." *Pharmaceutical Research* 11, no. 8 (1994): 1115-20.

Atwood, D. and A. T. Florence. In *Surfactant Systems* Chapman Hall (1983): 124- 228

Audus, K. L., R. L. Bartel, I. J. Hidalgo, and R. T. Borchardt. "The use of cultured epithelial and endothelial-cells for drug transport and metabolism studies." *Pharmaceutical Research* 7, no. 5 (1990): 435-51.

Augustijns, P. F., and R. T. Borchardt. "Transport and metabolism of delta sleep-inducing peptide in cultured human intestinal epithelial cell monolayers." *Drug Metabolism and Disposition* 23, no. 12 (1995): 1372-78.

Augustijns, P. F., T. P. Bradshaw, L. S. L. Gan, R. W. Hendren, and D. R. Thakker. "Evidence for a polarized efflux system in Caco-2 cells capable of modulating Cyclosporine-A transport." *Biochemical and Biophysical Research Communications* 197, no. 2 (1993): 360-365.

Aungst, B. J. "Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism." *Journal of Pharmaceutical Sciences* 82, no. 10 (1993): 979-87.

Azria M. "The value of biomarkers in detecting alterations in bone metabolism." - *Calcified Tissue International* 45 No 1 (1989): 7-11.

Bai, J. P., M. J. Hsu, and W. T. Shier. "Insulin-degrading enzyme in a human colon adenocarcinoma cell line (Caco-2)." *Pharmaceutical Research* 12, no. 4 (1995a): 513-7.

Bai, J. P. "The involvement of cytosolic chymotrypsin-like, trypsin- like, and cucumsin-like activities in degradation of insulin and insulin-like growth factor I by epithelial tissues." *Journal of Pharmacy and Pharmacology* 47, no. 8 (1995b): 674-7.

Bai, J. P. F., and G. L. Amidon. "Structural specificity of mucosal-cell transport and metabolism of peptide drugs - implication for oral peptide drug delivery." *Pharmaceutical Research* 9, no. 8 (1992): 969-78.

Bai, J. P., H. J. Hong, D. A. Rothenberger, W. D. Wong, and J. G. Buls. "The presence of insulin-degrading enzyme in human ileal and colonic mucosal cells." *Journal of Pharmacy and Pharmacology* 48, no. 11 (1996): 1180-4.

Banga, A.K. *Therapeutic peptides and proteins: formulation, processing and delivery systems* (Publishers Technomic Publishing Co. Inc.) 1996.

Baker, S. S., and R. D. Baker. "Antioxidant enzymes in the differentiated Caco-2 cell-line." *In Vitro Cellular & Developmental Biology-Animal* 28A, no. 9-10 (1992): 643-47.

Baranczykkuzma, A., J. A. Garren, I. J. Hidalgo, and R. T. Borchardt. "Substrate-specificity and some properties of phenolsulfotransferase from human intestinal Caco-2 cells." *Life Sciences* 49, no. 16 (1991): 1197-206.

Barclay, R. K., and M. A. Phillipps. "Inhibition of enkephalin-degrading aminopeptidase activity by certain peptides." *Biochemical and Biophysical Research Communications* 96, no. 4. (1980): 1732-8.

Baruch, E., D. Lichtenberg, P. Barak, and S. Nir. "Calcium-binding to bile-salts."

Chemistry and Physics of Lipids 57, no. 1 (1991): 17-27.

Baudyš, M., D. Mix, and S. W. Kim. "Stabilization and intestinal absorption of human calcitonin." *Journal of Controlled Release* 39, no. 2-3 (1996): 145-51.

Bendayan, M., E. Ziv, D. Gingras, R. Bensasson, H. Baron, and M. Kidron. "Biochemical and morpho-cytochemical evidence for the intestinal-absorption of insulin in control and diabetic rats -comparison between the effectiveness of duodenal and colon mucosae." *Diabetologia*" 37, no. 2 (1994): 119-26.

Bhat, M., D. Toledovelasquez, L. Wang, C. J. Malanga, J. K. H. Ma, and Y. Rojanasakul. "Regulation of tight junction permeability by calcium mediators and cell cytoskeleton in rabbit tracheal epithelium." *Pharmaceutical Research* 10, no. 7 (1993): 991-97.

Bjork, E., U. Isaksson, P. Edman, and P. Artursson. "Starch microspheres induce pulsatile delivery of drugs and peptides across the epithelial barrier by reversible separation of the tight junctions." *Journal of Drug Targeting* 2, no. 6 (1995): 501-7.

Blanchard, J., L. M. Tang, and M. E. Earle. "Reevaluation of the absorption of carbenoxolone using an *in-situ* rat intestinal technique." *Journal of Pharmaceutical Sciences* 79, no. 5 (1990): 411-14.

Boulenc, X., M. Bourrie, I. Fabre, C. Roque, H. Joyeux, Y. Berger, and G. Fabre. "Regulation of cytochrome-p450 gene-expression in a human intestinal-cell line, Caco-2." *Journal of Pharmacology and Experimental Therapeutics* 263, no. 3 (1992): 1471-78.

Boulenc, X., T. Breul, J. C. Gautier, P. Saudemon, H. Joyeux, C. Roques, Y. Berger, and G. Fabre. "Sodium lauryl sulfate increases tiludronate paracellular transport using human epithelial Caco-2 monolayers." *International Journal of Pharmaceutics* 123, no. 1 (1995): 71-83.

Brange, J., L. Andersen, E. D. Laursen, G. Meyn, and E. Rasmussen. "Toward understanding insulin fibrillation." *Journal of Pharmaceutical Sciences* 86, no. 5 (1997): 517-25.

Brange, J., L. Langkjaer, and J. Markussen. "Insulin structure and Stability." *Pharmaceutical Biotechnology, Stability and Characteristics of protein and Peptide Drugs: Case Histories* Vol 5 (1993) (Eds: Wang, Y.J. and Pearlman, R.) Plenum Press, New York, 315-350.

Brewster, M. E., M. S. Hora, J. W. Simpkins, and N. Bodor. "Use of 2-hydroxypropyl-β-cyclodextrin as a solubilizing and stabilizing excipient for protein drugs." *Pharmaceutical Research* 8, no. 6 (1991): 792-95.

Brown, D.T., C. Marriott and M.F. Beeson. "Antibiotic binding to purified mucus glycoprotein." *Journal of Pharmaceutical Pharmacology* 35, 80P (1983).

Burke, C. J., B. L. Steadman, D. B. Volkin, P. K. Tsai, M. W. Bruner, and C. R. Middaugh. "The adsorption of proteins to pharmaceutical container surfaces." *International Journal of Pharmaceutics* 86, no. 1 (1992): 89-93.

Burton, P. S., R. A. Conradi, and A. R. Hilgers. "Mechanisms of peptide and protein-absorption .2. Transcellular mechanism of peptide and protein-absorption - passive aspects." *Advanced Drug Delivery Reviews* 7, no. 3 (1991): 365-86.

Burton, P. S., R. A. Conradi, A. R. Hilgers, and N. F. H. Ho. "Evidence for a polarized efflux system for peptides in the apical membrane of CaCo-2 cells." *Biochemical and Biophysical Research Communications* 190, no. 3 (1993): 760-766.

Burton, P. S., R. A. Conradi, N. F. H. Ho, A. R. Hilgers, and R. T. Borchardt. "How structural features influence the biomembrane permeability of peptides." *Journal of Pharmaceutical Sciences* 85, no. 12 (1996): 1336-40.

Burton, P. S., J. T. Goodwin, R. A. Conradi, N. F. H. Ho, and a. R. Hilgers. "*In-vitro* permeability of peptidomimetic drugs: the role of polarized efflux pathways as additional barriers to absorption." *Advanced Drug Delivery Reviews* 23, no. 1-3 (1997): 143-56.

Buur, A., and N. Mork. "Metabolism of testosterone during *in-vitro* transport acrosscaco-2 cell monolayers - evidence for beta-hydroxysteroid dehydrogenase-activity in differentiated Caco-2 cells." *Pharmaceutical Research* 9, no. 10 (1992): 1290-1294.

Carey, M. C. and D. M. Small. "The characteristics of mixed micellar solutions with particular reference to bile." *American Journal of Medicine.* 49, Nov (1970): 590-608.

Carey, M. C. "Physical-chemical properties of bile acids and their salts". In *Sterols and bile acids* (Eds. Danielsson, H. and Sjovall, J.) (1985): 345- 503

Carino, G. P., and E. Mathiowitz. "Oral insulin delivery." *Advanced Drug Delivery Reviews* 35, no. 2-3 (1999): 249-57.

Caro, I., X. Boulenc, M. Rousset, V. Meunier, M. Bourrie, B. Julian, H. Joyeux, C. Rogues, Y. Berger, a. Zweibaum, and G. Fabre. "Characterization of a newly isolated Caco-2 clone (TC-7), as a model of transport processes and biotransformation of drugs." *International Journal of Pharmaceutics* 116, no. 2 (1995): 147-58.

Cereijido, M., L. González Mariscal, R. G. Contreras, J. M. Gallardo, R. García Villegas, and J. Valdés. "The making of a tight junction." *Journal of Cell Science* Suppl:17 (1993): 127-32.

Chantret, I., A. Rodolosse, A. Barbat, E. Dussaulx, E. Brotlaroche, a. Zweibaum, and M. Rousset. "Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell-line Caco-2 -evidence for glucose-dependent negative regulation." *Journal of Cell Science* 107 (1994): 213-25.

Charman, S. A., K. L. Mason, and W. N. Charman. "Techniques for assessing the effects of pharmaceutical excipients on the aggregation of porcine growth-hormone." *Pharmaceutical Research* 10, no. 7 (1993): 954-62.

Child, P., and J. Rafter. "Calcium enhances the hemolytic action of bile salts." *Biochimica Et Biophysica Acta* 855, no. 3 (1986): 357-64.

Chong, S. H., S. A. Dando, K. M. Soucek, and R. A. Morrison. "*In-vitro* permeability through CaCo-2 cells is not quantitatively predictive of *in-vivo* absorption for peptide-like drugs absorbed via the dipeptide transporter system." *Pharmaceutical Research* 13, no. 1 (1996): 120-123.

Claude, P. "Morphological factors influencing transepithelial permeability: a model for the resistance of the Zonula Occludens." *Journal of Membrane Biology* 39, no. 2-3 (1978): 219-32.

Cogburn, J. N., M. G. Donovan, and C. S. Schasteen. "A model of human small intestinal absorptive cells .1.transport barrier." *Pharmaceutical Research* 8, no. 2 (1991): 210-216.

Collares Buzato, C. B., G. T. A. Mcewan, M. A. Jepson, N. L. Simmons, and B. H. Hirst. "Paracellular barrier and junctional protein distribution depend on basolateral extracellular ca2+ in cultured epithelia." *Biochimica Et Biophysica Acta-Molecular Cell Research* 1222, no. 2 (1994): 147-58.

Conradi, R. A., A. R. Hilgers, N. F. H. Ho, and P. S. Burton. "The influence of peptide structure on transport across Caco-2 cells." *Pharmaceutical Research* 8, no. 12 (1991): 1453-60.

Conradi, R. A., A. R. Hilgers, N. F. H. Ho, and P. S. Burton. "The influence of peptide structure on transport across caco-2 cells .2. Peptide-bond modification, which results in improved permeability." *Pharmaceutical Research* 9, no. 3 (1992): 435-39.

Conradi, R. A., K. F. Wilkinson, B. D. Rush, A. R. Hilgers, M. J. Ruwart, and P. S. Burton. "*In-vitro in-vivo* models for peptide oral absorption -comparison of caco-2 cell-permeability with rat intestinal-absorption of renin inhibitory peptides." *Pharmaceutical Research* 10, no. 12 (1993): 1790-1792.

Corrigan, O. I. and C. T. Stanley. "Mechanism of drug dissolution rate enhancement from beta- cyclodextrin-drug systems." *Journal of Pharmacy and Pharmacology* 34, no. 10 (1982): 621-6.

Crane, C. W., and G. R. Luntz. "Absorption of insulin from the human small intestine." *Diabetes* 17, No. 10 (1968): 625-7.

Croyle, M. A., B. J. Roessler, C. P. Hsu, R. Sun, and G. L. Amidon. "Beta cyclodextrins enhance adenoviral-mediated gene delivery to the intestine." *Pharmaceutical Research* 15, no. 9 (1998): 1348-55.

Cruz, N., X. Alvarez, R. D. Specian, R. D. Berg, and E. A. Deitch. "Role of mucin, mannose, and beta-1 integrin receptors in *Escherichia coli* translocation across Caco-2 cell monolayers." *Shock* 2, no. 2 (1994): 121-26.

Dan, N., and D. F. Cutler. "Transcytosis and processing of intrinsic factor-cobalamin in CaCo-2 cells." *Journal of Biological Chemistry* 269, no. 29 (1994): 18849-55.

Dantzig, A. H. "Oral absorption of beta-lactams by intestinal peptide transport proteins." *Advanced Drug Delivery Reviews* 23, no. 1-3 (1997): 63-76.

Dantzig, A. H., and L. Bergin. "Uptake of the cephalosporin, cephalexin, by a dipeptide transport carrier in the human intestinal-cell line, Caco-2." *Biochimica Et Biophysica Acta* 1027, no. 3 (1990): 211-17.

Dantzig, A. H., D. C. Duckworth, and L. B. Tabas. "Transport mechanisms responsible for the absorption of loracarbef, cefixime, and cefuroxime axetil into human intestinal Caco-2 cells." *Biochimica Et Biophysica Acta-Biomembranes* 1191, no. 1 (1994): 7-13.

Dantzig, A. H., L. B. Tabas, and L. Bergin. "Cefaclor uptake by the proton-dependent dipeptide transport carrier of human intestinal Caco-2 Cells and comparison to cephalexin uptake." *Biochimica Et Biophysica Acta* 1112, no. 2 (1992): 167-73.

Damge, C., C. Michael, M. Aprahamian and P. Couvreur. "New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapules as drug carrier." *Diabetes*, 37 (1988): 246-51.

Daugherty, A. L., and R. J. Mrsny. "Regulation of the intestinal epithelial paracellular barrier." *Pharmaceutical Science & Technology Today* 2, no. 7 (1999): 281-87.

Daugherty, A. L., and R. J. Mrsny. "Transcellular uptake mechanisms of the intestinal epithelial barrier - Part One." *Pharmaceutical Science & Technology Today* 2, no. 4 (1999): 144-51.

Davis, S. S. "Overcoming barriers to the oral-administration of peptide drugs." *Trends in Pharmacological Sciences* 11, no. 9 (1990): 353-55.

Deftos, L. J., J. J. Nolan, B. L. Seely, P. L. Clopton, G. J. Cote, C. L. Whitham, L. J. Florek, T. A. Christensen, and M. R. Hill. "Intrapulmonary drug delivery of salmon calcitonin." *Calcified Tissue International* 61, no. 4 (1997): 345-47.

Delie, F., and W. Rubas. "A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model." *Critical Reviews in Therapeutic Drug Carrier Systems* 14, no. 3 (1997): 221-86.

Dharmsathaphorn, K., P. A. Huott, P. Vongkovit, G. Beuerlein, S. J. Pandol, and H. V. Ammon. "Cl^- secretion induced by bile salts. a study of the mechanism of action

based on a cultured colonic epithelial cell line." *Journal of Clinical Investigation* 84, no. 3 (1989): 945-53.

Dix, C. J., I. F. Hassan, H. Y. Obray, R. Shah, and G. Wilson. "The transport of vitamin-b12 through polarized monolayers of CaCo-2 cells." *Gastroenterology* 98, no. 5 (1990): 1272-79.

Dodane, V., M. A. Khan, and J. R. Merwin. "Effect of chitosan on epithelial permeability and structure." *International Journal of Pharmaceutics* 182, no. 1 (1999): 21-32.

Dodane, V., and V. D. Vilivalam. "Pharmaceutical applications of chitosan." *Pharmaceutical Science & Technology Today* 1, no. 6 (1998): 246-53.

Dollery, C. T. "Drug discovery and development in the molecular era." *British Journal of Clinical Pharmacology* 47, no. 1 (1999): 5-6.

Doluisio, J. T., N. F. Billups, L. W. Dittert, E. T. Sugita, and J. V. Swintosky. "Drug absorption. I. An in situ rat gut technique yielding realistic absorption rates." *Journal of Pharmaceutical Science* 58, no. 10 (1969): 1196-200.

Donovan, M. D., G. L. Flynn, and G. L. Amidon. "Absorption of polyethylene glycols 600 through 2000: the molecular weight dependence of gastrointestinal and nasal absorption." *Pharmaceutical Research* 7, no. 8 (1990): 863-8.

Drewe, J., G. Fricker, J. Vonderscher, and C. Beglinger. "Enteral absorption of octreotide - absorption enhancement by polyoxyethylene-24-cholesterol ether." *British Journal of Pharmacology* 108, no. 2 (1993): 298-303.

Duchene, D., D. Wouessidjewe, and G. Ponchel. "Cyclodextrins and Carrier Systems." *Journal of Controlled Release* 62, no. 1-2 (1999): 263-68.

Duncan, M. R., J. M. Lee, and M. P. Warchol. "Influence of surfactants upon protein peptide adsorption to glass and polypropylene." *International Journal of Pharmaceutics* 120, no. 2 (1995): 179-88.

Epand R.M. "Relationships among several different non-homologous polypeptide hormones." *Molecular Cell Biochemistry* 57 No 1 (1983): 41-7.

Falk, P., K. A. Roth,and J.I. Gordon. "Lectins are sensitive tools for defining the differentiation programs of mouse gut epithelial cell lineages." *American Journal of Physiology* (1994): G987-1005.

Fantini, J., D. G. Cook, N. Nathanson, S. L. Spitalnik, and F. Gonzalezscarano. "Infection of colonic epithelial-cell lines by Type-1 Human-Immunodeficiency-Virus is associated with cell-surface expression of galactosylceramide, a potential alternative Gp120 receptor." *Proceedings of the National Academy of Sciences of the United States of America* 90, no. 7 (1993): 2700-2704.

Farquhar,M.G. and G.E. Palade. "Junctional complexes in various epithelia." *Journal of cell Biology* 1 (1963): 375- 412.

Fasano, A., B. Baudry, D. W. Pumplin, S. S. Wasserman, B. D. Tall, J. M. Ketley, and J. B. Kaper. "Vibrio-Cholerae Produces a 2nd enterotoxin, which affects intestinal tight junctions." *Proceedings of the National Academy of Sciences of the United States of America* 88, no. 12 (1991): 5242-46.

Fath, M.J., L.H. Zhang, J. Rush, and R. Kolter. "Purification and characterization of Colicin V from *E. Coli* culture supernatants." *Biochemistry* 33, (1994): 6911-17.

Feldman, S.and M. Gibaldi. "Bile Salt-induced permeability changes in the isolated rat intestine." *Proceedings of Society of Experimental Biology and Medicine* 132, no. 3 (1969): 1031-3.

Feldman, S., M. Reinhard, and C. Willson. "Effect of Sodium taurodeoxycholate on biological membranes: release of phosphorus, phospholipid, and protein from everted rat small intestine." *Journal of Pharmaceutical Science* 62, no. 12. (1973): 1961-4.

Fletcher, K. "Drug Delivery-strategies and technologies." *Pharmaceutical Science and Technology Today* 1 (1998): 49-51.

Fogh, J., J.M. Fogh and T. Orefo. "One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice." *Journal of the national Cancer Institute* 59 (1977): 221- 226.

Fix J.A., K. Engle, P.A. Porter, P.S. Leppert, S.J. Selk, C.R. Gardner, and J. Alexander. "Acyl carnitines: Drug absorption-enhancing agents in the gastrointestinal tract." *American Journal of Physiology* 251(3 Pt 1) (1986): G332-40.

Franz, J.M., G., J.P. Vonderscher, and R. Voges. "Quantitative mechanistic studies in simultaneous fluid flow and intestinal absorption using steroids as model solutes." *International Journal of Pharmaceutics* 7, (1980): 19-28.

Fricker, G., J. Drewe, J. Vonderscher, T. Kissel, and C. Beglinger. "Enteral absorption of octreotide." *British Journal of Pharmacology* 105, no. 4 (1992): 783-86.

Fricker, G., A. Fahr, C. Beglinger, T. Kissel, G. Reiter, and J. Drewe. "Permeation enhancement of octreotide by specific bile salts in rats and Human Subjects: *in-vitro/in-vivo* correlations." *British Journal of Pharmacology* 117, no. 1 (1996): 217-23.

Fuller, S.D., and K.Simmons. "Transferrin receptor polarity and recycling accuracy in'tight' and 'leaky' strains of Madin-Darby Canine Kidney cells." *Journal of Cell Biology* 103, (1986): 1769-79.

Gan, L. S. L., and D. R. Thakker. "Applications of the Caco-2 model in the design and development of orally active drugs: elucidation of biochemical and physical barriers

posed by the intestinal epithelium." *Advanced Drug Delivery Reviews* 23, no. 1-3 (1997): 77-98.

Gonnella, P. A., and M. R. Neutra. "Membrane-bound and fluid-phase macromolecules enter separate pre-lysosomal compartments in absorptive cells of suckling rat ileum." *Journal of Cell Biology* 99, no. 3 (1984): 909-17.

Gottesman, M. M., and I. Pastan. "Biochemistry of multidrug-resistance mediated by the multidrug transporter." *Annual Review of Biochemistry* 62 (1993): 385-42

Güldütuna, S., G. Zimmer, M. Leuschner, S. Bhatti, a. Elze, B. Deisinger, M. Hofmann, and U. Leuschner. "The effect of bile salts and calcium on isolated rat liver mitochondria." *Biochimica et Biophysica Acta-Molecular Basis of Disease*, no. 3 (1999): 396-406.

Gumbiner, B. "Structure, biochemistry, and assembly of epithelial tight junctions." *American Journal of Physiology* 253, no. 6, Pt 1 (1987): C749-58.

Haeberlin, B., T. Gengenbacher, a. Meinzer, and G. Fricker. "Cyclodextrins - Useful Excipients for Oral Peptide administration?" *International Journal of Pharmaceutics* 137, no. 1 (1996): 103-10.

Hammad, M. A., and B. W. Muller. "Factors affecting solubility and stability of indomethacin in mixed micelles." *Pharmazie* 53, no. 11 (1998a): 790-794.

Hammad, M. A., and B. W. Muller. "Solubility and stability of clonazepam in mixed micelles." *International Journal of Pharmaceutics* 169, no. 1 (1998b): 55-64.

The Handbook of Biological Confocal Microscopy.

Hartman, F., R. Owen and D.M. Bissell. "The preparation of isolated intestinal enterocytes." *American Journal of Physiology*. 242, G147 (1982).

Harrison, D. D., and H. L. Webster. "The preparation of isolated intestinal crypt cells." *Experimental Cell Research* 55, no. 2 (1969): 257-60.

Hastewell, J., S. Lynch, R. Fox, I. Williamson, P. Skeltonstroud, and M. Mackay. "Enhancement of human calcitonin absorption across the rat colon *in-vivo*." *International Journal of Pharmaceutics* 101, no. 1-2 (1994): 115-20.

Hastewell, J., S. Lynch, I. Williamson, R. Fox, and M. Mackay. "Absorption of human calcitonin across the rat colon *in-vivo*." *Clinical Science* 82, no. 5 (1992): 589-94.

Hata, Y. S., S. Ota, T. Kawabe, A. Terano, M. Razandi, and K. J. Ivey. "Bile-salts stimulate mucous glycoprotein-secretion from cultured rabbit gastric-mucosal cells." *Journal of Laboratory and Clinical Medicine* 124, no. 3 (1994): 395-400.

Hayashi, M., T. Sakai, Y. Hasegawa, T. Nishikawahara, H. Tomioka, a. Iida, N. Shimizu,

M. Tomita, and S. Awazu. "Physiological Mechanism for Enhancement of Paracellular Drug transport." *Journal of Controlled Release* 62, no. 1-2 (1999): 141-48.

Hidalgo, I. J., P. Bhatnagar, C. P. Lee, J. Miller, G. Cucullino, and P. L. Smith. "Structural requirements for interaction with the oligopeptide transporter in Caco-2 cells." *Pharmaceutical Research* 12, no. 2 (1995): 317-19.

Hidalgo, I. J., and R. T. Borchardt. "Transport of a large neutral amino-acid (phenylalinine) in a human intestinal epithelial-cell line - Caco-2." *Biochimica et Biophysica Acta* 1028, no. 1 (1990a): 25-30.

Hidalgo, I. J., and R. T. Borchardt.. "Transport of bile-acids in a human intestinal epithelial-cell line, Caco-2." *Biochimica et Biophysica Acta* 1035, no. 1 (1990b): 97-103.

Hidalgo, I. J., K. M. Hillgren, G. M. Grass, and R. T. Borchardt. "Characterization of the unstirred water layer in caco-2 cell monolayers using a novel diffusion apparatus." *Pharmaceutical Research* 8, no. 2 (1991): 222-27.

Hidalgo, I. J., T. J. Raub, and R. T. Borchardt. "Characterization of the human colon carcinoma cell line (caco-2) as a model system for intestinal epithelial permeability." *Gastroenterology* 96, no. 3 (1989a): 736-49.

Hidalgo, I. J., A. Kato, and R. T. Borchardt. "Binding of epidermal growth factor by human colon carcinoma cell (CaCo-2)." *Biochimica et Biophysica Research Communications* 160, no. 1 (1989b): 317-324

Hillgren, K. M., A. Kato, and R. T. Borchardt. "*In-vitro* systems for studying intestinal drug absorption." *Medicinal Research Reviews* 15, no. 2 (1995): 83-109.

Hirai, S., K. Yashikia, and H. Minima. "Effects of surfactants on the nasal absorption of insulin in rats." *International Journal of Pharmaceutics* 9, (1981a): 165-172.

Hirai, S., K. Yashikia, and H. Minima "Mechanisms of enhancement of the nasal absorption of insulin by surfactants." *International Journal of Pharmaceutics* 9, (1981b): 173-184.

Ho, N. F. H., J.Y. Park, W. Morozwich, and W. L. Higuchi. "Physical model approach to the design of drugs with improved intestinal absorption". In *Design of Biopharmaceutical properties through Prodrugs and Analogues.* (Ed. Roche, E.B.) (1977)

Ho, N. F. H., J.Y. Park, P.F. Ni, and W. L. Higuchi. "Advancing quantitative and mechanistic approaches in interfacing gastrointestinal drug absorption studies in animals and humans". In *Animal models for Oral drug Delivery.* (Ed. Crouthamel, W. and Sarapu, A.C.) (1983)

Ho, N. F. H., P. S. Burton, R. A. Conradi, and C. L. Barsuhn. "Biophysical model of passive and polarized active-transport processes in Caco-2 cells - approaches to uncoupling apical and basolateral membrane events in the intact cell." *Journal of Pharmaceutical Sciences* 84, no. 1 (1995): 21-27.

Hochman, J., and P. Artursson. "Mechanisms of absorption enhancement and tight junction regulation." *Journal of Controlled Release* 29, no. 3 (1994a): 253-67.

Hochman, J., J. Fix and E.L. Le Cluyse. "*In-vivo* and *in-vitro* analysis of the mechanism of absorption enhancement by Palmitoylcarnitine." *Journal of Pharmacology and Experimental Therapeutics* 269, no. 2 (1994b): 813-21.

Hofmann, E.J., and K. J. Mysels. "Bile Acid solubility and precipitation in vitro and in vivo: the role of conjugation, pH, and Ca^{2+} ions." *Journal of Lipid Research* 30, no. 5 (1992): 617-26.

Hofmann, A.F. "Intestinal Absorption of Bile Acids and Biliary Constituents." Physiology of The Gastrointestinal Tract (Eds Johnson, C.R) 3rd Edition, Raven Press, New York (1994): 1037-67.

Hofmann, A.F. "Biliary Secretion and Excretion." Physiology of The Gastrointestinal Tract (Eds Johnson, C.R) 3rd Edition, Raven Press, New York (1994): 617-26.

Holohan, A. "Polyionic amphiphilic cyclodextrins." *Ph.D. Thesis, UCD* (1999)

Hoogdalem, A. F., A.G. de Boer and D.D. Breimer. "Intestinal drug absorption enhancement: an overview." *Pharmacology Therapy* 44, (1989): 407-443.

Horbett, T.A. "Adsorption of proteins and peptides at interfaces." In *Stability of Proteins Pharmaceuticals, Part A: Chemical and Physical pathways of Protein degradation* (Eds. Ahern, T.J. and Manning, M.C.) (1992).

Horspol, K. "Conferences; Delivering the message." *Pharmaceutical science and Technology Today* 1, no. 9 (1987): 364-366

Hoskins, J., S. V. Deherdt, R. E. Moore, and T. F. Bumol. "The development and characterization of vinca alkaloid-resistant Caco-2 human colorectal cell-lines expressing MDR-1." *International Journal of Cancer* 53, no. 4 (1993): 680-688.

Hosoya, K., K. J. Kim, and V. H. L. Lee. "Age-dependent expression of p-Glycoprotein Gp170 in Caco-2 cell monolayers." *Pharmaceutical Research* 13, no. 6 (1996): 885-90.

Høvgaard, L., and H. Brondsted. "Drug delivery studies in Caco-2 monolayers. IV. Absorption enhancer effects of cyclodextrins." *Pharmaceutical Research* 12, no. 9 (1995b): 1328-32.

Høvgaard, L., H. Brondsted and H.M. Nielsen. "Drug delivery studies in Caco-2

monolayers. II. Absorption enhancing effects of lysophosphatidylcholines." *International Journal of Pharmaceutics* 114, no. 1 (1995a): 141-149.

Howell, S., A. J. Kenny, and A. J. Turner. "A Survey of membrane peptidases in 2 human colonic cell-lines: Caco-2 and Ht-29." *Biochemical Journal* 284 (1992): 595-601.

Hu, M., and R. T. Borchardt. "Mechanism of L-Alpha-Methyldopa transport through a monolayer of polarized human intestinal epithelial-cells (Caco-2)." *Pharmaceutical Research* 7, no. 12 (1990): 1313-19.

Hu, M., J. Y. Chen, L. X. Zheng, a. H. Dantzig, and R. E. Stratford. "Uptake characteristics of loracarbef and cephalexin in the CaCo-2 Cell culture model: Effects of the proton gradient and possible presence of a distinctive second component." *Journal of Pharmaceutical Sciences* 85, no. 7 (1996): 767-72.

Hu, M., Y. Q. Li, C. M. Davitt, S. M. Huang, K. Thummel, B. W. Penman, and C. L. Crespi. "Transport and metabolic characterization of caco-2 cells expressing Cyp3a4 and Cyp3a4 plus oxidoreductase." *Pharmaceutical Research* 16, no. 9 (1999): 1352-59.

Huet, C., C. Sahuquillo Merino, E. Coudrier, and D. Louvard. "Absorptive and mucus-secreting subclones isolated from a multi-potent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation." *Journal of Cell Biology* 105, no. 1 (1987): 345-57.

Hunter, J., B. H. Hirst, and N. L. Simmons. "Drug absorption limited by p-Glycoprotein-mediated secretory drug transport in human intestinal epithelial Caco-2 cell-layers." *Pharmaceutical Research* 10, no. 5 (1993): 743-49.

Hussain, A., J. Faraj, Y. Aramaki, and J. E. Truelove. "Hydrolysis of leucine enkephalin in the nasal cavity of the rat--a possible factor in the low bioavailability of nasally administered peptides." *Biochemical and Biophysical Research Communications* 133, no. 3 (1985): 923-8.

Hussain, M. A., S. M. Rowe, A. B. Shenvi, and B. J. Aungst. "Inhibition of leucine enkephalin metabolism in rat-blood, plasma and tissues *in-vitro* by an aminoboronic acid-derivative." *Drug Metabolism and Disposition* 18, no. 3 (1990): 288-91.

Iiboshi, Y., R. Nezu, L. Cui, K. Chen, J. Khan, H. Yoshida, K. Sando, S. Kamata, Y. Takagi, and A. Okada. "Adhesive mucous gel layer and mucus release as intestinal barrier in rats." *Journal of Parenteral and Enteral Nutrition* 20, no. 2 (1996): 98-104.

Illum, L., N. F. Farraj, S. S. Davis, B. R. Johansen, and D. T. Ohagan. "Investigation of the nasal absorption of biosynthetic human growth-hormone in sheep - Use of a bioadhesive microsphere delivery system." *International Journal of Pharmaceutics* 63, no. 3 (1990): 207-11.

Inui, K., M. Yamamoto, and H. Saito. "Transepithelial transport of oral cephalosporins by

monolayers of intestinal epithelial-cell line Caco-2 - Specific transport-systems in apical and basolateral membranes." *Journal of Pharmacology and Experimental Therapeutics* 261, no. 1 (1992): 195-201.

Irie, T., and K. Uekama. "Cyclodextrins in peptide and protein delivery." *Advanced Drug Delivery Reviews* 36, no. 1 (1999): 101-23.

Irie, T., and K. Uekama. "Pharmaceutical applications of cyclodextrins .3. Toxicological issues and safety evaluation." *Journal of Pharmaceutical Sciences* 86, no. 2 (1997): 147-62.

Irie, T., K. Wakamatsu, H. Arima, H. Aritomi, and K. Uekama. "Enhancing effects of cyclodextrins on nasal absorption of insulin in rats." *International Journal of Pharmaceutics* 84, no. 2 (1992): 129-39.

Irwin, W. J., A. K. Dwivedi, P. A. Holbrook, and M. J. Dey. "The effect of cyclodextrins on the stability of peptides in nasal enzymatic systems." *Pharmaceutical Research* 11, no. 12 (1994): 1698-703.

Jacobsen, J., S. Bjerregaard, and M. Pedersen. "Cyclodextrin inclusion complexes of antimycotics intended to act in the oral cavity - drug super-saturation, toxicity on TR146 cells and release from a delivery system." *European Journal of Pharmaceutics and Biopharmaceutics* 48, no. 3 (1999): 217-24.

Jalal, F., C. Jumarie, W. Bawab, D. Corbeil, C. Malo, A. Berteloot, and P. Crine. "Polarized distribution of neutral endopeptidase 24.11 at the cell-surface of cultured human intestinal epithelial Caco-2 cells." *Biochemical Journal* 288 (1992): 945-51.

Jani, P., G. W. Halbert, J. Langridge, and A. T. Florence. "The uptake and translocation of latex nanospheres and microspheres after oral-administration to rats." *Journal of Pharmacy and Pharmacology* 41, no. 12 (1989): 809-&.

Jezyk, N., W. Rubas, and G. M. Grass. "Permeability characteristics of various intestinal regions of rabbit, dog, and monkey." *Pharmaceutical Research* 9, no. 12 (1992): 1580-1586.

Jørgensen, L., P. Artursson, and E. Bechgaard. "Toxicological and absorption enhancing effects of glycofurol-75 and sodium glycocholate in monolayers of human intestinal epithelial (Caco-2) cells." *International Journal of Pharmaceutics* 95, no. 1-3 (1993): 209-17.

Junginger, H.E. and J.C. Verhoef. "Macromolecules as safe penetration enhancers for hydrophilic compound- A fiction." *Pharmaceutical Science and Technology Today*, no. 1 (1998): 370-76.

Kakemi, K., H. Sezaki, R. Konishi, T. Kimura, and M. Murakami. "Effect of bile salts on the gastrointestinal absorption of drugs. I." *Chemical and Pharmaceutical Bulletin* 18, no. 2 (1970): 275-80.

Kararli, T. T. "Gastrointestinal absorption of drugs." *Critical Reviews in Thereapeutic Drug Carrier Systems* 6 no. 1 (1989): 39-86.

Karls, M. S., B. D. Rush, K. F. Wilkinson, T. J. Vidmar, P. S. Burton, and M. J. Ruwart. "Desolvation Energy - a major determinant of absorption, but not clearance, of peptides in rats." *Pharmaceutical Research* 8, no. 12 (1991): 1477-81.

Karlsson, J., and P. Artursson. "A method for the determination of cellular permeability coefficients and aqueous boundary-layer thickness in monolayers of intestinal epithelial (Caco-2) cells grown in permeable filter chambers." *International Journal of Pharmaceutics* 71, no. 1-2 (1991): 55-64.

Karlsson, J., A. L. Ungell, J. Grasjo, and P. Artursson. "Paracellular drug transport across intestinal epithelia:influence of charge and induced water flux." *European Journal of Pharmaceutical Sciences* 9, no. 1 (1999): 47-56.

Karlsson, J., S.M. Kuo, J. Ziemniak and P. Artursson. "Transport of celiprolol across human intestinal epithelial cells: mediation of secretion by multiple transporters including p-glycoprotein." *British Journal of Pharmacology* 110, (1993a): 1009-16.

Karlsson, J., A. Wikman, and P. Artursson. "The mucus layer as a barrier to drug absorption in monolayers of human intestinal epithelial Ht29-H goblet cells." *International Journal of Pharmaceutics* 99, no. 2-3 (1993b): 209-18.

Kearney, P. and C. Marriott. "The effects of mucus glycoproteins on the bioavailability of tetracycline: II Binding." *International Journal of Pharmaceutics* 35 (1987): 211- 217

Kelly, G. S. "Clinical applications of N-Acetylcysteine." *Alternative Medical Reviews* 3, no. 2. (1998): 114-27.

Kerneis, S., A Bogdanova, J. P. Kraehenbuhl, and E. Pringault. "Conversion by Peyer's Patch lymphocytes of human enterocytes into M Cells that transport bacteria." *Science* 277, no. 5328 (1997): 949-52.

Kerrs, S., A. Allen, and A. Garner. "A simple method for measuring thickness of the mucus gel layer adherent to rat, frog and human gastric mucosa: Influence of feeding, prostaglandin, N-Acetylcysteine and other agents." *Clinical Science* 63, no. 2 (1982): 187-95.

Kim, D. C., P. S. Burton, and R. T. Borchardt. "A correlation between the permeability characteristics of a series of peptides using an *in-vitro* cell culture model (Caco-2) and those using an *in-situ* perfused rat ileum model of the intestinal mucosae." *Pharmaceutical Research* 10, no. 12 (1993): 1710-4.

Kim, J. S., R. L. Oberle, D. A. Krummel, J. B. Dressman, and D. Fleisher. "Absorption of ACE inhibitors from small intestine and colon." *Journal of Pharmaceutical Sciences* 83, no. 9 (1994): 1350-6.

Klinkspoor, J. H., R. Kuver, C. E. Savard, D. Oda, H. Azzouz, G. N. Tytgat, A. K. Groen, and S. P. Lee. "Model bile and bile salts accelerate mucin secretion by cultured dog gallbladder epithelial cells." *Gastroenterology* 109, no. 1 (1995): 264-74.

Knipp, G. T., N. F. H. Ho, C. L. Barsuhn, and R. T. Borchardt. "Paracellular diffusion in Caco-2 cell monolayers: Effect of perturbation on the transport of hydrophilic compounds that vary in charge and size." *Journal of Pharmaceutical Sciences* 86, no. 10 (1997): 1105-10.

Kotze, A. F., B. J. De Leeuw, H. L. Luessen, a. G. De Boer, J. C. Verhoef, and H. E. Junginger. "Chitosans for enhanced delivery of therapeutic peptides across intestinal epithelia: in vitro evaluation in Caco-2 cell monolayers." *International Journal of Pharmaceutics* 159, no. 2 (1997): 243-53.

Kotze, A. F., H. L. Luessen, B. J. de Leeuw, A. G. de Boer, J. C. Verhoef, and H. E. Junginger. "Comparison of the effect of different chitosan salts and N-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (Caco-2)." *Journal of Controlled Release* 51, no. 1 (1998): 35-46.

Kotze, A. F., M. M. Thanou, H. L. Luebetaen, A. G. de Boer, J. C. Verhoef, and H. E. Junginger. "Enhancement of paracellular drug transport with highly quaternized n-trimethyl chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2)." *Journal of Pharmaceutical Sciences* 88, no. 2 (1999): 253-7.

Kramer, W., C. Dechent, F. Girbig, U. Gutjahr, and H. Neubauer. "Intestinal uptake of dipeptides and beta-lactam antibiotics .1.the intestinal uptake system for dipeptides and beta-lactam antibiotics is not part of a brush-border membrane peptidase." *Biochimica Et Biophysica Acta* 1030, no. 1 (1990): 41-49.

Kramer, W., G. Wess, a. Enhsen, E. Falk, A. Hoffmann, G. Neckermann, G. Schubert, and M. Urmann. "Modified bile acids as carriers for peptides and drugs." *Journal of Controlled Release* 46, no. 1-2 (1997): 17-30.

Krishnamoorthy, R., A. M. Volka, Z. Z. Shao, and A. K. Mitra. "Cyclodextrins as mucosal absorption promoters .4. evaluation of nasal muco-toxicity." *European Journal of Pharmaceutics and Biopharmaceutics* 41, no. 5 (1995): 296-301.

Lane, M. E. "Mechanistic studies on the absorption of hydrophilic molecules from the rat intestine." *Ph.D. Thesis, Trinity College, Dublin* (1997)

Lane, M. E., C. M. O'Driscoll, and O. I. Corrigan. "The relationship between rat intestinal permeability and hydrophilic probe size." *Pharmaceutical Research* 13, no. 10 (1996): 1554-8.

Langguth, P., V. Bonher, J. Heizmann, H. P. Merkle, S. Wolffram, G.I. Amidon and S. Yamashita. "The challenge of proteolytic enzymes in intestinal permeability." *Journal of Controlled Release* 46, no. 3 (1997): 39- 57.

Lapre, J. A., D. S. Termont, A. K. Groen, and R. Van der Meer. "Lytic effects of mixed micelles of fatty acids and bile acids." *American Journal of Physiology* 263, no. 3 Pt 1 (1992): G333-7.

Larhed, A. W., P. Artursson, and E. Björk. "The influence of intestinal mucus components on the diffusion of drugs." *Pharmaeutical Research* 15, no. 1 (1998): 66-71.

Larhed, A. W., P. Artursson, J. GrÁsjö, and E. Björk. "Diffusion of drugs in native and purified gastrointestinal mucus." *Journal of Pharmaceutical Science* 86, no. 6 (1997): 660-5.

Latta, R. K., H. Fiander, N. W. Ross, C. Simpson, and H. Schneider. "Toxicity of Bile-Acids to Colon-Cancer Cell-Lines." *Cancer Letters* 70, no. 3 (1993): 167-73.

Law, S. L., and C. L. Shih. "Adsorption of Calcitonin to Glass." *Drug Development and Industrial Pharmacy* 25, no. 2 (1999): 253-56.

Lee, K. C., Y. J. Lee, H. M. Song, C. J. Chun, and P. P. Deluca. "Degradation of synthetic salmon-calcitonin in aqueous-solution." *Pharmaceutical Research* 9, no. 11 (1992): 1521-23.

Lee, K. C., S. C. Moon, M. O. Park, J. T. Lee, D. H. Na, S. D. Yoo, H. S. Lee, and P. P. Deluca. "Isolation, characterization, and stability of positional isomers of mono-pegylated salmon calcitonins." *Pharmaceutical Research* 16, no. 6 (1999): 813-18.

Lee, V.H.L. "Enzymatic barriers to peptide and protein absorption." *Critical Reviews in Therapeutic Drug Carrier Systems* 5, no. 2 (1988): 69-97.

Lee, V.H.L., A. Yamamoto, and U. B. Kompella. "Mucosal penetration enhancers for facilitation of peptide and protein drug absorption." *Critical Reviews in Therapeutic Drug Carrier Systems* 8, no. 2 (1991): 91-192.

Lee, V.H.L., S. Dodda-Kashi, G.M. Grass, and W. Rubas "Oral route of peptide and protein drug delivery." In *Protein and Peptide Drug Delivery* (Eds Lee, V.H.L.) (1992)

Lee, Y. H., B. A. Perry, S. Labruno, H. S. Lee, W. Stern, L. M. Falzone, and P. J. Sinko. "Impact of regional intestinal ph modulation on absorption of peptide drugs: oral absorption studies of salmon calcitonin in beagle dogs." *Pharmaceutical Research* 16, no. 8 (1999): 1233-39.

Leibach, F. H., and V. Ganapathy. "Peptide transporters in the intestine and the kidney." *Annual Review of Nutrition* 16 (1996): 99-119.

Lennernäs, H. "Does fluid flow across the intestinal mucosae affect quantitative oral drug absorption? Is it time for a reevaluation? " *Pharmaceutical Research* 12, no. 11 (1995): 1573-82.

Lennernäs, H. "Gastrointestinal absorption mechanisms - a comparison between animal and human models." *European Journal of Pharmaceutical Sciences* 2, no. 1-2 (1994): 39-43.

Lennernäs, H. "Human intestinal permeability." *Journal of Pharmaceutical Sciences* 87, no. 4 (1998): 403-10.

Lennernäs, H., K. Palm, U. Fagerholm, and P. Artursson. "Comparison between active and passive drug transport in human intestinal epithelial (Caco-2) cells *in-vitro* and human jejunum *in-vivo*." *International Journal of Pharmaceutics* 127, no. 1 (1996): 103-7.

Lesuffleur, T., A. Kornowski, C. Augeron, E. Dussaulx, A. Barbat, C. Laboisse, and A. Zweibaum. "Increased growth adaptability to 5-fluorouracil and methotrexate of Ht-29 subpopulations selected for their commitment to differentiation." *International Journal of Cancer* 49, no. 5 (1991a): 731-37.

Lesuffleur, T., A. Kornowski, C. Luccioni, M. Muleris, A. Barbat, J. Beaumatin, E. Dussaulx, B. Dutrillaux, and A. Zweibaum. "Adaptation to 5-fluorouracil of the heterogeneous human colon-tumor cell-line Ht-29 results in the selection of cells committed to differentiation." *International Journal of Cancer* 49, no. 5 (1991b): 721-30.

Levine J.S., R.H Allen., D.H Alpers., and B. Seetharam. "- Immunocytochemical localization of the intrinsic factor- cobalamin receptor in dog-ileum: distribution of intracellular receptor during cell maturation." *Journal of Cell Biology* 98, no. 3 (1984): - 1111-8.

Lewin, M. R., S. H. El Masri, and C. G. Clark. "Effects of bile acids on mucus secretion in the dog colon." *European Surgical Research* 11, no. 6 (1979): 392-9.

Li, C. Y., C. L. Zimmerman, and T. S. Wiedmann. "Diffusivity of bile salt phospholipid aggregates in mucin." *Pharmaceutical Research* 13, no. 4 (1996): 535-41.

Lindmark, T., T. Nikkila, and P. Artursson. "Mechanisms of absorption enhancement by medium chain fatty acids in intestinal epithelial Caco-2 cell monolayers." *Journal of Pharmacology & Experimental Therapeutics* 275, no. 2 (1995): 958-64.

Lindmark, T., N. Schipper, L. Lazorova, A. G. de Boer, and P. Artursson. "Absorption enhancement in intestinal epithelial caco-2 monolayers by sodium caprate: assessment of molecular weight dependence and demonstration of transport routes." *Journal of Drug Targeting* 5, no. 3 (1998): 215-23.

Lloyd, A. W. "Monitor: Progress and Profiles." *Pharmaceutical Science & Technology Today* 1, no. 2 (1998): 83-86.

Loftsson, T., and M. E. Brewster. "Pharmaceutical applications of cyclodextrins .1. Drug solubilization and stabilization." *Journal of Pharmaceutical Sciences* 85, no. 10

(1996): 1017-25.

Louvard, D., M. Arpin, E. Coudrier, C. Huet, E. Pringault, S. Robine and C. Sahuquillo-Merino. "Experimental manipulation of intestinal cell differentiation using a human adenocarcinoma cell line (Ht29)." *Journal of Cell Biology,* 99, 6a (1984)

Lowe, P. J. and C. S. Temple. "Calcitonin and Insulin in Isobutylcyanoacrylate Nanocapsules -Protection Against Proteases and Effect on Intestinal-Absorption in Rats." *Journal of Pharmacy and Pharmacology* 46, no. 7 (1994): 547-52.

Lu, R. H., P. Kopeckova, and J. Kopecek. "Degradation and Aggregation of Human Calcitonin in Vitro." *Pharmaceutical Research* 16, no. 3 (1999): 359-67.

Lundin, S., and P. Artursson. "Absorption of a vasopressin analog, 1-Deamino-8-D-Arginine-Vasopressin (dDAVP), in a human intestinal epithelial-cell line, Caco-2." *International Journal of Pharmaceutics* 64, no. 2-3 (1990): 181-86.

Lundin, S., J. Moss, H. Bundgaard, and P. Artursson. "Absorption of thyrotropin-releasing-hormone (TRH) and a TRH prodrug in a human intestinal-cell line (Caco-2)." *International Journal of Pharmaceutics* 76, no. 3 (1991): R1-R4.

Lutz, K. L., and T. J. Siahaan. "Molecular structure of the apical junction complex and its contribution to the paracellular barrier." *Journal of Pharmaceutical Sciences* 86, no. 9 (1997): 977-84.

Lyritis, G. P., N. Tsakalakos, B. Magiasis, T. Karachalios, a. Yiatzides, and M. Tsekoura. "Analgesic effect of salmon-calcitonin in osteoporotic vertebral fractures - a double-blind placebo-controlled clinical-study." *Calcified Tissue International* 49, no. 6 (1991): 369-72.

Madara, J. L. "Intestinal Absorptive Cell Tight Junctions Are Linked to Cytoskeleton." *American Journal of Physiology* 253, no. 1 Pt 1 (1987): C171-5.

Madara, J. L., and K. Dharmsathaphorn. "Occluding Junction Structure-Function Relationships in a Cultured Epithelial Monolayer." *Journal of Cell Biology* 101, no. 6 (1985): 2124-33.

Madara, J. L., and J. Stafford. "Interferon-Gamma directly affects barrier function of cultured intestinal epithelial monolayers." *Journal of Clinical Investigation* 83, no. 2 (1989): 724-7.

Madara, J. L., and J.R. Trier. "The functional morphology of the mucosa of the small intestine." In *Physiology of the Gastrointestinal Tract* (Eds: Johnson, C.R.) 3rd Edition, Raven Press, New York (1994): 1577-622.

Madara, J. L., J. Stafford, D. Barenberg, and S. Carlson. "Functional coupling of tight junctions and microfilaments in T84 monolayers." *American Journal of Physiology* 254, no. 3 Pt 1 (1988): G416-23.

Mah, S.M., and W. Rubas. "Vinblastine, a substrate for p-glycoprotein, flux across CaCo-2 cell monolayers and rabbit jejunum." *Proceedings of the International Symposium for Controlled Release Bioactive Materials* 23, #344 (1996).

Manning, M. C., K. Patel and R.T. Borchardt "Stability of Pharmaceuticals." *Pharmaceutical Research* 6, no. 11 (1989): 903-18.

Marriott, C. "Drug-Mucus actions and interactions." *Symposium for the Society of Experimental Biology* 43 (1989): 163-77.

Martin, G. P., C. Marriott, and I. W. Kellaway. "Direct effect of bile salts and phospholipids on the physical properties of mucus." *Gut* 19, No. 2 (1978): 103-7.

Martindale, The Extra Pharmacopeia, 32nd Ed. (1998)

Martinez-Palomo, A., I. Meza, G. Beaty, and M. Cereijido. "Experimental modulation of occluding junctions in a cultured transporting epithelium." *Journal of Cell Biology* 87, no. 3 Pt 1 (1980): 736-45.

Marx S.J., C. Woodward, G.D. Aurbach, H. Glossmann, and H.T. Keutmann." Renal receptors for calcitonin. Binding and degradation of hormone. "*Journal of Biology and Chemistry* 178 (1973): 4797-802.

Marx S.J., C. Woodward and G.D Aurbach. "Calcitonin receptors of kidney and bone." *Science;* 178 No 64(1972): 999-1001.

Matsubara, K., Y. Ando, T. Irie, and K. Uekama. "Protection afforded by maltosyl-beta-cyclodextrin against alpha-chymotrypsin-catalyzed hydrolysis of a luteinizing hormone-releasing hormone agonist, buserelin acetate." *Pharmaceutical Research* 14, no. 10 (1997a): 1401-5.

Matsubara, K., T. Irie, and K. Uekama. "Spectroscopic characterization of the inclusion complex of a luteinizing hormone-releasing hormone agonist, buserelin acetate, with dimethyl-beta-cyclodextrin." *Chemical & Pharmaceutical Bulletin* 45, no. 2 (1997b): 378-83.

Matsumoto, S., H. Saito, and K. Inui. "Transcellular transport of oral cephalosporins in human intestinal epithelial-cells, Caco-2 - Interaction with dipeptide transport-systems in apical and basolateral membranes." *Journal of Pharmacology and Experimental Therapeutics* 270, no. 2 (1994): 498-504.

Matsumoto, S., H. Saito, and K. Inui. "Transport characteristics of ceftibuten, a new cephalosporinantibiotic, via the apical H^+/dipeptide cotransport system in human intestinal cell line Caco-2 - regulation by cell-growth." *Pharmaceutical Research* 12, no. 10 (1995): 1483-87.

Matuszewska, B., M. Keogan, D. M. Fisher, K. A. Soper, C. M. Hoe, A. C. Huber, and J. V. Bondi. "Acidic fibroblast growth-factor - evaluation of topical formulations in a

diabetic mouse wound-healing model." *Pharmaceutical Research* 11, no. 1 (1994): 65-71.

MacAdam, A. "The effect of gastrointestinal mucus on drug absorption." *Advanced Drug Delivery Reviews* 11, (1993): 313- 318

Mackay, M., I. Williamson and J. Hastewell. "Cell biology of epithelia." *Advanced Drug Delivery Reviews* 7 (1991): 427-44.

McRoberts, J. A., R. Aranda, N. Riley, and H. Kang. "Insulin Regulates the Paracellular Permeability of Cultured intestinal Epithelial-Cell Monolayers." *Journal of Clinical Investigation* 85, no. 4 (1990): 1127-34.

McRoberts, J. A., and N. E. Riley. "Regulation of T84 Cell Monolayer Permeability by Insulin-Like Growth-Factors." *American Journal of Physiology* 262, no. 1 (1992): C207-C213.

Meaney, C.M. "Use of intestinal cell culture models to assess the absorption enhancing potential of micellar systems". *PhD Thesis, Trinity College, Dublin* (1997)

Meaney, C.M., and C.M. O'Driscoll. "Mucus as a barrier to the permeability of hydrophilic and lipophilic compounds in the absence and presence of sodium taurocholate micellar systems using cell culture models." *European Journal of Pharmaceutical Sciences* 8, no. 3 (1999): 167-75.

Merkus F.W.H.M., J. C. Verhoef, S. G. Romeijn, and N. G. M. Schipper. "Absorption enhancing effect of cyclodextrins on intra-nasally administered insulin in rats." *Pharmaceutical Research* 8, no. 5 (1991): 588-92.

Mesiha,M., F. Plakogiannis, S. Vejosoth.. "Enhanced oral absorption of insulin from desolvated fatty acid- sodium glycocholate emulsions." *International Journal of Pharmaceutics* 111, (1994): 213-16.

Meza, I., G. Ibarra, M. Sabanero, A. Martinez-Palomo, and M. Cereijido. "Occluding junctions and cytoskeletal components in a cultured transporting epithelium." *Journal of Cell Biology* 87, no. 3 Pt 1 (1980): 746-54.

Mossman, T. J. "Rapid colourimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays." *Journal of Immunological Methods* 65 (1983): 55-63.

Murakami T., Y. Sasaki, R. Yamajo and N. Yata. "Effect of bile salts on the rectal absorption of sodium ampicillin in rats." *Chemistry and Pharmaceutical Bulletin (Tokyo)* 32, no 5 (1984):1948-55.

Muranishi, S. "Absorption enhancers." *Critical Reviews Therapeutic Drug Carrier Systems.* 7, no. 1. (1990): 1-33.

Muranishi, S., A. Sakai, K. Yamada, M. Murakami, K. Takada, and Y. Kiso. "Lipophilic peptides: Synthesis of lauroyl thyrotropin-releasing hormone and its biological activity." *Pharmaceutical Research* 8., no. 5 (1991): 649-52.

Muranushi, N., N. Takagi, S. Muranishi, and H. Sezaki. "Effect of Fatty Acids and Monoglycerides on Permeability of Lipid Bilayer." *Chemistry & Physics of Lipids* 28, no. 3 (1981): 269-79.

Nellans, H. N. "Mechanisms of peptide and protein-absorption .1. Paracellular intestinal transport - modulation of absorption." *Advanced Drug Delivery Reviews* 7, no. 3 (1991): 339-64.

Nerurkar, M. M., P. S. Burton, and R. T. Borchardt. "The use of surfactants to enhance the permeability of peptides through Caco-2 Cells by inhibition of an apically polarized efflux system." *Pharmaceutical Research* 13, no. 4 (1996): 528-34.

Nerurkar, M. M., N. F. H. Ho, P. S. Burton, T. J. Vidmar, and R. T. Borchardt. "Mechanistic roles of neutral surfactants on concurrent polarized and passive membrane transport of a model peptide in CaCo-2 cells." *Journal of Pharmaceutical Sciences* 86, no. 7 (1997): 813-21.

Neutra, M. R. "Current Concepts in Mucosal Immunity - V. Role of M cells in transepithelial transport of antigens and pathogens to the mucosal immune system." *American Journal of Physiology-Gastrointestinal and Liver Physiology* 37, no. 5 (1998): G785-G791.

Newbury, R. S., N.C. Turner, G.P. Martin and C. Marriott, "Gastric ulceration: the effects of bile salts and bilary phospholipids in the rat." *Journal of Pharmacy and Pharmacology* 36, 44P (1984).

Ng, K. Y., and R. T. Borchardt. "Biotin transport in a human intestinal epithelial-cell line (Caco-2)." *Life Sciences* 53, no. 14 (1993): 1121-27.

Nicklin, P., B. Irwin, I. Hassan, I. Williamson, and M. Mackay. "Permeable support type influences the transport of compounds across Caco-2 cells." *International Journal of Pharmaceutics* 83, no. 1-3 (1992): 197-209.

Nielsen, H. M., and M. R. Rassing. "TR146 cells grown on filters as a model of human buccal epithelium: III. Permeability enhancement by different pH values, different osmolality values, and bile salts." *International Journal of Pharmaceutics* 185, no. 2 (1999): 215-25.

Niibuchi, J.J, Y. Aramaki and S. Tsuchiya. "Binding of antibiotics to rat intestinal mucus." *International Journal of Pharmaceutics* 30, (1986): 181- 187

Nimmerfall, F., and J. Rosenthaler. "Significance of the goblet-cell mucin layer, the outermost luminal barrier to passage through the gut wall." *Biochemical & Biophysical Research Communications.* 94, no.3 (1980): 960-66

Noach, A. B. J., M. Sakai, M. C. M. Blomroosemalen, H. R. Dejonge, A. G. Deboer, and D. D. Breimer. "Effect of an isotonic conditions on the transport of hydrophilic model compounds across monolayers of human colonic cell-lines." *Journal of Pharmacology and Experimental Therapeutics* 270, no. 3 (1994): 1373-80.

Ohtani, Y., T. Irie, K. Uekama, K. Fukunaga, and J. Pitha. "Differential effects of alpha-, beta- and gamma-cyclodextrins on human erythrocytes." *European Journal of Biochemistry* 186 no.1-2 (1989): 17-22.

Okimoto, K., R. A. Rajewski, K. Uekama, J. A. Jona, and V. J. Stella. "The interaction of charged and uncharged drugs with neutral (Hp-Beta-CD) and anionically charged (Sbe7-Beta-Cd) Beta-Cyclodextrins." *Pharmaceutical Research* 13, no. 2 (1996): 256-64.

O'Donnell, C.M., Ph.D. Thesis under submission at Trinity College, Dublin, 2000

O'Donnell, C.M., O. I. Corrigan, and C. M. O'Driscoll. "Assessment of the stability of the peptide, salmon calcitonin, sCT in rat perfusions and homogenates." *QUB/TCD Colloquium,* Belfast 1996

O'Reilly, J. R., O. I. Corrigan, and C. M. O'Driscoll. "The effect of mixed micellar systems, bile-salt fatty-acids, on the solubility and intestinal-absorption of clofazamine (B663) in the anesthetized rat." *International Journal of Pharmaceutics* 109, no. 2 (1994a): 147-54.

O'Reilly, J. R., O. I. Corrigan, and C. M. O'Driscoll. "The effect of simple micellar systems on the solubility and intestinal-absorption of clofazamine (B663) in the anesthetized rat." *International Journal of Pharmaceutics* 105, no. 2 (1994b): 137-46.

Pade, V., and S. Stavchansky. "Estimation of the relative contribution of the transcellular and paracellular pathway to the transport of passively absorbed drugs in the Caco-2 Cell culture model." *Pharmaceutical Research* 14, no. 9 (1997): 1210-1215.

Palm, K., K. Luthman, A. L. Ungell, G. Strandlund, and P. Artursson. "Correlation of drug absorption with molecular surface properties." *Journal of Pharmaceutical Sciences* 85, no. 1 (1996): 32-39.

Pappenheimer, J. R., and K. Z. Reiss. "Contribution of solvent drag through intercellular junctions to absorption of nutrients by the small intestine of the rat." *Journal of Membrane Biology* 100, no. 2 (1987): 123-36.

Patro, S. Y., and T. M. Przybycien. "Simulations of reversible protein aggregate and crystal structure." *Biophysical Journal* 70, no. 6 (1996): 2888-902.

Patton, J. S., P. Trinchero, and R. M. Platz. "Bioavailability of pulmonary delivered peptides and proteins -alpha-interferon, calcitonins and parathyroid hormones." *Journal of Controlled Release* 28, no. 1-3 (1994): 79-85.

Paul, E. C. A., J. Hochman, and A. Quaroni. "Conditionally immortalized intestinal epithelial-cells - novel-approach for study of differentiated enterocytes." *American Journal of Physiology* 265, no. 1 (1993): C266-C278.

Pauletti, G. M., S. Gangwar, G. T. Knipp, M. M. Nerurkar, F. W. Okumu, K. Tamura, T. J. Siahaan, and R. T. Borchardt. "Structural requirements for intestinal absorption of peptide drugs." *Journal of Controlled Release* 41, no. 1-2 (1996): 3-17.

Pauletti, G. M., S. Gangwar, T. J. Siahaan, J. Aube and R. T. Borchardt. "Improvement of oral peptide bioavailability: peptidometics and prodrug strategies." *Advanced Drug Delivery Reviews* 27, no. 1-2 (1997): 235-56.

Peralta Soler, A., R.D. Miller, K.V. Laughlin, N.Z. Carp, D.M. Klurfeld and J.M. Mullin "Increased tight junctional permeability is associated with the development of colon cancer." *Carcinogenesis* 20, no. 8 (1999): 1425-39.

Peters, W. H. M., C. E. W. Boon, H. M. J. Roelofs, T. Wobbes, F. M. Nagengast, and P. G. Kremers. "Expression of drug-metabolizing-enzymes and p-170-glycoprotein in colorectal-carcinoma and normal mucosa." *Gastroenterology* 103, no. 2 (1992): 448-55.

Pettit, D. K., and W. R. Gombotz. "The development of site-specific drug-delivery systems for protein and peptide biopharmaceuticals." *Trends in Biotechnology* 16, no. 8 (1998): 343-49.

Peppas, N.A., P.J. Hansen and P.A. Buri. "A theory of molecular diffusion in intestinal mucus." *International Journal of Pharmaceutics* 20, (1984): 107- 118.

Pinto, M., M.D. Appay, P. Simon-Assan, G. Chevalier, N. Dracopoli, J. Fogh and A. Zweibaum. "Enterocyctic differentiation of cultured human colon cancer cells by replacement of the glucose by galactose in the medium." *Biology of the Cell* 47, (1982): 193- 196

Pinto, M., S. Robine-Leon, and M.D. Appay. "Enterocyte-like differentiation and polarization of the human colon carcinoma cell line CaCo-2 in culture." *Biology of the Cell* 47, (1983): 323- 330

Poelma, F. G. J., R. Breas, and J. J. Tukker. "Intestinal-Absorption of Drugs .4. The Influence Of taurocholate and L-Cysteine on the Barrier Function of Mucus." *International Journal of Pharmaceutics* 64, no. 2-3 (1990): 161-69.

Poelma, F. G., J. J. Tukker, and D. J. Crommelin. "Intestinal Absorption of Drugs. I: The influence of taurocholate on the absorption of dantrolene in the small intestine of the rat." *Journal of Pharmaceutical Sciences* 78, no. 4 (1989): 285-9.

Polli, J. E., and M. J. Ginski. "Human drug absorption kinetics and comparison to Caco-2 monolayer permeabilities." *Pharmaceutical Research* 15, no. 1 (1998): 47-52.

Raeissi, S. D., Z. Guo, G. L. Dobson, P. Artursson, and I. J. Hidalgo. "Comparison of CYP3A activities in a subclone of Caco-2 Cells (TC7) and human intestine." *Pharmaceutical Research* 14, no. 8 (1997): 1019-25.

Rafter, J. J., V. W. Eng, R. Furrer, A. Medline, and W. R. Bruce. "Effects of Calcium and pH on the Mucosal Damage Produced by Deoxycholic Acid in the Rat Colon." *Gut* 27, no. 11 (1986): 1320-9.

Rajagopalan, N., and S. Lindenbaum. "Counter-Ion Binding by Bile Acid Solutions." *Hepatology* 4 Suppl 5 (1984): 110S-4S.

Rajewski, R. A., and V. J. Stella. "Pharmaceutical applications of cyclodextrins .2. *In-vivo* drug delivery." *Journal of Pharmaceutical Sciences* 85, no. 11 (1996): 1142-69.

Ramphul, M. "Amphiphilic Cyclodextrins and their Drug Complexes." *Ph.D Thesis, UCD* (1998)

Reginster, J. Y. "Calcitonin and Bone-Diseases." *Lancet* 339, no. 8800 (1992a): 1064-65.

Reginster, J. Y., M. Azria, S. Gaspar, M. Bleicher, N. Franchimont, M. Behhar, A. Albert, and P. Franchimont. "Endogenous production of specific antibodies does not decrease hypocalcaemia response to calcitonin in young-rabbits." *Calcified Tissue International* 50, no. 6 (1992b): 518-20.

Reid, L., and J. R. Clamp. "The biochemical and histochemical nomenclature of mucus." *British Medical Bulletin* 34, no. 1 (1978): 5-8.

Rhodes J.M., and Ching C.K. "The application of lectins to the study of mucosal glycoproteins. " *Methods in Molecular Biology* 14 (1993):247-62.

Ribadeneira, M.D., B.J. Aungst, C.J. Eyermann, and S.M. Huang. "Effects of structural modifications on the intestinal permeability of Angiotensin II receptor antagonists and the correlation of *in-vivo, in-vitro* and *in-situ* absorption." *Pharmaceutical Research* 13, no. 2 (1996): 227-33.

Rosenberg, D. W., and T. Leff. "Regulation of Cytochrome P450 in cultured human colonic cells." *Arch Biochimica Et Biophysica* 300, no. 1 (1993): 186-92.

Rouge, N., P. Buri, and E. Doelker. "Drug absorption sites in the gastrointestinal tract and dosage forms for site-specific delivery." *International Journal of Pharmaceutics* 136, no. 1-2 (1996): 117-39.

Rousset, M., M. Laburthe, M. Pinto, G. Chevalier, C. Rouyer Fessard, E. Dussaulx, G. Trugnan, N. Boige, J. L. Brun, and A. Zweibaum. "Enterocytic differentiation and glucose utilization in the human colon tumor cell line Caco-2: Modulation by Forskolin." *Journal of Cell Physiology* 123, no. 3 (1985): 377-85.

Rubas, W., M. E. M. Cromwell, Z. Shahrokh, J. Villagran, T. N. Nguyen, M. Wellton, T.

H. Nguyen, and R. J. Mrsny. "Flux measurements across CaCo-2 monolayers may predict transport in human large intestinal tissue." *Journal of Pharmaceutical Sciences* 85, no. 2 (1996): 165-69.

Rubas, W., N. Jezyk, and G. M. Grass. "Comparison of the permeability characteristics of a human colonic epithelial (Caco-2) cell-line to colon of rabbit,monkey, and dog intestine and human drug absorption." *Pharmaceutical Research* 10, no. 1 (1993): 113-18.

Rubas, W., J. Villagran, M. Cromwell, a. Mcleod, J. Wassenberg, and R. Mrsny. "Correlation of solute flux across caco-2 monolayers and colonic tissue *in-vitro*." *Stp Pharma Sciences* 5, no. 1 (1995): 93-97.

Runkel, L., W. Meier, R. B. Pepinsky, M. Karpusas, A. Whitty, K. Kimball, M. Brickelmaier, C. Muldowney, W. Jones, and S. E. Goelz. "Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-beta (Ifn-Beta)." *Pharmaceutical Research* 15, no. 4 (1998): 641-49.

Russell-Jones, G. J., S. W. Westwood, and a. D. Habberfield. "Vitamin-B-12 mediated oral delivery systems for granulocyte-colony-stimulating factor and erythropoietin." *Bioconjugate Chemistry* 6, no. 4 (1995): 459-65.

Rutten, M. J., P. Harmon, and D. R. Campbell. "Insulin enhances epidermal growth factor-alpha-stimulated and transforming growth factor-alpha-stimulated growth in primary cultures of guinea-pig gastric mucous epithelial-cells." *Scandinavian Journal of Gastroenterology* 26, no. 9 (1991): 965-73.

Saarinen-Savolainen, P., T. Jarvinen, K. Araki-Sasaki, H. Watanabe, and A. Urtti. "Evaluation of cytotoxicity of various ophthalmic drugs, eye drop excipients and cyclodextrins in an immortalized human corneal epithelial cell line." *Pharmaceutical Research* 15, no. 8 (1998): 1275-80.

Said, H. M., R. Redha, and W. Nylander. "- A carrier-mediated, Na^+ gradient-dependent transport for biotin in human intestinal brush-border membrane vesicles." *American Journal of Physiology:* 253 (1987): G631-6.

Saito, H., and K. Inui. "Dipeptide transporters in apical and basolateral membranes of the human intestinal-cell line Caco-2." *American Journal of Physiology* 265, no. 2 (1993): G289-G294.

Sakai, M., T. Imai, H. Ohtake, and M. Otagiri. "Cytotoxicity of absorption enhancers in Caco-2 cell monolayers." *Journal of Pharmacy and Pharmacology* 50, no. 10 (1998): 1101-8.

Sakai, M., T. Imai, H. Ohtake, H. Azuma and M. Otagiri. "Effect of absorption enhancers on the transport of model compounds in CaCo-2 cell monolayers: Assessment by confocal laser scanning microscopy." *Journal of Pharmaceutical Sciences* 86, no. 7 (1997): 779-85.

Samanen, J., G. Wilson, P. L. Smith, C. P. Lee, W. Bondinell, T. Ku, G. Rhodes, and A. Nichols. "Chemical approaches to improve the oral bioavailability of peptiodergic molecules." *Journal of Pharmacy and Pharmacology* 48, no. 2 (1996): 119-35.

Sarkadi, B., M. Muller, L. Homolya, Z. Hollo, J. Seprodi, U. A. Germann, M. M. Gottesman, E. M. Price, and R. C. Boucher. "Interaction of bioactive hydrophobic peptides with the human multidrug transporter." *FASEB Journal* 8, no. 10 (1994): 766-70.

Sayani, a. P., and Y. W. Chien. "Systemic delivery of peptides and proteins across absorptive mucosae." *Critical Reviews in Therapeutic Drug Carrier Systems* 13, no. 1-2 (1996): 85-184.

Schasteen, C. S., M. G. Donovan, and J. N. Cogburn. "A novel *in-vitro* screen to discover agents which increase the absorption of molecules across the intestinal epithelium." *Journal of Controlled Release* 21, no. 1-3 (1992): 49-62.

Schilling, R. J., and a. K. Mitra. "Intestinal mucosal transport of insulin." *International Journal of Pharmaceutics* 62, no. 1 (1990): 53-64.

Schipper, N. G. M., Hermens Wajj, S. G. Romeyn, J. Verhoef, and F.W.H.M Merkus. "Nasal absorption of 17-beta-estradiol and progesterone from a dimethyl-cyclodextrin inclusion formulation in rats." *International Journal of Pharmaceutics* 64, no. 1 (1990): 61-66.

Schipper, N. G. M., S. G. Romeijn, J. Verhoef, and F.W.H.M Merkus. "Hypocalcemic effect of salmon-calcitonin following single and repeated nasal and intravenous administration in young-rabbits." *Calcified Tissue International* 54, no. 1 (1994): 50-55.

Schipper, N. G. M., J. C. Verhoef, L. M. Delannoy, S. G. Romeijn, J. H. Brakkee, V. M. Wiegant, W. H. Gispen, and F.W.H.M Merkus. "Nasal administration of an ACTH (4-9) peptide analog with dimethyl-beta-cyclodextrin as an absorption enhancer - pharmacokinetics and dynamics." *British Journal of Pharmacology* 110, no. 4 (1993): 1335-40.

Schipper, N. G. M., J. Verhoef, S. G. Romeijn, and F.W.H.M Merkus. "Absorption enhancers in nasal insulin delivery and their influence on nasal ciliary functioning." *Journal of Controlled Release* 21, no. 1-3 (1992): 173-85.

Schmiedlin-Ren, P., K. E. Thummel, J. M. Fisher, M. F. Paine, K. S. Lown, and P. B. Watkins. "Expression of enzymatically active Cyp3a4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1 Alpha,25-Dihydroxyvitamin D_3." *Molecular Pharmacology* 51, no. 5 (1997): 741-54.

Schneyer C.R. "Calcitonin and the Treatment of Osteoporosis." *Medical Journal* 40 (1991): 469-73.

Schurgers, N., J. Bijdendijk, J. J. Tukker, and D. J. Crommelin. "Comparison of four experimental techniques for studying drug absorption kinetics in the anesthetized rat in situ." *Journal of Pharmaceutical Science* 75, no. 2. (1986): 117-9.

Sergent-Engelen, T., C. Halleux, E. Ferrain, H. Hannot, R. Legras and Y. J. Schneider. "Improved cultivation of polarized animal cells on culture inserts with new transparent polyethylene terephthalate or polycarbonate microporous membranes." *Biotechnology Technology* 4 (1990): 89-94.

Shao, Z. H., Y. P. Li, T. Chermak, and A. K. Mitra. "Cyclodextrins as mucosal absorption promoters of insulin .2.effects of beta-cyclodextrin derivatives on alpha-chymotryptic degradation and enteral absorption of insulin in rats." *Pharmaceutical Research* 11, no. 8 (1994): 1174-79.

Shao, Z. H., Y. P. Li, R. Krishnamoorthy, T. Chermak, and A. K. Mitra. "Differential-effects of anionic, cationic, nonionic, and physiological surfactants on the dissociation, alpha-chymotryptic degradation, and enteral absorption of insulin hexamers." *Pharmaceutical Research* 10, no. 2 (1993): 243-51.

Shao, Z. H., R. Krishnamoorthy, and A. K. Mitra. "Cyclodextrins as nasal absorption promoters of insulin -mechanistic evaluations." *Pharmaceutical Research* 9, no. 9 (1992): 1157-63.

Sheffner, A. L. "The Mucolytic Action of Acetylcysteine." *Tubercology & Thoracic Diseases* 23, no. 2 (1966): 31-3.

Shekels, L. L., C. T. Lyftogt, and S. B. Ho. "Bile acid-induced alterations of mucin production in differentiated human colon cancer cell lines." *International Journal of Biochemistry & Cell Biology* 28, no. 2 (1996): 193-201.

Shen, W. C., J. S. Wan, and H. Ekrami. "Enhancement of polypeptide and protein-absorption bymacromolecular carriers via endocytosis and transcytosis" *Advanced Drug Delivery Reviews* 8, no. 1 (1992): 93-113.

Shiau, Y. F., R. J. Kelemen, and M. A. Reed. "Acidic Mucin Layer Facilitates Micelle Dissociation and Fatty-Acid Diffusion." *American Journal of Physiology* 259, no. 4 (1990): G671-G675.

Shiau, Y. F., and G. M. Levine. "pH Dependence of Micellar Diffusion and Dissociation." *American Journal of Physiology* 239, no. 3 (1980): G177-82

Shimizu, M., M. Tsunogai, and S. Arai. "Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2." *Peptides* 18, no. 5 (1997): 681-87.

Shiotani, K., K. Uehata, T. Irie, K. Uekama, D. O. Thompson, and V. J. Stella. "Differential-Effects of sulfate and sulfobutyl ether of beta-cyclodextrin on erythrocyte-membranes in-vitro." *Pharmaceutical Research* 12, no. 1 (1995): 78-84.

Singer, S. J., and G. L. Nicolson. "The fluid mosaic model of the structure of cell membranes." *Science* 175, no. 23 (1972): 720-31.

Sinko, P. J., P. Hu, E. J. Wagner, A. Sturmer, L. M., J. P. Gilligan, and W. Stern.. "Determination of the intestinal permeability of recombinant, salmon calcitonin." *Pharmaceutical Research* 10, Suppl: S293 (1993)

Sinko, P. J., Y. H. Lee, V. Makhey, G. D. Leesman, J. P. Sutyak, H. Yu, B. Perry, C. L. Smith, P. Hu, E. J. Wagner, L. M. Falzone, L. T. McWhorter, J. P. Gilligan, and W. Stern. "Biopharmaceutical approaches for developing and assessing oral peptide delivery strategies and systems: *in-vitro* permeability and *in-vivo* oral absorption of salmon calcitonin (sCT)." *Pharmaceutical Research* 16, no. 4 (1999): 527-33.

Small, D. M. "The physical chemistry of the cholanic acids." In *The Bile acids, chemistry, physiology and metabolism.* (Eds. Nair, D.P. and Kritchevsk, D.) Vol. 1 (1971): 249- 356.

Smith S.P., D.J.W. Grant, J. Hadgraft and G.D. Parr. "Cyclodextrins in Pharmaceutical Science." *Acta Pharmaceutica Technologica* 30, No. 3 (1984).

Soergel, K. H. "Showdown at the Tight Junction." *Gastroenterology* 105, no. 4 (1993): 1247-50.

Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. "Endocytosis and the recycling of plasma membrane." *Journal of Cell Biology* 96, no. 1 (1983): 1-27.

Stella, V. J., H. K. Lee, and D. O. Thompson. "The Effect of Sbe4-Beta-Cd on IV methylprednisolone pharmacokinetics in rats - Comparison to a co-solvent solution and to water-soluble prodrugs." *International Journal of Pharmaceutics* 120, no. 2 (1995): 189-95.

Stella, V. J., and R. A. Rajewski. "Cyclodextrins: Their Future in Drug Formulation and Delivery." *Pharmaceutical Research* 14, no. 5 (1997): 556-67.

Stella, V. J., V. M. Rao, E. A. Zannou, and V. Zia. "Mechanisms of drug release from cyclodextrin complexes." *Advanced Drug Delivery Reviews* 36, no. 1 (1999): 3-16.

Stenberg, P., K. Luthman, and P. Artursson. "Prediction of membrane permeability to peptides from calculated dynamic molecular surface properties." *Pharmaceutical Research* 16, no. 8 (1999): 1324-1326.

Stewart, B. H., O. H. Chan, R. H. Lu, E. L. Reyner, H. L. Schmid, H. W. Hamilton, B. A. Steinbaugh, and M. D. Taylor. "Comparison of Intestinal Permeabilities Determined in Multiple *in-vitro* and *in-situ* Models - Relationship to Absorption in humans." *Pharmaceutical Research* 12, no. 5 (1995): 693-99.

Stratford Jr., R.E. and V.H.L. Lee. "Amimopeptidase activity in albino rabbit extraocular tissue relative to the small intestine." *Journal of Pharmaceutical Science* 74, (1985):

731-34.

Stratford Jr., R.E. and V.H.L. Lee. "Aminopeptidase activity in Homogenates of various absorptive mucosae in the albino rabbit: implications in peptide delivery." *International journal Of Pharmaceutical Science* 30, (1986): 73-82.

Strattan, C. E. "Cyclodextrins and Biological Macromolecules." *Biopharm-the Technology & Business of Biopharmaceuticals* 4, no. 10 (1991): 44-51.

Swan, P. W., and J. J. Tucker. "Molecular determinants of recognition for the intestinal peptide carrier." *Journal of Pharmaceutical Science* 86, no. 5 (1997): 590-602.

Swenson, E. S., and W. J. Curatolo. "Intestinal Permeability Enhancement for Proteins, Peptides and other Polar Drugs - Mechanisms and Potential Toxicity .2." *Advanced Drug Delivery Reviews* 8, no. 1 (1992): 39-92.

Szejtli, J. *Cyclodextrin Technology* (1988) (Eds. Davies, E.D.). Kluwer Academic Publishers, Budapest, Hungary.

Szente, L., and J. Szejtli. "Highly Soluble Cyclodextrin Derivatives: Chemistry, Properties and Trends in Development." *Advanced Drug Delivery Reviews* 36, no. 1 (1999): 17-28.

Tada, H., O. Shiho, K. Kuroshima, M. Koyama, and K. Tsukamoto. "An Improved Colorimetric Assay for Interleukin 2." *Journal of Immunological Methods* 93, no. 2 (1986): 157-65.

Takanaga, H., I. Tamai, and A. Tsuji. "pH-Dependent and carrier-mediated transport of salicylic-acid across caco-2 cells." *Journal of Pharmacy and Pharmacology* 46, no. 7 (1994): 567-70.

Tamura, K., C. P. Lee, P. L. Smith, and R. T. Borchardt. "Metabolism, uptake, and transepithelial transport of the stereoisomers of Val-Val-Val in the human intestinal cell line, Caco-2." *Pharmaceutical Research* 13, no. 11 (1996): 1663-67.

Tan, X. Y., and S. Lindenbaum. "Studies on complexation between beta-cyclodextrin and bile-salts." *International Journal of Pharmaceutics* 74, no. 2-3 (1991): 127-35.

Tanaka, Y., Y. Taki, T. Sakane, T. Nadai, H. Sezaki, and S. Yamashita. "Characterization of drug transport through tight-junctional pathway in caco-2 monolayer - comparison with isolated rat jejunum and colon." *Pharmaceutical Research* 12, no. 4 (1995): 523-28.

Tavelin, S., V. Milovic, G. Ocklind, S. Olsson, and P. Artursson. "A Conditionally Immortalized Epithelial Cell Line for Studies of Intestinal Drug Transport." *Journal of Pharmacology Experimental Therapeutics* 290, no. 3 (1999): 1212-21.

Tengamnuay, P., and A. K. Mitra. "Bile salt-fatty acid mixed micelles as nasal absorption

promoters of peptides. II. *In-vivo* nasal absorption of insulin in rats and effects of mixed micelles on the morphological integrity of the mucosae." *Pharm Res* 7, no. 4 (1990): 370-75.

TenHoor, C.N. and J.B. Dressman. "Oral absorption of peptides and proteins." *S.T.P. Pharmaceutical Science* 2, (1992): 301- 312.

Thornburg, W., R. K. Rao, L. M. Matrisian, B. E. Magun, and O. Koldovský. "Effect of maturation on gastrointestinal absorption of epidermal growth factor in rats." *American Journal of Physiology* 253, no. 1 Pt 1 (1987): G68-71.

Thwaites, D. T., C. D. A. Brown, B. H. Hirst, and N. L. Simmons. "Transepithelial glycylsarcosine transport in intestinal caco-2cells mediated by expression of h+-coupled carriers at both apical and basal membranes." *Journal of Biological Chemistry* 268, no. 11 (1993): 7640-7642.

Thwaites, D. T., M. Cavet, B. H. Hirst, and N. L. Simmons. "Passive transepithelial absorption of thyrotropin-releasing-hormone (TRH) via a paracellular route in cultured intestinal and renal epithelial-cell lines." *Pharmaceutical Research* 10, no. 5 (1993): 674-81.

Thwaites, D. T., M. Cavet, B. H. Hirst, and N. L. Simmons. "Substrate-specificity of the di/tripeptide transporter in human intestinal epithelia (Caco-2) - Identification of substrates that undergo h+-coupled absorption." *British Journal of Pharmacology* 113, no. 3 (1994): 1050-1056.

Tomita, M., M. Hayashi, and S. Awazu. "Absorption-enhancing mechanism of EDTA, Caprate, and decanoylcarnitine in Caco-2 Cells." *Journal of Pharmaceutical Sciences* 85, no. 6 (1996): 608-11.

Tomita, M., M. Hayashi, and S. Awazu. "Absorption-Enhancing Mechanism of Sodium Caprate and decanoylcarnitine in Caco-2 Cells." *Journal of Pharmacology and Experimental Therapeutics* 272, no. 2 (1995): 739-43.

Tomita, M., M. Hayashi, and S. Awazu. "Comparison of Absorption-Enhancing Effect Between Sodium caprate and Disodium Ethylenediamine tetraacetate in Caco-2 cells." *Biological & Pharmaceutical Bulletin* 17, no. 5 (1994): 753-55.

Toth, I. "A novel chemical approach to drug-delivery - Lipidic Amino-Acid conjugates." *Journal of Drug Targeting* 2, no. 3 (1994a): 217-39.

Toth, I., N. Flinn, A. Hillery, W. A. Gibbons, and P. Artursson. "Lipidic Conjugates of Luteinizing-Hormone-Releasing Hormone (LHRH) and Thyrotropin-Releasing-Hormone (TRH) that release and protect the native hormones in homogenates of human intestinal epithelial (Caco-2) cells." *International Journal of Pharmaceutics* 105, no. 3 (1994b): 241-47.

Toth, I., A. Hillery, I. P. Wood, C. Magnusson, and P. Artursson. "Oral absorption of

lipidic amino-acid conjugates." *International Journal of Pharmaceutics* 102, no. 1-3 (1994c): 223-30.

Totterman, A. M., N. G. M. Schipper, D. O. Thompson, and J. P. Mannermaa. "Intestinal safety of water-soluble beta-cyclodextrins in paediatric oral solutions of spironolactone: effects on human intestinal epithelial Caco-2 cells." *Journal of Pharmacy and Pharmacology* 49, no. 1 (1997): 43-48.

Tozaki H, T. Odoriba, T. Iseki, T. Taniguchi, T. Fujita, M. Murakami, S. Muranishi and A. Yamamoto. " Use of protease inhibitors to improve calcitonin absorption from the small and large intestine in rats." *Journal of Pharmacy and Pharmacology* 50, No. 8 (1998): - 913-20.

Tsuji, A., T. Terasaki, I. Tamai, and H. Hirooka. "H^+ gradient-dependent and carrier-mediated transport of cefixime, a new cephalosporin antibiotic, across brush- border membrane vesicles from rat small intestine." *Journal of Pharmacology Experimental Therapeutics* 241, no. 2 (1987): 594-601.

Tucker, S. P., L. R. Melsen, and R. W. Compans. "Migration of polarized epithelial cells through permeable membrane substrates of defined pore-size." *European Journal of Cell Biology* 58, no. 2 (1992): 280-290.

Uchiyama K., A. Kotani, T. Kishida, H. Tatsumi, A. Okamoto, T. Fujita, M. Murakami, S. Muranishi and S. Yamamoto. "Effects of various protease inhibitors on the stability and the permeability of [D-Ala^2, D-Leu^5]enkephalin in rat intestine: comparison with leucine enkephalin." *Journal of Pharmaceutical Sciences* 87, No. 4 (1997): 447-452.

Uekama K, F. Hirayama and T. Irie. "Applications of cyclodextrins." *In Drug Targeting Delivery* 3 (1994): 411-456.

Uekama K., H. Arima, T. Irie, K. Matsubara, and T. Kuriki. "Sustained release of buserelin acetate, a luteinizing hormone- releasing hormone agonist, from an injectable oily preparation utilizing ethylated beta-cyclodextrin." *Journal of Pharmacy and Pharmacology* 41, (1989): 874-6

Ungell, A. L. "In vitro absorption studies and their relevance to absorption from the GI tract." *Drug Development and Industrial Pharmacy* 23, no. 9 (1997): 879-92.

Van Greevenbroek, M. M., W. F. Voorhout, D. W. Erkelens, G. van Meer, and T. W. de Bruin. "Palmitic acid and linoleic acid metabolism in Caco-2 cells: different triglyceride synthesis and lipoprotein secretion." *Journal of Lipid Research* 36, no. 1 (1995): 13-24.

Verhoef, J. C., N. G. M. Schipper, S. G. Romeijn, and F.W.H.W Merkus. "The potential of cyclodextrins as absorption enhancers in nasal delivery of peptide drugs." *Journal of Controlled Release* 29, no. 3 (1994): 351-60.

Walter, E., and T. Kissel. "Transepithelial transport and metabolism of thyrotropin-

releasing-hormone (TRH) in monolayers of a human intestinal-cell line (Caco-2) - evidence for an active-transport component." *Pharmaceutical Research* 11, no. 11 (1994): 1575-80.

Walter, E., T. Kissel, and P. Raddatz. "Transport of peptidomimetic renin inhibitors across monolayers of a human intestinal-cell line (Caco-2) - evidence for self-enhancement of paracellular transport route." *Pharmaceutical Research* 12, no. 11 (1995): 1801-5.

Wang, W. "Oral Protein Drug Delivery." *Journal of Drug Targeting*, no. 4. (1996): 195-232.

Weiser, M. M. "Intestinal epithelial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation." *Journal of Biology and Chemistry* 248, no. 7 (1973): 2536-41.

Werner, U., T. Kissel, and M. Reers. "Effects of Permeation Enhancers on the transport of a peptidomimetic Thrombin Inhibitor (CRC 220) in a Human intestinal Cell Line (Caco-2)." *Pharmaceutical Research* 13, no. 8 (1996): 1219-27.

Wesley, A. W., J. F. Forstner, and G. G. Forstner. "Structure of intestinal-mucus glycoprotein from human post- mortem or surgical tissue: inferences from correlation analyses of sugar and sulfate composition of individual mucins." *Carbohydrate Research* 115 (1983): 151-63.

Westergaard, H., and J. M. Dietschy. "Delineation of the dimensions and permeability characteristics of the two major diffusion barriers to passive mucosal uptake in the rabbit intestine." *Journal of Clinical Investigation* 54, no. 3 (1974): 718-32.

Wikman, A., J. Karlsson, I. Carlstedt, and P. Artursson. "A drug absorption model based on the mucus layer producing human intestinal goblet cell line HT29-H." *Pharmaceutical Research* 10, no. 6 (1993): 843-52.

Wikman-Larhed, A., and P. Artursson. "Co-cultures of human intestinal goblet (Ht29-H) and absorptive (Caco-2) cells for studies of drug and peptide absorption." *European Journal of Pharmaceutical Sciences* 3, no. 3 (1995): 171-83.

Wils, P., S. Legrain, E. Frenois, and D. Scherman. "Ht29-18-C1 intestinal cells - a new model for studying the epithelial transport of drugs." *Biochimica Et Biophysica Acta* 1177, no. 2 (1993): 134-38.

Wils, P., A. Warnery, V. Phungba, S. Legrain, and D. Scherman. "High lipophilicity decreases drug transport across intestinal epithelial-cells." *Journal of Pharmacology and Experimental Therapeutics* 269, no. 2 (1994): 654-58.

Wilson, G. "Cell-culture techniques for the study of drug transport." *European Journal of Drug Metabolism and Pharmacokinetics* 15, no. 2 (1990): 159-63.

Wilson, G., C. Washington and N. Washington. "Overview of epithelial cell barriers and drug transport." In *Physiological Pharmaceutics, Biological barrier to drug absorption.* Ellis Horwood, Chichester, (1989)

Wimalawansa, S. J. "Amylin, calcitonin gene-related peptide, calcitonin, and adrenomedullin: a peptide superfamily." *Critical Reviews in Neurobiology* 11, no. 2-3 (1997): 167-239.

Wimalawansa, S. J. "Long-Term and short-term side-effects and safety of calcitonin in man - a prospective-study." *Calcified Tissue International* 52, no. 2 (1993): 90-93.

Wimalawansa, S. J and I. MacIntyre. "Calcitonin." In *Therapeutic Drugs; A clinical pharmacopoeia* (Ed. Dollery, C.T.) (1991).

Woodcock, S., I. Williamson, I. Hassan, and M. Mackay. "Isolation and characterization of clones from the caco-2 cell-line displaying increased taurocholic acid transport." *Journal of Cell Science* 98 (1991): 323-32.

Wu, S. J., and J. R. Robinson. "Transport of human growth hormone across CaCo-2 cells with novel delivery agents: evidence for P-Glycoprotein involvement." *Journal of Controlled Release* 62, no. 1-2 (1999): 171-77.

Yamahara, H., K. Morimoto, V. H. L. Lee, and K. J. Kim. "Effects of protease inhibitors on vasopressin transport across rat alveolar epithelial-cell monolayers." *Pharmaceutical Research* 11, no. 11 (1994): 1617-22.

Yamamoto, A., T. Taniguchi, K. Rikyuu, T. Tsuji, T. Fujita, M. Murakami, and S. Muranishi. "Effects of various protease inhibitors on the intestinal-absorption and degradation of insulin in rats." *Pharmaceutical Research* 11, no. 10 (1994): 1496-500.

Yamashita, S., M. Masada, T. Nadai, and T. Kimura. "Effect of adjuvants on charge-selective permeability and electrical-resistance of rat jejunal membrane." *Journal of Pharmaceutical Sciences* 79, no. 7 (1990): 579-83.

Yamashita, S., Y. Tanaka, Y. Endoh, Y. Taki, T. Sakane, T. Nadai, and H. Sezaki. "Analysis of drug permeation across Caco-2 Monolayer: implication for predicting *in-vivo* drug absorption." *Pharmaceutical Research* 14, no. 4 (1997): 486-91.

Yang, C.Y., A.H. Dantzig and C. Pidgeon. "Intestinal peptide transport systems and oral drug availability." *Pharmaceutical Research* 16, no. 9 (1999): 1331- 43

Yazdanian, M., S. L. Glynn, J. L. Wright, and A. Hawi. "Correlating partitioning and Caco-2 Cell permeability of structurally diverse small molecular weight compounds." *Pharmaceutical Research* 15, no. 9 (1998): 1490-1494.

Yen, W. C., and V. H. L. Lee. "Penetration enhancement effect of Pz-peptide, a paracellularly transported peptide, in rabbit intestinal segments and CaCo-2cell

monolayers." *Journal of Controlled Release* 36, no. 1-2 (1995): 25-37.

Yuasa, H., G. L. Amidon, and D. Fleisher. "Peptide carrier-mediated transport in intestinal brush-border membrane-vesicles of rats and rabbits - Cephradine uptake and inhibition." *Pharmaceutical Research* 10, no. 3 (1993): 400-404.

Zapt, A., D Hsu, and J.M. Olefsky. "Comparison of the intracellular itineraries of insulin-like growth factor-I and insulin and their receptors in rat-I fibroblasts." *Endocrinology* 134, No. 6 (1994): 2445- 52.

Zhang, Y. and L. Z. Benet. "Characterization of p-glycoprotein mediated transport of KO2, novel vinylsulphone peptidometic cysteine protease inhibitor, across MDR1, MDCK and CaCo-2 cell monolayers." *Pharmaceutical Research* 15, no. 10 (1998): 1520-24.

Zheng, L. X., J. Y. Chen, Y. P. Zhu, H. Yang, W. Elmquist, and M. Hu. "Comparison of the transport characteristics of d-methionine and l-methionine in a human intestinal epithelial model (Caco-2) and in a perfused rat intestinal model." *Pharmaceutical Research* 11, no. 12 (1994): 1771-76.

Zhou, X. H. "Overcoming enzymatic and absorption barriers to non-parenterally administered protein and peptide drugs." *Journal of Controlled Release* 29, no. 3 (1994): 239-52.

Zhou, X. H., and A. L. W. Po. "Peptide and Protein Drugs .1. Therapeutic applications,absorption and parenteral administration." *International Journal of Pharmaceutics* 75, no. 2-3 (1991): 97-115.

Zweibaum, A., M. Pinto, G. Chevalier, E. Dussaulx, N. Triadou, B. Lacroix, K. Haffen, J. L. Brun, and M. Rousset. "Enterocytic differentiation of a subpopulation of the human colon tumor cell line HT-29 selected for growth in sugar- free medium and its inhibition by glucose." *Journal of Cell Physiology* 122, no. 1 (1985): 21-9.

Zweibaum, A., M. Laburthe, E. Grasset and D. Louvard. "Use of cultured cell lines in studies of intestinal cell differentiation and function." In *Handbook of Physiology: The Gastrointestinal System* (Ed. Raimer, B.B.) (1991).