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Additional comments included for clarification -

One examiner raised a question about the use of reduced temperature to elucidate the presence or absence of active transport given that the permeability of passive markers was also affected by the reduced temperature (studies described in Chapter Six). The use of reduced temperature is an accepted method of assessing the potential for active transport (for discussion see lines 9-12, page 253) although there is little information available in the literature regarding the absolute permeability of passively transported compounds at low temperature. The temperature comparisons were not drawn from the absolute permeability coefficients for each compound, rather from the relative effects of the carrier, SNAC-2, at each temperature. Even though the absolute permeability of the transcellular marker diazepam was reduced at 10°C, the relative effect of the carrier on diazepam permeability was the same at 10°C and 37°C consistent with the transport of this marker being via a passive process (lines 13-16, page 257). On the other hand, both the relative carrier effects and the absolute permeability coefficients of the passive paracellular markers, PEG 4000 and mannitol, were reduced at 10°C which was attributed to the contraction of tight junctions at low temperature (see lines 16-20, page 257). While the permeation rate of hGH in the absence of carrier was very low, it was also markedly reduced at the lower temperature and, similar to the observation made for the passive transcellular marker diazepam, the relative effect of the carrier on hGH permeation was the same at 10°C and 37°C (lines 10-13, page 257) suggesting that the carrier effects on the absolute permeation of hGH did not occur via active transport but instead were consistent with a passive transcellular process.

While the initial aim of the project was the elucidation of the mechanism of hGH permeation enhancement by SNAC-2, the development and validation of a suitable model for measuring hGH permeation in the presence of SNAC-2 required considerably more time than was originally anticipated. The *in situ* intestinal perfusion model was briefly evaluated and deemed unsuitable owing to extensive enzymatic degradation of hGH (Chapter Four). While the modified Ussing chamber model was successfully established for the determination of hGH permeability in the presence of the carrier (Chapter Five and Chapter Six), there were a number of issues, including the unforseen problem of an intestinal parasitic infection in the rabbits used to obtain gut tissue segments, that had to be addressed in order to achieve this goal. Unfortunately, time constraints did not permit further assessment of the physical interaction between hGH and the carrier (other than the CD studies described in Chapter Three), although microcalorimetry (which was not readily available during the course of this work) would provide a suitable, non-spectroscopic technique for this purpose. Despite these difficulties, important mechanistic data regarding hGH permeation pathways in the presence of the carrier was gained from comparative permeability studies conducted using hGH and marker compounds (diazepam, mannitol and PEG 4000) with histology and reversibility studies providing supportive data for these experiments. These studies have also highlighted the importance of taking into account the unique requirements of protein drugs when addressing model development and validation issues for this class of compounds.

The development of the Emisphere carrier compounds represents a significant area of research for the potential oral delivery of protein therapeutics, with the current study demonstrating a significant increase in hGH intestinal permeation in the presence of a model carrier compound. Numerous Emisphere publications (described in Chapter One) also attest to the efficacy of other similar carrier compounds for enhancing the bioavailability of macromolecules. Notwithstanding these promising data, more efficient carrier compounds combined with the use of optimal formulation strategies are still required to produce sufficient permeation enhancement such that an oral formulation would be feasible for hGH.

INTESTINAL ABSORPTION OF HUMAN GROWTH HORMONE IN THE PRESENCE OF A NOVEL CARRIER COMPOUND

A thesis submitted for the degree

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by

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This thesis is dedicated to my parents,

Don and Jacqui McIntosh.

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CHAPTER SIX: INVESTIGATION OF THE INTESTINAL PERMEABILITY OF hGH IN THE PRESENCE OF THE NOVEL CARRIER COMPOUND SNAC-2

ABSTRACT

The development of an oral formulation for human growth hormone (hGH) would substantially increase patient acceptability and compliance and would reduce the treatment costs associated with the currently available parenteral hGH preparations. A New York-based biotechnology company, Emisphere Technologies Inc., recently developed a family of novel carrier compounds which have been demonstrated to improve the oral absorption of hGH in rats and primates. An investigation of the mechanism of hGH oral absorption enhancement by a representative Emisphere carrier, SNAC-2, is described in this thesis.

The hypothesis that altered intestinal permeability is responsible for the observed increase in hGH oral absorption was the primary focus of these studies. Alternative hypotheses included increased uptake by the intestinal lymphatics and proteolytic enzyme inhibition, any of which could be the consequence of a non-native hGH conformation induced by SNAC-2.

In vivo studies were initially conducted in rats in order to confirm the utility of SNAC-2 for improving the oral delivery of hGH. The bioavailability of hGH following the oral administration of a simple solution of hGH combined with SNAC-2 in buffer was observed to be at least five-fold higher and maximum serum concentrations approximately fourteen times greater than those measured for control animals dosed orally with hGH in buffer alone.

A second *in vivo* rat study addressed the possible contribution of intestinal lymphatic transport to hGH absorption in the presence of SNAC-2. No hGH was detected in lymph following the intraduodenal administration of an hGH/SNAC-2 solution to lymph-cannulated rats. Given that the assay method was capable of detecting the presence of as little as 0.1% of the administered dose in lymph, it was concluded that the observed oral absorption enhancement of hGH by SNAC-2 was unlikely to be the result of increased lymphatic transport.

The role of altered intestinal permeability in the absorption enhancement of hGH by SNAC-2 was investigated firstly through the use of an *in situ* rat intestinal perfusion model, however significant proteolytic degradation of hGH within the *in situ* model precluded the

reliable determination of hGH permeation. Interestingly, enzymatic degradation of hGH was unaffected by the presence of SNAC-2 suggesting that proteolytic enzyme inhibition was not primarily responsible for the carrier-induced improvement in hGH oral absorption.

A modified Ussing chamber model incorporating isolated rabbit intestinal tissue was subsequently established and validated for examining hGH intestinal permeability. The addition of SNAC-2 was shown to increase the permeability coefficients (P_{app}) of the paracellular markers, mannitol and PEG 4000, by up to approximately three-fold in rabbit ileum, indicating an effect of the carrier on the paracellular pathway. In comparison, hGH ileal permeability increased nine-fold in the presence of SNAC-2. Since the MW of hGH (22 kDa) is five-fold larger than that of PEG 4000, these results indicated that SNAC-2 is either more specific for hGH, or that the hGH permeation does not occur predominantly via the paracellular route. Partial reversibility of carrier effects was demonstrated for mannitol and hGH permeability and histological studies on tissues which had been exposed to SNAC-2 verified the absence of significant damage.

The possible involvement of active transport in the enhancement of hGH permeability by SNAC-2 was evaluated at low temperature (10°C) in the modified Ussing chamber. The relative change in hGH permeability in the presence of the carrier remained the same at 10°C and 37°C, while a reduced effect of SNAC-2 on the permeability of the paracellular markers, mannitol and PEG 4000, was observed. The temperature independence of the carrier effect on hGH suggested that active transport was not responsible for the increased permeability of hGH in the presence of SNAC-2. The reduced effect of SNAC-2 on the paracellular markers was attributed to a contraction of tight junctions at low temperature and suggested that it was unlikely that the carrier-induced increase in hGH permeation occurred via the paracellular route alone. It is therefore postulated in this thesis that the results obtained using the *in vitro* permeability model may reflect the involvement of a passive transcellular process in the oral absorption of hGH in the presence of the model carrier, SNAC-2.

Statement of Originality

The Registrar

Victorian College of Pharmacy

Monash University

I hereby certify that the work contained in this thesis has not been submitted by myself or any other person for a degree of Monash University (Victorian College of Pharmacy) or of any other institution.

[redacted]

Kylie Anne McIntosh

May, 2002

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PUBLICATIONS AND COMMUNICATIONS

1. K.A. McIntosh, W.N. Charman, S. Milstein and S.A. Charman, 1998. Development and validation of an *in vitro* model to measure the intestinal permeability of potential oral formulations of human growth hormone. Proceedings of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists incorporating the Annual Scientific Meeting of the Australasian Pharmaceutical Science Association, 13th-16th December 1998, Hobart, Australia.

2. K.A. McIntosh, W.N. Charman and S.A. Charman, 1999. A modified Ussing chamber model for the evaluation of rhGH transepithelial absorption. Proceedings of the Annual Meeting of the American Association of Pharmaceutical Scientists, 14-18th November 1999, New Orleans, LA.

3. K.A. McIntosh, W.N. Charman and S.A. Charman, 2000. The intestinal permeability of human growth hormone in the presence of a novel carrier compound. Proceedings of GPEN (Globalisation of Pharmaceutics Education Network) Meeting, 13-15th September 2000, Uppsala, Sweden.

4. K.A. McIntosh, W.N. Charman and S.A. Charman, 2001. Effect of a novel carrier compound on the intestinal permeability of human growth hormone. Proceedings of APSA (Australasian Pharmaceutical Science Association) Annual Scientific Meeting, 9-12th December 2001, Melbourne, Australia.

5. K.A. McIntosh, W.N. Charman and S.A. Charman, 2002. Development of an *in vitro* model for the determination of the intestinal permeability of recombinant human growth hormone. (manuscript in preparation)

6. K.A. McIntosh, W.N. Charman and S.A. Charman, 2002. Intestinal permeability of recombinant human growth hormone in the presence of a novel carrier compound. (manuscript in preparation)

CHAPTER ONE

INTRODUCTION

1.1 STATEMENT OF THE PROBLEM

Human growth hormone (hGH) is a physiologically and pharmacologically important peptide hormone used in the treatment of paediatric hypopituitary dwarfism. Negligible oral bioavailability of hGH, a consequence of a number of factors including poor membrane permeation, proteolytic degradation and physical instability, currently necessitates its parenteral administration. This is an unpleasant prospect for the intended paediatric patient population and one that frequently leads to poor patient compliance. Development of an oral formulation would be of benefit in improving patient acceptability and in reducing treatment costs.

A New York-based biotechnology company, Emisphere Technologies, Inc., has recently developed a family of novel carrier compounds, which have been demonstrated to markedly improve the oral bioavailability of various macromolecules, including hGH (Leone-Bay et al., 1995b, Leone-Bay et al., 1996a, Leone-Bay et al., 1998a). Significant serum levels of hGH were achieved in rats and primates following the oral administration of hGH combined with selected carrier compounds (Leone-Bay et al., 1996a). The mechanism(s) responsible for this increased absorption are currently unknown, although an alteration in hGH conformation has been proposed to account for the increased membrane permeation (Milstein et al., 1998).

The novel carrier, SNAC-2, is one of the compounds shown by the Emisphere in-house screening program to improve the oral absorption of hGH in rats. The aim of the studies described in this thesis was to probe the mechanism of hGH absorption enhancement in the presence of SNAC-2 as a model carrier. Specifically, there were three main hypotheses regarding the possible mechanism of absorption enhancement effected by SNAC-2: improved intestinal permeation, proteolytic enzyme inhibition and increased uptake by the intestinal lymphatics, any of which could be the consequence of an altered hGH conformation. Studies described in this dissertation primarily focussed on investigating the hypothesis of altered intestinal permeation being

responsible for the observed oral absorption enhancement of hGH, with both an *in situ* rat intestinal perfusion model and a modified Ussing chamber model employed for this purpose. Supportive circular dichroism spectroscopy measurements were conducted in an attempt to probe possible alterations to hGH conformation induced by SNAC-2. Furthermore, the contribution of intestinal lymphatic transport to hGH absorption in the presence of the carrier was also briefly examined. The overall objective of this work was to gain insight into the mechanism(s) of absorption enhancement to assist in the identification of conditions necessary for the model carrier, SNAC-2, or other carrier compounds to effect the maximal absorption enhancement of hGH, thereby aiding the development of an oral hGH formulation.

1.2 ORAL DELIVERY OF PROTEIN DRUGS

Since the advent of recombinant DNA technology in the mid-1980's, there has been a dramatic increase in the number of therapeutic protein drugs developed. For the first time, the ready availability of commercial quantities of proteins promised significant advances in the treatment of many disease states including diabetes, hepatitis, multiple sclerosis, hypopituitary dwarfism, osteoporosis and chronic anaemia associated with renal failure and various cancers. Additionally, the commercial incentive to further explore and optimise the formulation and delivery of this class of compounds is significant, with biotechnology product sales in the United States alone estimated to rise to US$42b by the year 2008 (Prosser, 1999).

While the oral route of drug delivery remains the most acceptable and convenient for most patients, the delivery of therapeutic proteins is at present limited to the parenteral route of administration. The effects of enzymatic degradation, poor intestinal permeation characteristics and chemical and physical instability combine to present a formidable challenge to the oral delivery of protein drugs.

1.2.1 Challenges to the Successful Oral Delivery of Proteins

1.2.1.1 Enzymatic Degradation

Enzymatic degradation of biomacromolecules in the gut is an essential component of the normal physiological process of dietary protein digestion. Proteolytic enzymes are ubiquitous throughout the gastrointestinal tract, located both within the lumen of the gut and in the brush border membrane (BBM) and cytosol of enterocytes. Following oral ingestion, proteins (dietary or therapeutic) are partially digested by pepsin proteases in the stomach and subsequently further degraded by luminally secreted pancreatic enzymes (e.g. trypsin and α-chymotrypsin) in the small intestine. The remaining enzymatic degradation of proteins in the gut takes place in the BBM and in the cytosol, following uptake into the enterocyte. This highly efficient digestive process results in 94-98% of protein being completely digested/degraded and absorbed in normal human beings (Langguth et al., 1997). Consequently, it is not surprising that gut proteases present such a significant impediment to the absorption of protein drugs.

It is widely recognised that the extensive hydrolysis of protein drugs by gut proteases plays a substantial role in severely limiting the oral bioavailability of this class of drugs (Lee, 1988, Zhou, 1994, Langguth et al., 1997). Perhaps the best known and characterised example of enzymatic degradation of a protein drug would be that of insulin (MW ~6000). The rapid loss of insulin determined using intestinal homogenates and other *in vitro* testing methods (Lee, 1988, Yamamoto et al., 1990, Yamamoto et al., 1994) has implicated enzymatic proteolysis as a significant contributing factor in its low oral bioavailability (Danforth and Moore, 1959).

1.2.1.2 Chemical and Physical Stability

Proteins targeted for oral delivery are subjected to a number of chemical degradation processes through their exposure to the harsh environment of the gastrointestinal tract. By

definition, chemical degradation involves structural modification through the formation or destruction of covalent bonds. Decomposition reactions inherent to biomacromolecules include hydrolysis, deamidation and oxidation. Protein deamidation involves the hydrolysis of Asn and Gln residues to the corresponding carboxylic acids (Asp and Glu), while oxidation of Met residues may lead to the formation of the corresponding sulfoxide (Becker et al., 1988, Manning et al., 1989).

Physical instability is also an important consideration in the area of protein drug delivery. The physical degradation of proteins is a consequence of non-covalent structural modifications, which are mostly unique to this class of molecules due to their ability to adopt higher order structures. Degradative processes for proteins include denaturation, aggregation, precipitation and adsorption. Denaturation refers to the loss of tertiary (and often secondary) structures, typically resulting in the formation of hydrophobic non-native conformations which may more readily adsorb to surfaces, aggregate with other protein molecules in solution and ultimately precipitate out of solution. The process of denaturation may or may not be reversible and is triggered by factors such as increased or decreased temperature, extremes of solution pH (e.g. the acidic environment of the stomach) or agitation (e.g. mixing/gastric emptying).

Studies conducted to investigate these aspects of protein drug stability have typically focussed on the *in vitro* degradation of biomacromolecules as it pertains to formulation issues or the *in vivo* degradation of proteins following parenteral administration, since this is the conventional route of administration for these compounds. Consequently, while it is known that nonenzymatic protein deamidation may occur at a rapid rate *in vivo* (Cleland et al., 1993), it is difficult to assess the contribution of this and other chemical and physical degradation processes to the overall degradation of proteins in the gut. Furthermore, as discussed in Section 1.2.1.1, it is likely that enzymatic proteolysis is responsible for the vast majority of protein degradation in the gastrointestinal tract.

1.2.1.3 Intestinal Permeability

In addition to the challenges presented by enzymatic and non-enzymatic degradation, oral delivery of proteins is also significantly limited by poor intestinal permeation - a problem resulting primarily from their size, charge and hydrophilicity. Even though intestinal permeability has been extensively reviewed in the literature (Conradi et al., 1993, Hochman and Artursson, 1994, Grass, 1997, Lecluyse and Sutton, 1997, Artursson et al., 2001), a description of the more fundamental aspects of this area have been included in this thesis in order to provide a more complete background to the studies described.

Intestinal permeation is dependent upon both the nature of the intestinal wall (primarily lipidic) and the physicochemical characteristics of the permeant (Csaky, 1984). The permeant characteristics which impact on transit through the intestinal membrane include lipophilicity, molecular size and shape, ionisation and hydrogen-bonding potential (van de Waterbeemd, 2000). The ability of a substance to traverse the intestinal wall is reflected in its apparent permeability coefficient (P_{app}), a quantitative measure of the rate of permeation (generally expressed as distance per time), with higher P_{app} values associated with drugs which more readily permeate the intestinal membrane.

Principally, there are two routes of molecular permeation across the intestinal mucosa: the paracellular route (between adjacent enterocytes) and the transcellular route (through the enterocyte) (Daugherty and Mrsny, 1999). Small, lipophilic drugs typically permeate via the transcellular route, readily partitioning into and out of the lipid membrane barrier, whereas small, hydrophilic drugs generally traverse the intestinal barrier via the paracellular route. In specialised cases, particularly for the absorption of dietary nutrients (e.g. D-glucose), molecules may permeate by means of an active transport mechanism. It is known that small quantities of proteins are absorbed intact (Gardner, 1988), although as mentioned above, permeation of proteins through the intestinal membrane is extremely limited by their hydrophilicity, size, and charge.

Transcytosis has been suggested to account for the permeation of selected proteins, including horseradish peroxidase (Walker et al., 1972), β-lactoglobulin (Marcon Genty et al., 1989) and hGH (Mlynek et al., 2000), while the permeation of the smaller peptide, thyrotropin releasing hormone, is thought to occur primarily via the paracellular route (Thwaites et al., 1993, Gan et al., 1993). Only small quantities of protein drug are typically detected in such studies.

The intestinal permeability coefficients (P_{app}) of a series of proteins have been measured in rabbit colon, with values for rhDNAse and rhGH of $0.04 \pm 0.00 \times 10^{-6}$ cm/sec and $0.12 \pm 0.01 \times 10^{-6}$ cm/sec, respectively (Rubas et al., 1995). The authors suggested that these low P_{app} values were highly unlikely to translate to *in vivo* intestinal absorption to any significant degree. Based upon the effects of unfavourable physicochemical properties, coupled with low permeability coefficients, it is evident that poor intestinal permeation contributes significantly to the negligible oral bioavailability of proteins.

1.2.2 Strategies for Improving Oral Absorption of Protein Drugs

The successful development of oral protein formulations requires strategies specifically aimed at overcoming the significant biological and physicochemical barriers presented to orally administered proteins. Efforts to achieve this target have included the use of protease inhibitors, absorption enhancers, specialised formulations, chemical modification, carrier compounds, or a combination of several of these approaches.

1.2.2.1 Protease Inhibitors

The utility of protease inhibitors is an approach which has attracted significant scientific interest, since it has been demonstrated that co-administration of enzyme inhibitors with protein drugs can markedly increase oral bioavailability (Zhou, 1994, Bernkop-Schnurch, 1998).

As early as 1958, Laskowski et al. showed that jejunal administration of insulin and trypsin inhibitor to Sprague-Dawley rats lead to a significant reduction in blood glucose levels, whereas jejunal administration of insulin alone had no measurable effect (Laskowski et al., 1958). More recently, Bai and Chang observed a dramatic increase in insulin transport across rat ileum in the presence of specific inhibitors of insulin-degrading enzyme (Bai and Chang, 1996), while Yamamoto et al. described *in vitro* studies of insulin proteolysis in rabbit mucosal homogenates with prevention of proteolysis by various protease inhibitors (Yamamoto et al., 1990). In another example, the absorption of recombinant human granulocyte colony stimulating factor following intra-duodenal administration in rats was increased five- to six-fold in the presence of soybean trypsin inhibitor and chicken egg white trypsin inhibitor (Ushirogawa et al., 1992).

While this approach for improving the oral bioavailability of protein drugs appears promising, consideration must also be given to the potential problems associated with the chronic use of enzyme inhibitors, including toxic side effects of the inhibitors themselves, the effect on normal dietary protein digestive processes and the possibility of inducing hypersecretion of proteolytic enzymes (Watanabe et al., 1992, Reseland et al., 1996).

1.2.2.2 Novel Formulation Approaches

Specialised formulations function to enhance the oral absorption of protein drugs by protecting against enzymatic and/or chemical degradation in the gastrointestinal tract or targeting delivery to a specific route. Liposomes, although themselves subject to degradation in the gut, generally serve to protect proteins from proteolysis and have been shown to improve the oral delivery of drugs including insulin (Chien and Banga, 1989, Iwanaga et al., 1999) and hGH (Dean et al., 1999). The enhanced uptake of liposomal vaccine formulations has also been attributed to transport across Peyer's patches (Chen et al., 1996). Further, it is likely that nanoparticulate (particle size up to approximately 10 μm) formulations target this route of absorption, although

the limited capacity of intestinal lymphatic uptake via Peyer's patches restricts the applicability of this approach to very potent compounds (e.g. vaccine delivery) (Swenson and Curatolo, 1992).

Mucoadhesive polymers and polymer-inhibitor conjugates increase the oral absorption of protein drugs by several mechanisms: prevention of unwanted dilution effects, increased gut residence time and protection against proteolysis (Bernkop-Schnurch, 1998, Bernkop-Schnurch and Thaler, 2000). These systems have gained interest in the area of oral protein drug delivery, with one study observing that the administration of insulin formulated in a polymer-inhibitor conjugate to diabetic mice resulted in significantly lowered glucose levels (Marschutz et al., 2000).

Other novel formulation approaches have included the use of pH-responsive gels (insulin) (Lowman et al., 1999), microemulsions (calcitonin and insulin) (Gomez-Orellana and Paton, 1998) and microspheres (insulin) (Morishita et al., 1992).

1.2.2.3 Chemical Modification

Appropriate structural modification of protein drugs can result in greater stability to enzymatic and chemical degradation in the gut and/or improved intestinal permeability characteristics. Chemical alterations which have been investigated for this purpose include replacement of L-amino acids with unnatural D-amino acids (Friedman and Amidon, 1991, Krondahl et al., 1997), N-methylation of amide bonds (Conradi et al., 1991), glycosylation (Nomoto et al., 1998), cyclisation (Langguth et al., 1997), pegylation (Jensen-Pippo et al., 1996) and derivatisation with fatty acids (Hashimoto et al., 1989, Uchiyama et al., 2000).

A specific example of a successful chemical modification is one reported by Friedman and Amidon, who observed that the replacement of the second amino acid in leu-enkephalin with D-alanine lead to significantly reduced proteolysis in rat intestinal perfusate (Friedman and Amidon, 1991). More recently, Jensen-Pippo et al. described the increase in enteral bioavailability

of pegylated human granulocyte colony stimulating factor (G-CSF) in rats relative to unmodified G-CSF (Jensen-Pippo et al., 1996). However, while stability and permeability characteristics of protein drugs may be enhanced through chemical modification, there is a considerable associated risk of reduced pharmacological activity with this approach (Wang, 1996).

1.2.2.4 Absorption Enhancers

The use of absorption enhancers for improving the oral delivery of peptides and proteins is a key area of research and has been extensively reviewed (Lee et al., 1991, Swenson and Curatolo, 1992, Zhou, 1994, Fix, 1996, Aungst et al., 1996, Wang, 1996, Gomez-Orellana and Paton, 1998, Aungst, 2000). Absorption enhancers generally function non-specifically by one or a number of putative mechanisms including altering membrane fluidity, opening intercellular tight junctions, reducing viscosity of the intestinal mucus layer, inhibiting intestinal proteases and increasing protein thermodynamic activity (Wang, 1996).

Absorption enhancers (with some exceptions) may be classified into five groups: (i) surfactants such as Triton X-100, (ii) bile salts such as sodium glycocholate, (iii) chelating agents such as EDTA, (iv) fatty acids such as capric acid, and (v) medium-chain glycerides. Some notable examples of increased protein absorption in the presence of enhancers include the increased permeation of insulin in the presence of a variety of absorption-enhancing compounds across rat intestine as described by Uchiyama and co-workers (Uchiyama et al., 1999). The authors identified sodium glycocholate, sodium caprate and n-lauryl-beta-D-maltopyranoside as the most useful compounds due to efficient enhancement and relatively low toxicity. Another study demonstrated the absorption enhancement of insulin in the presence of sodium 5-methoxysalicylate from the upper gastrointestinal tract of rats, producing a bioavailability of approximately 10% (Nishihata et al., 1981).

While it is evident that absorption enhancers can significantly improve the oral absorption of protein drugs, their use warrants serious consideration of toxicological issues. The primary concern regarding the use of penetration enhancing compounds is the potential for membrane damage upon acute and/or chronic ingestion of the enhancer and the unwanted concomitant increase in the absorption of toxins/other drugs. The ideal absorption enhancer should be non-toxic and have a rapid and reversible effect on the intestinal membrane in order to facilitate the passage of reproducible, therapeutic concentrations of drug across the intestine (Swenson and Curatolo, 1992).

1.2.2.5 Carrier Compounds

The family of carrier compounds developed by Emisphere Technologies, Inc. has been demonstrated to improve the oral delivery of a number of macromolecular drugs (Leone-Bay et al., 1995a, Leone-Bay et al., 1995b, Milstein, 1995, Milstein et al., 1998, Leone-Bay and Paton, 1999). It has been suggested that the carriers improve oral absorption by means of a specific interaction with the protein that facilitates the formation of an altered protein conformation which is capable of translocating lipid membranes (Leone-Bay et al., 1996b, Milstein et al., 1998, Stoll et al., 2000). The term "carrier compounds" distinguishes these novel molecules from conventional penetration enhancers, which are known to improve oral bioavailability by non-specific membrane permeabilising effects (described in Section 1.2.2.4). Efforts are continuing to expand this class of compounds in addition to probing their precise mechanism of action. The development and use of the Emisphere carriers for increasing the oral bioavailability of macromolecules is described in detail in Section 1.4.

1.3 HUMAN GROWTH HORMONE (hGH)

Endogenous hGH is secreted by the pituitary gland and is responsible for promoting growth in the body functioning directly by interacting with target receptors, and indirectly by stimulating the release of other growth factors such as insulin-like growth factor-I (IGF-I). In addition to growth effects, hGH also induces altered insulin sensitivity, hyperglycaemia, improved lean body mass, stimulation of protein synthesis and improved bone density (Mehta and Hindmarsh, 2000). The isolation, *in vivo* effects, physicochemical characteristics and amino acid composition of hGH were first described in 1956 by Li and Papkoff (Li and Papkoff, 1956).

1.3.1 Physicochemical Characteristics

hGH is a 22 kDa polypeptide hormone that comprises a single chain of 191 amino acids and two intramolecular disulphide bonds. Circular dichroism (CD) studies have shown the secondary structure of hGH to consist primarily of α-helix (50-60%) (Bewley and Li, 1972), with the folded structure existing as a four-helix bundle (Pearlman and Bewley, 1993). In solution, hGH behaves as a typical globular protein, being tightly folded and approximately spherical (Stokes radius 25-30 Å (Brems et al., 1990)) with hydrophobic domains buried within the core of the molecule. hGH solubility ranges from a minimum of approximately 1 mg/mL at its pI of 5.3, up to greater than 25 mg/mL at pH 7.4 (Pearlman and Bewley, 1993).

Degradation of hGH occurs principally via three mechanisms: deamidation, oxidation and aggregation. While hGH contains 22 potential sites of deamidation (9 Asn and 13 Gln residues), the primary site of deamidation is at Asn-149 with a minor site at Asn-152. Of the three Met residues which can be oxidised, the oxidation of Met-14 to the corresponding sulfoxide is the most significant (Becker et al., 1988). Both the desamido and sulfoxide derivatives were observed to retain full biological activity.

It is widely recognised that hGH exhibits a strong tendency to aggregate in solution, either by covalent or non-covalent interactions, particularly in the presence of a high air/water interface such as that induced by agitation/mixing (Hagenlocher and Pearlman, 1989, Brems et al., 1990, Pikal et al., 1991, Katakam et al., 1995, Bam et al., 1998). Katakam and co-workers reported a 67% loss of hGH as insoluble aggregates following vortex mixing for 1 min (Katakam et al., 1995) and Bam et al. described the "nearly complete loss" of monomeric hGH over a 10 h period upon shaking an hGH solution in the absence of additives (Bam et al., 1998). The consequences of hGH aggregation with regard to formulation and delivery include precipitation of drug, possible increased immunogenicity upon injection (Moore, 1978) and a loss of biological activity, with one study reporting a dimeric form of hGH as "essentially inactive" in the rat bioassay (Becker et al., 1987).

1.3.2 Clinical Use

Prior to the mid-1980's, hGH for clinical use was obtained from human cadaver pituitary tissue, thus supply was generally limited and variable. Reports linking the pituitary-derived hGH to deaths from the virus infection Creutzfeld Jakob syndrome (CJS) led to its removal from the US market in 1985, an event coinciding with the timely development of recombinant hGH (Jorgensen, 1991, Lippe and Nakamoto, 1993). Recombinant DNA technology offered the advantage of producing a potentially unlimited supply of hGH, employing a genetically modified strain of *E. coli*, a process not involving the use of human tissue and therefore providing a product free of contaminating pathogens such as CJS (Pearlman and Bewley, 1993). Pituitary derived hGH and recombinant hGH possess identical amino acid sequences and consequently it has been demonstrated that the pharmacological effects and pharmacokinetics of the two are comparable (Jorgensen, 1991).

The primary clinical use of hGH is in the treatment of paediatric hypopituitary dwarfism, which arises as a result of the pituitary being unable to secrete adequate quantities of endogenous growth hormone (Lippe and Nakamoto, 1993, Pearlman and Bewley, 1993, Mehta and Hindmarsh, 2000). Diagnosis of the condition is complex and involves an evaluation of clinical features and biochemical investigations, including the stimulation of hGH release by agents such as arginine and insulin in order to assess pituitary function. Treatment typically consists of a daily dose of hGH (0.024 mg/kg, otherwise expressed as 0.071 IU/kg) administered by subcutaneous injection in the evening in order to mimic endogenous GH physiology (Mehta and Hindmarsh, 2000). Duration of therapy varies and may be determined according to the achievement of a particular target height, cessation of growth or fusion of the long bones (epiphyses). Early diagnosis is essential for successful treatment and can bring about normalised growth and the attainment of the target adult height in most patients.

Other accepted paediatric indications of hGH include the treatment of short stature due to chronic renal failure and Turner syndrome, a genetic disorder characterised by a missing or defective X chromosome in females (Mehta and Hindmarsh, 2000). Improvements have also been observed with hGH replacement therapy in the metabolic status of growth hormone deficient adults, although the long term benefits in this condition are unclear (Kearney and Johnston, 2000). Less conventional uses of hGH are many and varied and chiefly result from its metabolic effects promoting protein anabolism and lipid catabolism and include the treatment of obesity, aging, ovulation induction and critical injury e.g. severe burns (Lippe and Nakamoto, 1993). Further, the anabolic effects of hGH combined with difficulties in its detection by routine drug testing procedures have also lead to its widespread illicit use amongst elite athletes (Healy and Russell-Jones, 1997, Ehrnborg et al., 2000).

1.3.3 Current Formulation Approaches

Currently available commercial hGH preparations are designed exclusively for parenteral administration, presented as solutions or lyophilised powder for reconstitution and SC injection (e.g. Humatrope®) using either a conventional needle and syringe or a pen injection device such as HumatroPen™. As described in Section 1.1, the requisite long-term therapy, generally comprising daily subcutaneous injections of hGH over a period of several years, is an undesirable and unpleasant experience for the predominantly paediatric patient group (Verrips et al., 1998). In addition to poor patient acceptability, the costs associated with the manufacture of injectable drug formulations and the associated dosage administration costs surpass those of simpler and more convenient non-parenteral dosage forms such as oral tablets or capsules. Consequently, various studies have been conducted to investigate the feasibility of alternative drug delivery options for hGH including sustained-release depot injections (Cleland and Jones, 1996) and non-parenteral delivery methods including nasal (Baldwin et al., 1990, O'Hagan et al., 1990, Hedin et al., 1993, Kagatani et al., 1998), pulmonary (Colthorpe et al., 1995), colonic and oral administration (Moore et al., 1986, Hertz et al., 1991, Leone-Bay et al., 1996a, Dean et al., 1999).

The most convenient and acceptable non-parenteral route of drug delivery remains the oral route of administration. Studies have been conducted to assess the feasibility of hGH administration by this route, with the use of absorption enhancing compounds (Moore et al., 1986, Hertz et al., 1991) or specialised formulation approaches (Dean et al., 1999) employed in an attempt to overcome the formidable barriers of proteolytic enzymes and poor membrane permeability (discussed in Section 1.2.1).

The absorption enhancement of hGH from the rat gastrointestinal tract has been described by Moore and co-workers (Moore et al., 1986). Formulations containing hGH and one of salicylate, mineral oil or a combination of salicylate and mineral oil, were administered to the

stomach, duodenum, ileum and colon of male rats. Co-administration of hGH with the combined salicylate and mineral oil resulted in an increase in hGH bioavailability from 0.7 ± 0.4% to 7.0 ± 4.7% in the ileum and from 0.2 ± 0.2% to 9.5 ± 4.3% in the colon. While the mechanism of absorption enhancement was not defined, the authors speculated that altered intestinal permeability may have been responsible for the observed increase in bioavailability.

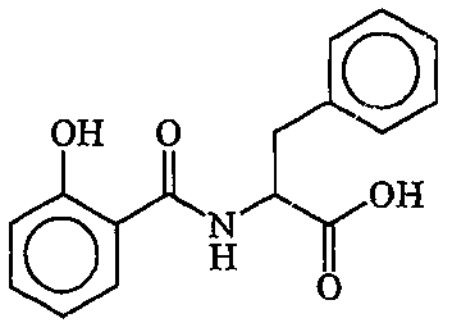
Studies by Dean and co-workers employed polymerised phospholipid liposomes as a vehicle for the oral delivery of hGH in mice (Dean et al., 1999). Serum hGH concentrations in mice administered 3 μg hGH subcutaneously and mice administered 30 μg hGH orally in polymerised liposomes were found to be comparable, while hGH was undetectable in the serum of control mice receiving oral hGH in buffer. The authors concluded that hGH formulated in polymerised liposomes was orally absorbed, demonstrating a bioavailability of approximately 10% relative to subcutaneous administration.

Recently, a series of novel carrier compounds developed by a New York biotechnology company (Emisphere Technologies, Inc.) was reported to enhance the oral bioavailability of hGH (Leone-Bay et al., 1996a). As discussed in Section 1.1, an investigation of the oral absorption of hGH in the presence of one of the Emisphere carriers formed the basis of the studies described in this thesis. The following section (Section 1.4) provides relevant background information regarding the development and use of this novel class of compounds, the physicochemical characteristics of SNAC-2 (the model carrier compound employed in these studies) and a brief review of *in vivo* studies previously conducted to demonstrate the utility of the carriers for improving the oral absorption of hGH and other macromolecules.

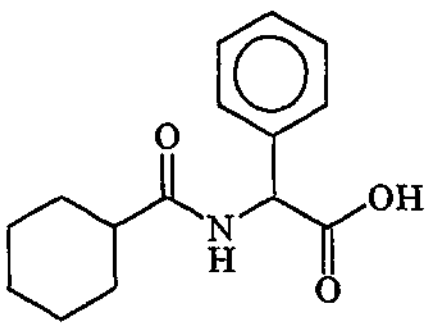
1.4 NOVEL CARRIER COMPOUNDS FOR IMPROVING THE ORAL BIOAVAILABILITY OF MACROMOLECULES

There is a growing body of literature describing the development by Emisphere Technologies, Inc. of a unique family of carrier compounds which have been shown to facilitate the absorption of macromolecules. The carriers are derivatised amino acids and have been demonstrated to improve the oral bioavailability of hGH (Leone-Bay et al., 1996a), human interferon-α (hIFN) (Leone-Bay et al., 1995b), salmon calcitonin (sCT) (Leone-Bay et al., 1995a, Leone-Bay et al., 1995b), parathyroid hormone (PTH) (Leone-Bay et al., 2001), heparin (Rivera et al., 1997, Leone-Bay et al., 1998a, Leone-Bay et al., 1998b) and other macromolecules in rats and primates.

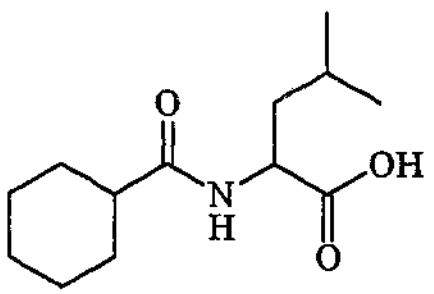
Research by Steiner and Rosen in the late 1980's lead to the fortuitous discovery that microspheres prepared from thermally condensed α-amino acid mixtures could improve the oral delivery of drugs (Santiago et al., 1993, Leone-Bay et al., 1995b). This first generation of carriers, described as "proteinoids", proved difficult to isolate and subsequent studies employed more defined derivatised α-amino acids (Leone-Bay et al., 1995b, Leone-Bay et al., 1995a). A hydrophobic amino acid component of the early mixtures was found to be responsible for oral absorption enhancement of macromolecules and consequently, the development of additional carriers was aimed at synthesising hydrophobic amino acid derivatives. Figure 1.1 depicts three representative structures from the second carrier series, with both the chemical name and designated abbreviations (where used) listed for each. Where naturally occurring amino acids have been utilised for derivatisation, those possessing the more hydrophobic side chains were chosen, including phenylalanine and leucine, which appear in HPA and N-cyclohexanoylleucine, respectively.



N-salicyloylphenylalanine *(HPA)*



N-cyclohexanoylphenylglycine



N-cyclohexanoylleucine

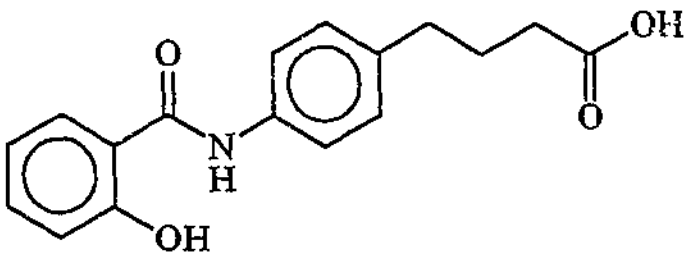
Figure 1.1 Structures of selected derivatised α-amino acids (second carrier series).

Figure 1.2 illustrates selected carriers from the third series, the derivatised non-α-amino acids, of which SNAC-2 (the model carrier examined in the current study) is a member. The incorporation of hydrophobic moieties into the carriers of this series included the 8-carbon acid side chain present in SNAC and 4-MOAC, while additional phenyl groups linked to 4-carbon acid side chains are found in SABA and 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid. Extensive *in vivo* studies have been conducted in rats and primates to demonstrate the utility of these and other closely related novel carriers for effecting the oral delivery of macromolecules, with a heparin/SNAC combination currently undergoing human clinical trials (Baughman et al., 1998). Results of *in vivo* studies published by Emisphere are described in Section 1.4.2.

1.4.1 Physicochemical Characteristics of the Carrier, SNAC-2

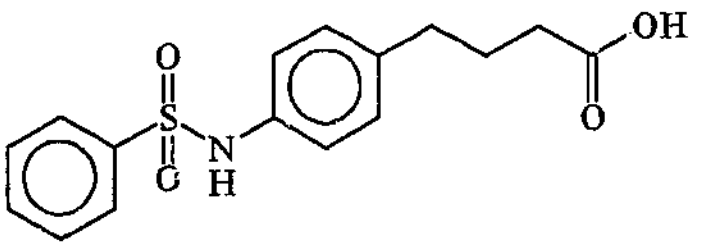
The general structure of the Emisphere novel carrier compound provided for use in these studies, SNAC-2, is illustrated in Figure 1.3 and is similar that of SNAC and 4-MOAC (see Figure 1.2 for structures of SNAC and 4-MOAC). The exact structure of SNAC-2 cannot be presented for confidentiality reasons. In the current studies, the sodium salt of SNAC-2 (MW 301.3) was employed owing to its more favourable water solubility relative to the free acid (>150 mg/mL compared to a predicted 0.9 mg/mL[1]). SNAC-2 has a pKa of 4.77[1], which reflects its weakly acidic nature, and a log D (pH 7.4) of 0.10[1]. Based upon the structure, SNAC-2 would be expected to self-associate in solution and studies to measure the CMC are presented in Section 6.3.3.

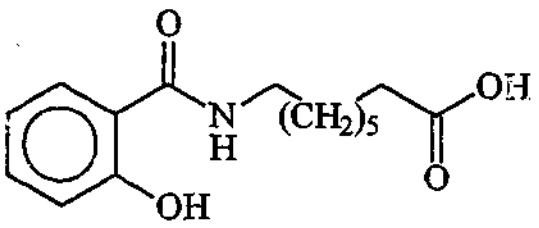
[1] physicochemical characteristics of SNAC-2 predicted using ACD/log D Suite software, version 4.55, Advanced Chemistry Development, Inc., Ontario, Canada.



N-salicyloyl-4-(4-aminophenyl) butyric acid *(SABA)*

4-(4-(N-phenylsulfonyl)amino phenyl)butyric acid





8-(N-salicyloyl)aminocaprylic acid *(SNAC)*

N-[8-(2-hydroxy-4-methoxy)benzoyl] amino caprylic acid *(4-MOAC)*

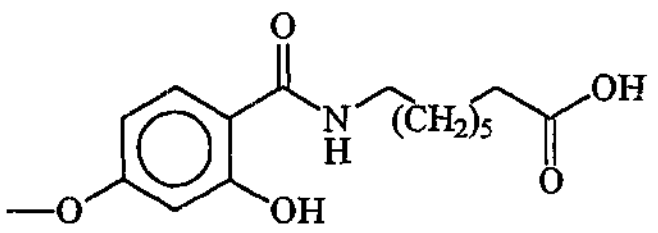


Figure 1.2 Structures of selected derivatised non-α-amino acids (third carrier series).

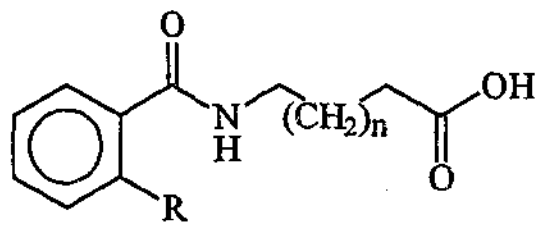


Figure 1.3 General structure of the novel carrier compound, SNAC-2.

1.4.2 Bioavailability Studies of Macromolecules in Rodents and Primates - Background Data

To date, a large number of novel carrier compounds have been synthesised and screened by Emisphere for use in promoting the absorption of various macromolecules. The following description of published background data is intended to provide an overview of *in vivo* studies, focussing on selected efficacious carriers (carrier structures have been presented in Figures 1.1 and 1.2), and is by no means an exhaustive review of all carriers investigated by Emisphere. Early studies employed carrier microspheres to improve oral drug delivery, although it was later observed that enhanced absorption could be achieved following dosing of drug as either microsphere suspensions or aqueous solutions, indicating that microspheres were not necessary for drug delivery (Leone-Bay et al., 1995b). Consequently, dosing solutions utilised in the studies described in this overview were simple aqueous solutions of drug and carrier administered as a single dose. It is also noteworthy that several of the results were acquired following intracolonic (IC) dosing in rats. IC dosing was frequently employed as it was found to be a suitable screening technique for oral drug delivery in the presence of carriers (Leone-Bay et al., 1996a).

A series of N-cyclohexanoyl acid amides, including N-cyclohexanoylphenylglycine, was evaluated for use in enhancing the oral absorption of hIFN (20 kDa protein drug used in the treatment of hepatitis and hairy cell leukaemia) in rats. Table 1.1 presents data obtained in rats following the oral administration of hIFN with selected carrier compounds. N-cyclohexanoylphenylglycine was found to consistently produce the greatest peak serum concentrations of hIFN (approximately 3800 pg/mL) in 118 rats. Based on the efficacy of N-cyclohexanoylphenylglycine in rodents, it was used to demonstrate oral delivery of hIFN in a primate model. Oral administration of hIFN/N-cyclohexanoylphenylglycine to cynomolgus monkeys lead to a C_{max} of around 3000 pg/mL. A series of derivatised phenylglycines was

Table 1.1 Maximum serum concentration (C_{max}) data obtained following the oral administration of a single dose of hIFN with selected novel carrier compounds to rats (Leone-Bay et al., 1995b). Carrier structures for N-cyclohexanoylphenylglycine and N-cyclohexanoylleucine are presented in Figures 1.1 and 1.2.

Carrier	*Carrier dose*	*hIFN dose*	*C_{max} (pg/mL)*[a]
nil	-	1 mg/kg	688 ± 173
N-cyclohexanoylphenylglycine	800 mg/kg	1 mg/kg	3808 ± 3203
N-cyclohexanoylphenylglycine	400 mg/kg	500 μg/kg	2598 ± 1423
N-cyclohexanoylphenylglycine	800 mg/kg	1 mg/kg	2988 ±1179[b]
N-(benzoylphenyl)glycine	800 mg/kg	1 mg/kg	8427 ± 2838
N-cyclohexanoylleucine	800 mg/kg	1 mg/kg	1124 ± 763

[a] mean ± SD
[b] experiments conducted in monkeys

prepared next, with N-(benzoylphenyl)glycine producing the highest peak concentration of hIFN (approximately 8400 pg/mL) from this group of carriers. Notwithstanding species differences, it is likely that the measured hIFN serum concentrations from these studies represent therapeutic levels, as it has previously been determined that clinically relevant hIFN levels in humans range from 90-580 pg/mL (Leone-Bay et al., 1995b).

N-cyclohexanoylleucine, another N-cyclohexanoyl derivative, was tested for use with hIFN in rats and was observed to produce a C_{max} of less than a third of that produced by N-cyclohexanoylphenylglycine at the same drug/carrier dose. However, this carrier proved to be more efficient for promoting the oral delivery of sCT, a 3500 Da polypeptide hormone involved in the regulation of blood calcium concentration. Oral administration of N-cyclohexanoylleucine in conjunction with sCT to rats lead to a peak reduction in serum calcium levels of approximately 22%, the most significant effect of the range of carriers examined for use with this macromolecule. A reduction in serum calcium could not be measured following the administration of unformulated sCT. Interestingly, N-cyclohexanoylleucine was also shown to be effective in improving the oral absorption of the significantly smaller anti-allergy agent, cromolyn sodium (MW 468), increasing the oral bioavailability of cromolyn in rats from less than 0.5% to 4.6% (Leone-Bay et al., 1996c).

Emisphere have conducted extensive studies on the carrier mediated oral absorption of heparin. Heparin is a 20 kDa mucopolysaccharide employed in the prevention and treatment of deep vein thrombosis (DVT) and pulmonary embolism. The anticoagulant effect of heparin is monitored using the activated partial thromboplastin time (APTT) assay, a determination of blood clotting time. Therapeutic heparin levels are reached when the APTT is 1.5-2.5 times that of baseline. Table 1.2 describes the APTT values obtained following the oral administration of heparin with selected carriers. The APTT values are all significantly above baseline (baseline values are approximately 20 sec in rats and monkeys) and also well above the therapeutic range.

Table 1.2 APTT values obtained following the oral administration of a single dose of heparin with selected novel carrier compounds to rats (Rivera et al., 1996, Rivera et al., 1997, Leone-Bay et al., 1998a, Leone-Bay et al., 1998b). Carrier structures are presented in Figures 1.1 and 1.2.

Carrier	*Carrier dose*	*Heparin dose*	*APTT (sec)*[a]
nil	-	nil	~21
nil	-	100 mg/kg	25.0 ± 1.7
SNAC	300 mg/kg	nil	23.5 ± 0.5
SNAC	300 mg/kg	100 mg/kg	101.9 ± 12.0
SNAC	50 mg/kg	25 mg/kg	125 ± 24[b]
SNAC	150 mg/kg	15 mg/kg	80 ± 54[c]
SABA	300 mg/kg	100 mg/kg	129 ± 50
4-MOAC	50 mg/kg	25 mg/kg	76 ± 16[b]

[a] mean ± SEM
[b] data obtained following intracolonic drug administration
[c] experiments conducted in monkeys

The oral administration of heparin together with either SNAC or SABA in rats produced comparable APTT values, and oral heparin/SNAC in primates led to an APTT four times higher than control, equating to a bioavailability of 8.3% relative to SC administration. IC dosing to rats with heparin and either SNAC or 4-MOAC showed SNAC to be a more efficient carrier of the anticoagulant, although 4-MOAC still increased the APTT above the target therapeutic range. Evaluation of the heparin/SNAC combination in a rat model of DVT indicated that effective treatment of the condition was achieved with orally administered heparin/SNAC (Gonze et al., 2000). Furthermore, clinical trials conducted in healthy male human volunteers confirmed that anticoagulant doses of heparin can be delivered to humans by oral heparin/SNAC (Baughman et al., 1998).

The third generation carriers, 4-MOAC and SNAC, were screened for use with parathyroid hormone (PTH), a 9600 Da polypeptide indicated in the treatment of osteoporosis (Leone-Bay et al., 2001). Significant serum levels of PTH were obtained in rats following the oral administration of PTH combined with either 4-MOAC or SNAC, and oral dosing of PTH/4-MOAC in a primate model resulted in a bioavailability of 2.1% relative to SC administration. Additionally, the biological activity of PTH after oral dosing of PTH/4-MOAC was successfully demonstrated in a rat model of osteoporosis, thereby confirming the feasibility of PTH oral delivery.

The use of various compounds from each of the three generations of Emisphere carriers has been investigated for improving the oral delivery of hGH (Leone-Bay et al., 1996a, Carozza et al., 1997), with selected data from these studies presented in Table 1.3. Soy hydrolysate, which contains a mixture of poorly defined α-amino acids, was administered orally to rats at a dose of 1400 mg/kg combined with 1 mg/kg hGH, resulting in maximum hGH serum concentrations of approximately 19 ng/mL. The second generation α-amino acid derivative, HPA, at a slightly lower carrier dose (1200 mg/kg) and ten-fold lower hGH dose (1 mg/kg), produced

Table 1.3 Maximum serum concentrations (C_{max}) obtained following the oral administration of a single dose of hGH with selected novel carrier compounds to rats (Leone-Bay et al., 1996a, Carozza et al., 1997). Carrier structures are presented in Figures 1.1 and 1.2.

Carrier	*Carrier dose*	*hGH dose*	*C_{max} (ng/mL)*[a]
Nil	-	6 mg/kg	ND[b]
Nil	-	1 mg/kg	ND[b,c]
Soy hydrolysate	1400 mg/kg	10 mg/kg	19 ± 4
HPA	1200 mg/kg	1 mg/kg	107 ± 13
SABA	300 mg/kg	6 mg/kg	49.7 ± 13.5
SABA	25 mg/kg	1 mg/kg	64.7 ± 5.5[e]
SABA	800 mg/kg	6 mg/kg	57[d,e]
4-(4-(N-phenylsulfonyl)amino phenyl)butyric acid	500 mg/kg	6 mg/kg	28.0 ± 20.3
4-(4-(N-phenylsulfonyl)amino phenyl)butyric acid	250 mg/kg	6 mg/kg	2.6 ± 3.1

[a] mean ± SD
[b] not detected
[c] data obtained following intracolonic drug administration
[d] experiments conducted in monkeys
[e] mean reported only

hGH serum levels in excess of 100 ng/mL in rats with a resulting bioavailability of 9.8% relative to SC administration. SABA, a third generation carrier, was identified as a lead compound from a series of seventy non-α-amino acid carriers tested for enhancing the oral absorption of hGH in rats (Leone-Bay et al., 1996a). SABA was observed to produce a C_{max} for hGH of around 50 ng/mL following the oral administration of 6 mg/kg hGH together with 300 mg/kg SABA giving an hGH bioavailability of 4.85% relative to SC administration. It is of interest that the second and third generation carriers were much more efficient than the soy hydrolysate (first generation carriers) for facilitating hGH oral delivery (Carozza et al., 1997).

The bioavailability of hGH following IC dosing of 1 mg/kg hGH and 25 mg/kg SABA was 28.5%. In comparison, oral administration of 6 mg/kg hGH in combination with 250 and 500 mg/kg of the carrier, 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid, gave rise to 2.6 ng/mL and 28 ng/mL, respectively, indicating that this carrier is significantly less efficient than SABA in enhancing hGH oral absorption in rats. SABA was subsequently employed to study the oral delivery of hGH in a second species, with oral administration of hGH/SABA to cynomolgus monkeys producing hGH serum levels of up to 57 ng/mL (Leone-Bay et al., 1996a, Carozza et al., 1997).

From the *in vivo* studies conducted thus far by Emisphere, it is evident that the carrier compounds can significantly enhance the oral absorption of a number of macromolecules. Structure activity relationships (SAR) have been proposed in relation to the different carrier structures and their varying efficiencies for improving the delivery of individual drugs (Leone-Bay et al., 1995b, Leone-Bay et al., 1996a, Leone-Bay et al., 1998a, Leone-Bay et al., 1998b, Leone-Bay et al., 2001), with a specific drug/carrier interaction speculated to be the mechanism responsible for the carrier-mediated absorption enhancement (Leone-Bay et al., 1996a, Milstein et al., 1998). The current studies cover the investigation of one carrier only and, consequently, carrier SAR will not be further addressed in this dissertation, but potential mechanisms of hGH

absorption enhancement in the presence of the model carrier, SNAC-2, and means by which these mechanisms may be explored, are described in the following section.

1.5 POSSIBLE MECHANISM(S) OF INCREASED ORAL BIOAVAILABILITY AND METHODS FOR PROBING THESE MECHANISMS

It was hypothesised that there are three mechanisms by which the oral bioavailability of protein drugs may be increased: proteolytic enzyme inhibition, altered intestinal permeability characteristics and/or enhanced intestinal lymphatic transport. The mechanism(s) by which the Emisphere model carrier, SNAC-2, promotes the oral absorption of hGH remain to be elucidated, although it has been suggested that a carrier-induced altered protein conformation, which is more readily able to traverse lipid membranes, may be responsible for the observed oral absorption enhancement of macromolecules by carrier compounds (Milstein et al., 1998).

1.5.1 Inhibition of Proteolytic Enzymes

Degradation of proteins by proteolytic enzymes constitutes a formidable barrier to the oral delivery of protein drugs, as discussed in Section 1.2.1.1. Since coadministration of enzyme inhibitors with peptide and protein drugs can improve the intestinal absorption of these drugs (Ziv et al., 1987, Owens et al., 1988, Ushirogawa et al., 1992, Bai and Chang, 1996), it was hypothesised that carriers could function as enzyme inhibitors, thereby promoting the oral absorption of hGH.

Studies conducted by Emisphere on N-cyclohexanoylleucine and N-cyclohexanoylphenylglycine (see Figure 1.1), effective carriers for sCT and hIFN, respectively, showed that the carriers exhibited approximately 200-fold less enzyme inhibitory activity against trypsin compared to Bowman Burke inhibitor, a known inhibitor of trypsin (Leone-Bay et al.,

1995b). Based upon the minimal enzyme inhibitory activity demonstrated by N-cyclohexanoylleucine and N-cyclohexanoylphenylglycine in this study, it is likely that the Emisphere carriers act through an alternate mechanism to facilitate oral absorption.

In the current studies, the hypothesis of enzyme inhibition was addressed indirectly, with stability studies and the assessment of the potential for enzymatic degradation essential components of the establishment and validation of the *in situ* and *in vitro* models for assessing hGH intestinal permeability (Chapters Four and Five).

1.5.2 Conformationally Altered Form

There is strong evidence to suggest that partially folded protein conformations, sometimes referred to as 'molten globules' (MG), are capable of translocating lipid membranes (Bychkova et al., 1988, Banuelos and Muga, 1995, Lala et al., 1995, Mach and Middaugh, 1995, Johnston et al., 1998). While the MG state retains a reasonably compact, ordered secondary structure, it lacks rigid tertiary structure, with the exposure of more hydrophobic amino acids and the accompanying alterations in surface charge characteristics providing an environment more likely to undergo favourable interactions with lipid membranes. Bychkova et al. reviewed evidence for the involvement of the non-native state of proteins in their membrane translocation, citing the translocation of incompletely folded proteins, the binding of partially denatured proteins to model membranes and spectroscopic evidence that the "partly unfolded" or "molten globule" states exhibit native-like secondary structure, but lack rigid tertiary structure (Bychkova et al., 1988). The authors argued that the involvement of the partly unfolded state with membrane translocation was "consistent with the observed phenomena". More recently, Mach and Middaugh described the binding of a "molten globule-like state" of acidic fibroblast growth factor (aFGF) to negatively charged phospholipid vesicles and suggested that this interaction may play a part in the membrane

permeation of aFGF (Mach and Middaugh, 1995). Similar observations were made by Johnston and co-workers, who demonstrated the enhanced transport of guanidine-unfolded human basic fibroblast growth factor across rabbit buccal mucosa (Johnston et al., 1998).

Microcalorimetry studies have demonstrated a reduction in the transition temperature (T_m) of proteins in the presence of the carriers which is indicative of protein destabilisation (Leone-Bay et al., 1996b, Milstein et al., 1998). Milstein et al. observed varying reductions in the T_m of hGH in the presence of selected carriers, with the more effective carriers *in vivo* producing a greater reduction in the T_m value *in vitro*. Similar results have been described for hIFN. Based on the microcalorimetry data, Milstein proposed that a carrier-induced alteration in conformation is responsible for the observed oral absorption enhancement. Consequently, it was hypothesised that the improved ability of a partially folded hGH conformation in the presence of the model carrier, SNAC-2, may be responsible for the increased hGH permeation.

1.5.2.1 Techniques Used to Monitor Protein Conformation

Spectroscopic techniques, including circular dichroism (CD), UV absorbance, Fourier transform infrared (FTIR), fluorescence and NMR have been extensively reviewed for use in monitoring protein conformation (Greenfield, 1996, Jung, 2000, Ferentz and Wagner, 2000, Eftink, 2000, Pelton and McLean, 2000). These techniques offer a sensitive means of examining protein conformation and conformational changes and provide valuable information regarding protein stability under a variety of solution conditions. Advantages of spectroscopic studies include small sample requirements and the capability of selected techniques (e.g. CD spectroscopy) to monitor both the secondary and tertiary structure of proteins. The use of CD spectroscopy is described in detail in Chapter Three, since this technique was employed in the current studies for monitoring hGH conformation in the absence and presence of the carrier.

Microcalorimetry is a highly sensitive analytical technique for monitoring protein conformational stability, and it is commonly used as an alternative to spectroscopic measurements. It allows for the direct determination of thermodynamic parameters associated with the conformational stability of proteins based on the analysis of temperature-induced changes in partial heat capacity. Valuable information may be obtained regarding the nature of protein/excipient interactions: for example, a reduction in the thermal transition temperature (T_m) is indicative of reduced conformational stability. While microcalorimetry provides a well-established method for evaluating additive effects on protein conformational stability (Boctor and Mehta, 1992, Bell et al., 1995, Jain and Ahluwalia, 1996, Ghirlando et al., 1999, Hendrix et al., 2000) and also the practical advantage of minimal sample requirements, it does not allow the assessment of protein conformation and conformational changes.

An alternative approach is the investigation of the interaction of altered protein conformations with model membranes by examining the association of the protein of interest with phospholipid vesicles (liposomes) in solution. Such studies typically employ spectroscopic techniques, such as fluorescence spectroscopy, as an indirect means to detect and characterise protein-membrane complexes (Lala et al., 1995, Banuelos and Muga, 1995). Alternatively, the leakage of a fluorescent probe from phospholipid vesicles following their exposure to partially folded proteins in solution may be used as a measure of lipid bilayer disruption resulting from the protein/membrane interaction (Mach and Middaugh, 1995).

An interesting variation of this technique was recently reported by Shin et al., who described the use of a flotation-gradient centrifugation method for assessing the binding of acetylcholinesterase (AChE) to dimyristoylphosphatidylcholine liposomes (Shin et al., 1996). Liposomes were incubated with either native AChE or guanidine unfolded AChE and subsequently loaded onto a sucrose gradient and centrifuged in an ultracentrifuge. It was observed that native AChE stayed at the bottom of the gradient, while guanidine unfolded AChE floated to

the top together with the liposomes, suggesting that the unfolded protein associated with the liposomes. Feasibility studies to investigate the use of this method for examining the interaction of hGH with a model membrane in the absence and presence of SNAC-2 are presented in Chapter Three.

1.5.3 Changes in Membrane Permeation

Studies to address the hypothesis of altered intestinal permeation of hGH in the presence of the model carrier, SNAC-2, being responsible for the observed oral absorption enhancement of hGH constituted the major component of the work described in this thesis. Improved oral absorption of protein drugs achieved via strategies such as the use of absorption enhancing compounds or chemical modification is known to be a direct consequence of increased membrane permeation. Simplistically, increased intestinal permeation of protein drugs may occur by two means: firstly, as a result of altered physicochemical characteristics of the drug itself, or secondly, as a result of a non-specific alteration to the barrier properties of the intestinal membrane. The use of conventional absorption enhancers, such as sodium caprate, is an example of the latter, while increased lipophilicity conferred on appropriately chemically modified proteins is an example of the former.

Mlynek and co-workers described the intestinal permeation of ^{125}I-hGH in the presence of the Emisphere carrier, 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid (referred to as E198 by Mlynek), reporting a two-fold increase in ^{125}I-hGH permeability in rabbit duodenum upon the addition of 150 mg/mL 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid (Milstein et al., 1998, Mlynek et al., 2000). 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid has been previously demonstrated to improve the oral absorption of hGH in rats following oral administration (see Table 1.1), although not as efficiently as other carriers, such as SABA. An essential consideration

in such *in vitro* studies is the potential for *in vitro* tissue damage to artefactually contribute to measurements of increased permeation. However, since the permeability of well-defined marker compounds was unchanged in the presence of 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid and histological assessments showed no abnormalities, Mlynek suggested that the increased permeation of ^{125}I-hGH was not likely to be the result of tissue damage. In a separate series of studies conducted using cultured cell monolayers (Caco-2), Wu and Robinson examined the permeability of ^{125}I-hGH in the presence of the Emisphere carriers, SNAC (37.5 mg/mL, referred to by Wu and Robinson as E414) and SABA (25 mg/mL, referred to by Wu and Robinson as E352), observing twelve– and nine-fold increases, respectively, in the P_{app} value of ^{125}I-hGH (Wu and Robinson, 1999a, Wu and Robinson, 1999b).

There are a variety of *in situ* and *in vitro* techniques that may be employed to examine intestinal permeability including the rat *in situ* intestinal perfusion model and *in vitro* models incorporating either isolated tissues or Caco-2 cell monolayers. Following is an overview of these methods, their advantages and disadvantages and the manner in which they may be employed to examine intestinal permeation and mechanisms of permeation.

1.5.3.1 In Situ Rat Intestinal Perfusion

The *in situ* rat intestinal perfusion model is a well-established method for evaluating intestinal permeability (Schurgers et al., 1986, Stewart et al., 1995, Fagerholm et al., 1996, Lennernas, 1997, Stewart et al., 1997). Previous studies have demonstrated that intestinal permeabilities measured using the single pass *in situ* rat intestinal perfusion model are predictive of human intestinal absorption, irrespective of transport mechanisms (Amidon et al., 1988).

The surgical procedures for this method typically involve the isolation and cannulation of a segment of gut and may also include the cannulation of the jugular and/or portal veins to facilitate blood sampling (Griffiths et al., 1996). A solution containing the drug of interest

(perfusate) is pumped through the isolated gut segment of the anaesthetised rat, and the loss of drug from perfusate monitored over time, thereby allowing the calculation of the effective permeability coefficient of the drug. Intestinal viability/damage may be monitored by measuring the release of markers of cell damage, such as LDH, and histological assessments (Swenson et al., 1994). Furthermore, altering the perfusate composition by modifying drug concentration, solution pH or the inclusion of additives can provide an assessment of the effect of such variables on intestinal permeability.

Advantages of the *in situ* model over *in vitro* techniques include the capability to study absorption in the whole animal and to differentiate between absorption to the blood of the systemic circulation and the hepatic portal blood, while the intact blood supply aids gut viability within the *in situ* preparation. However, there are some practical disadvantages, as surgery for this model may be relatively complex and additionally, only a single permeability measurement can be obtained from each animal.

Studies to investigate the use of the *in situ* rat intestinal perfusion model for determining the intestinal permeability of hGH in the presence of the model carrier, SNAC-2, are described in Chapter Four.

1.5.3.2 Caco-2 Cell Monolayers

The permeability of passively transported drugs in Caco-2 cell monolayers, a differentiated human intestinal cell-line derived from colorectal carcinoma, has been demonstrated to correlate well with the *in vivo* absorption of drugs (Artursson et al., 2001) and consequently, this *in vitro* model has found increasing use in studies evaluating drug absorption and absorption mechanisms in the absence and presence of absorption enhancing compounds (Anderberg and Artursson, 1993, Anderberg et al., 1993, Hurni et al., 1993, Tomita et al., 1996, Batrakova et al., 1998, Duizer et al., 1998, Kotze et al., 1998, Lindmark et al., 1998). In addition, the use of Caco-

2 cells for determining the permeability of hGH in the presence of carriers has been described previously (Wu and Robinson, 1999a, Wu and Robinson, 1999b).

The experimental determination of permeability using Caco-2 monolayers is relatively straightforward. Cell monolayers are placed into diffusion chambers and bathed in physiological, oxygenated buffer maintained at 37°C. The drug of interest is spiked into the apical (donor) chamber and the flux of drug into the basolateral (receiver) chamber measured over a period of approximately one to two hours. The permeability coefficient is directly related to drug flux and is corrected for the initial drug concentration in the donor chamber, the volume of liquid in the half-chamber and the surface area for absorption. The integrity of the monolayer is generally confirmed either through the use of a marker compound such as ^{14}C-mannitol, or the measurement of transepithelial electrical resistance (TEER). These experiments can provide valuable information regarding permeation mechanisms such as the route of permeation (paracellular or transcellular) and the possible involvement of active transport processes.

As well as not requiring the use of animals, the greater viability of cultured cell monolayers relative to that of isolated tissues used in modified Ussing chamber experiments (described in Section 1.5.3.3) is a significant advantage of this model.

1.5.3.3 Modified Ussing Chamber

In contrast to *in vitro* studies employing Caco-2 cells, the modified Ussing chamber method, originally developed in the early 1950s by Ussing and Zerahn for investigating the electrical properties of frogskin (Ussing and Zerahn, 1950), utilises isolated intestinal tissues for permeability measurements. The modified Ussing chamber technique has found widespread use in the study of intestinal permeability, with a number of different animal models employed, including rat (Yodoya et al., 1994, Ungell et al., 1998, Uchiyama et al., 1999), rabbit (Grass and Sweetana,

1988, Smith et al., 1988, Swaan et al., 1994), monkey (Grass and Sweetana, 1989, Jezyk et al., 1992) and human (Soderholm et al., 1998).

Permeability experiments using the modified Ussing chamber model are conducted in a similar manner to that described for Caco-2 cells (Section 1.5.3.2), and as such provide similar information regarding absorption mechanisms. However, tissue freshly obtained from animals and humans provides the most realistic model of intestinal absorption, especially with regard to factors including the presence of intestinal mucus and transporter systems, both of which may be absent or altered in cultured cell monolayers. Consideration of tissue viability imposes some limitations, such as the length of the experimental time period over which permeability may be measured. For example, incomplete recovery of tissue observed following exposure to an absorption-enhancing compound may be due to a time dependent recovery process, which is longer than that which can be monitored for a viable tissue.

The establishment of a modified Ussing chamber model incorporating rabbit intestinal tissue for assessing hGH permeation and the use of this model to examine absorption mechanisms in the presence of the model carrier, SNAC-2, are described in Chapters Five and Six.

1.5.4 Enhanced Lymphatic Uptake

The final hypothesis regarding the mechanism of oral absorption enhancement of hGH by the model carrier, SNAC-2, was that of increased intestinal lymphatic transport. It is known that small quantities of intact proteins are taken up into the intestinal lymphatics following oral protein administration (Rubas and Grass, 1991). This has been demonstrated to occur via transcytosis through gut-associated lymphoid tissue (Peyer's patches) (Keljo and Hamilton, 1983) and may also theoretically occur via the paracellular pathway, although this is unlikely to be significant in the absence of absorption enhancers (Muranishi et al., 1997). Absorption of

macromolecules into the lymphatic system is severely limited by extensive enzymatic degradation, molecular size and hydrophilicity, coupled with an inability to readily pass the significant barrier of the small intestine. Consequently, lymphatic uptake of enterally administered proteins and peptides is generally only around 0.1% (Muranishi et al., 1997).

Strategies to target macromolecules to the intestinal lymphatics have included the concomitant use of absorption enhancing compounds, the development of prodrugs and the use of novel formulation approaches (e.g. mixed micelles and nanoparticulates) (Porter, 1997). For example, the oral administration of a 10 mg dose of ovalbumin (MW 45 kD) to conscious rats resulted in the appearance of 0.00071% of the dose in lymph, with lymphatic uptake increased (although only very slightly) to 0.0018% through the use of a liposome formulation (Tsume et al., 1996). The mechanism of enhanced uptake was attributed to protection of the ovalbumin from enzymatic degradation in the gut.

The unconscious lymph cannulated rat described in detail by Porter and Charman provides an established small animal model capable of readily assessing the lymphatic transport of drugs (Porter and Charman, 1996). Briefly, the duodenum, mesenteric lymph duct and jugular vein (or carotid artery) of a male Sprague-Dawley rat are cannulated in order to facilitate dosing and sampling of lymph and blood, respectively. Dosing solutions are infused into the duodenum, after which sampling of blood and lymph may be conducted in the anaesthetised animal. By and large, the mesenteric lymph duct collects all lymph leaving the small intestine and therefore analysis of drug in the collected lymph samples permits the calculation of the total amount of drug absorbed via the intestinal lymphatics. Studies conducted using the triple cannulated rat model to assess the potential for hGH intestinal lymphatic transport in the presence of the model carrier, SNAC-2, are described in Chapter Two.

1.6 THESIS STRUCTURE

This thesis describes studies conducted to investigate the oral absorption enhancement of hGH in the presence of the model carrier, SNAC-2. The introductory chapter (Chapter One) has detailed the hypotheses to be addressed and has provided relevant background information regarding the means by which this may be accomplished. Chapter Two describes *in vivo* studies conducted in rats to demonstrate the effect of SNAC-2 on the oral absorption of hGH and the assessment of the possible contribution of intestinal lymphatic transport. The potential effect of SNAC-2 on the conformation of hGH is explored in Chapter Three. Chapters Four, Five and Six describe the establishment and use of an *in situ* model (Chapter Four) and an *in vitro* model (Chapters Five and Six) for the assessment of hGH permeation in the presence of SNAC-2. Finally, Chapter Seven provides a summary of the findings from this work and includes a discussion of possible future works that may be of benefit in the elucidation of carrier mechanisms.

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CHAPTER TWO

IN VIVO STUDIES TO INVESTIGATE THE ORAL ABSORPTION OF hGH

2.1 ORAL BIOAVAILABILITY OF hGH IN THE PRESENCE OF SNAC-2

2.1.1 Goals

As discussed in Section 1.4.2, numerous publications have described the use of Emisphere novel carrier compounds to facilitate the oral absorption of macromolecules including heparin (Leone-Bay et al., 1997, Rivera et al., 1997, Leone-Bay et al., 1998a, Leone-Bay et al., 1998b), salmon calcitonin (Leone-Bay et al., 1995), interferon (Milstein et al., 1998) and hGH (Leone-Bay et al., 19[illegible]one-Bay et al., 1996b). The model carrier provided for use in the current investigation, SNAC-2, is one of a series of Emisphere carriers known to improve the oral bioavailability of [illegible] (Leone-Bay et al., 1996a). The specific goal of the *in vivo* rat study presented in Section 2.1 was to demonstrate the utility of SNAC-2 for enhancing the absorption of hGH following oral administration.

The study involved the oral administration of a simple solution of either hGH in buffer or hGH combined with SNAC-2 in buffer to two parallel groups of male Sprague-Dawley rats. A third group of rats received a subcutaneous (SC) injection of a solution of hGH in buffer in order to demonstrate blood levels using the conventional route of hGH administration. The concentration of hGH in serum was determined using an ELISA.

2.1.2 Experimental

2.1.2.1 Materials

Human growth hormone (hGH) was a gift from Eli Lilly & Co. (Indianapolis, IN) and the model carrier, SNAC-2, was donated by Emisphere Technologies Inc. (Tarrytown, NY). hGH ELISA kits were purchased from Genzyme Diagnostics (San Carlos, CA) and horse serum was obtained from Gibco BRL, Life Technologies (Auckland, NZ). Sodium dihydrogen orthophosphate dihydrate and disodium hydrogen orthophosphate anhydrous were obtained from Ajax Chemicals (New South Wales, Australia). All other reagents were of at least analytical

grade or higher. Water was obtained from a Milli-Q (Millipore, Bedford, MA) water purification system and was used throughout.

2.1.2.2 Study design

The experimental procedures were approved and conducted in accordance with the guidelines from the Institutional Animal Experimentation Ethics Committee. The study involved three groups of animals, with 3-4 animals per group. One group was dosed orally with hGH (3 mg/kg) and SNAC-2 (300 mg/kg), one group was dosed orally with hGH (3 mg/kg) in buffer, and one group was dosed SC with hGH (0.125 mg/kg) in buffer. Formulation variables, such as the inclusion of additional excipients or variations in dose and dose volume, were not investigated in this brief series of experiments. Oral doses were based on previously determined effective doses of hGH with SNAC-2 (personal communication with Dr Sam Milstein, Emisphere Technologies, Inc.). Blood levels of hGH were determined using the ELISA, as described in Section 2.1.2.3.5.

2.1.2.3 Methods

2.1.2.3.1 hGH Solution Preparation

Lyophilised hGH was weighed into a polypropylene tube and dissolved in 5 mM phosphate buffer (pH 7.0) to give a protein concentration of approximately 8-9 mg/mL. Prior to use, the hGH solution was filtered through a 0.22 μm cellulose acetate syringe filter (Lida Corp., Kenosha, WI).

The concentration of hGH in aqueous stock solutions was determined by UV absorbance (Cary 3 spectrometer, Varian Instruments) using the baseline corrected maximum absorbance at 277 nm and a molar extinction coefficient of 17,700 $M^{-1}cm^{-1}$ (Pearlman and Bewley, 1993). Stock solutions were diluted to less than 1 mg/mL hGH to ensure that the

absorbance was in the linear range (approximately 0.1 to 1.0 mg/mL). The absorbance reading at 340 nm was subtracted from that measured at 277 nm in order to correct for possible light scattering errors (Winder and Gent, 1971) which were found to be minimal in these studies.

hGH stock solutions were stored under refrigerated conditions (4-8°C) for a maximum of two weeks. Previous studies conducted in our laboratories have shown that degradation of hGH is minimal under these conditions (S.A. Charman and W.N. Charman, unpublished results). Oral dosing solutions containing hGH (1.25 mg/mL) with or without SNAC-2 (125 mg/mL) were freshly prepared for each experiment by appropriate dilution of hGH and SNAC-2 stock solutions (described in Section 2.1.2.3.2) with 5 mM phosphate buffer. Subcutaneous dosing solutions were prepared in a similar manner and contained 0.075 mg/mL hGH.

2.1.2.3.2 SNAC-2 Solution Preparation

Stock solutions of SNAC-2 (150 mg/mL) were prepared using the sodium salt (MW 301.3), with the concentration of solutions reported as the mass of the acid per mL. SNAC-2 was prepared in 5 mM phosphate buffer and adjusted to a pH of approximately 8.0 with 1 M NaOH. The solution was sonicated for 15-20 min to aid the dissolution of the carrier.

2.1.2.3.3 Surgical Method

Surgery was performed on the day prior to dosing on fasted male Sprague-Dawley rats (approximately 300 g). Animals were anaesthetised with isoflurane (3%), placed on a warming pad (37°C) to maintain body temperature, and the external throat area cleaned and shaved. A small incision was made just above the left clavicle and the exposed connective and adipose tissue pushed aside to isolate the carotid artery. The artery was clamped and ligated before inserting a previously prepared polyethylene cannula (PE-50 tubing, Bioscience, Victoria, Australia) and flushing with heparinised saline (2 U/mL, 1 mL). The cannula was then

exteriorised by tunnelling subcutaneously to emerge above the scapulae. Following surgery, animals were allowed to recover overnight, with normal grooming, drinking and sleeping behaviour resumed within 1 h of surgery. Rats were housed in cages fitted with swivel attachments to enable free movement while protecting the integrity and patency of the cannula.

2.1.2.3.4 Experimental Dosing Procedures

Dosing solutions were prepared by gently mixing together hGH and SNAC-2 stock solutions with 5 mM phosphate buffer, pH 7.9 (test solution) or hGH stock solution with 5 mM phosphate buffer, pH 7.9 (control solution) to provide a final hGH concentration of 1.25 mg/mL (test and control solutions) and a final SNAC-2 concentration of 125 mg/mL (test solution). A solution pH of 7.9 was required in order to prevent the precipitation of SNAC-2 at the relatively high concentrations utilised (stock solution concentration 150 mg/mL). Doses were 3 mg/kg hGH and 300 mg/kg SNAC-2 in a final volume of 720 µL. The subcutaneous dosing solutions were prepared in a similar manner, but were administered at a 24-fold lower dose of hGH (0.125 mg/kg) in a 0.5 mL volume.

For the oral administrations, rats were lightly anaesthetised with isoflurane to allow insertion of a 3″, 18G gavage tube. The dosing solution was drawn up into a syringe, attached to the gavage tube and the dose administered directly into the stomach. SC injections were given into the side of the abdomen, also following light anaesthesia with isoflurane.

The duration of each experiment was six hours, with blood samples (250 µL) withdrawn from the carotid cannula at time zero (pre-dose), 20, 40, 60, 90, 120, 180, 240, 300 and 360 min. Samples were immediately transferred to a polypropylene tube without anticoagulant, allowed to clot for 5-10 min and then centrifuged for 15 min in a microcentrifuge. The serum was subsequently separated, placed in a clean Eppendorf and stored at –20°C prior to

analysis for hGH by ELISA (described in Section 2.1.2.3.5). Following the final time point, animals were euthanased by a lethal injection of pentobarbitone (1 g/kg) via the carotid cannula.

2.1.2.3.5 hGH ELISA for the Determination of Serum Concentrations

A commercially available hGH ELISA kit was used to determine the concentration of hGH in rat serum samples. Standards supplied with the kit were prepared as directed and diluted further with horse serum if required. A quality control (QC) standard prepared in buffer and diluted with horse serum to give a final concentration of approximately 10 ng/mL was included with each assay. Rat serum samples were diluted a minimum of 1:2 with horse serum to fall within the range of the standard curve and also to give an equivalent response to that of samples in a matrix of pure horse serum.

The ELISA kit was a double antibody sandwich ELISA and was used as per the manufacturer's instructions. Briefly, the assay involved the addition of 50 µL of sample (or standard) followed by 100 µL of enzyme conjugate reagent (mouse monoclonal anti-hGH antibody conjugated to horseradish peroxidase) to a microwell plate coated with capture antibody (sheep polyclonal anti-hGH antibody). The plate was then gently shaken to ensure thorough mixing and incubated for 45 min at ambient temperature. The liquid contents of the plate were removed by inversion and the plate washed five times with water using a Nunc-Immuno Wash 8 plate washer (Nunc, Roskilde, Denmark). A volume of 200 µL of a 1:1 mixture of hydrogen peroxide in acetate buffer and 3,3',5,5'-tetramethylbenzidine (TMB) in methanol, DMSO and glycerol (concentrations of each not specified by the manufacturer) was added to each well and the plate incubated in the dark for 15 min resulting in the development of a blue colour. Following this incubation period, the enzyme reaction was quenched by the addition of 50 µL of 2 M hydrochloric acid and the intensity of the resulting yellow colour read at 450 nm using a Titertek® Multiskan MC plate reader (Helsinki, Finland). The concentration of hGH was

proportional to the colour intensity of the sample over the range of the calibration curve (1-30 ng/mL).

Calibration curves were constructed over the range of 1 to 30 ng/mL by plotting the absorbance at 450 nm versus hGH concentration. Sigmaplot for Windows software Version 4.00 (SPSS Inc., Chicago, IL) was used to fit a three parameter sigmoidal function (Equation 2.1.1) to the data:

$$A_{450} = a \times \frac{(x/c)^b}{1 + (x/c)^b} \qquad (2.1.1)$$

where A_{450} is the absorbance at 450 nm, a is the asymptotic maximum, b is the slope parameter, c is the hGH concentration at the inflection point and x is the concentration of hGH. The concentration of hGH in test samples (x) was then calculated using Equation 2.1.2 (Equation 2.1.1 solved for x):

$$x = \left(\frac{c^b \times A_{450}}{a - A_{450}} \right)^{1/b} \qquad (2.1.2)$$

Intra-assay precision (expressed as the % coefficient of variation, CV) was evaluated by analysing hGH standards in triplicate, while inter-assay precision was assessed by analysing standards over three separate days. Accuracy was determined by back calculation of the standard concentration, which was then compared to the known concentration of the standard. The limit of quantitation (LOQ) for the ELISA was taken to be the lowest standard concentration (1.5 ng/mL). Blank rat serum was analysed to demonstrate the absence of any cross-reacting species. The response of samples prepared in rat serum versus horse serum was evaluated by diluting hGH of a known concentration (in 5 mM phosphate buffer) with 100% rat serum, 100% horse serum, 50:50 rat serum:horse serum or 25:75 rat serum:horse serum and comparing the results for the different matrices. Possible interference with the ELISA by SNAC-2 present in the matrix was investigated by diluting the reference standards with SNAC-2 to give a final

concentration of 200 µg/mL (estimated maximum serum concentration of carrier) and comparing the result to that obtained when the standards were diluted with blank matrix.

The QC samples were stored at -20°C along with the standards and samples and were included in each assay. The performance of the QC samples over time was used as an indication of hGH stability at -20°C. Individual runs were accepted if the results for QC samples were within ± 20% of the known concentration.

The short-term stability of hGH in rat serum was evaluated by spiking freshly collected rat serum with approximately 40-50 ng/mL hGH and then monitoring the concentration of hGH over time. Fresh blood was collected by cardiac puncture into tubes containing no anticoagulant, allowed to clot for 5-10 min, centrifuged and the serum separated for immediate use in the experiment. The mixture was incubated at 37°C for 6 h and samples were withdrawn at time zero and then at 0.5, 1, 2, 3, 4 and 6 h and immediately frozen at -20°C prior to analysis. For analysis by ELISA, samples were rapidly thawed and assayed immediately as described above.

2.1.2.4 Data Analysis

The area under the serum concentration versus time curve (AUC) was calculated by the linear trapezoidal method between 0 and 360 min and was used to determine the effect of SNAC-2 on the serum levels of hGH following oral dosing. These calculations incorporated only serum concentrations which were greater than the assay LOQ (1.5 ng/mL). There were an insufficient number of time points in the terminal portion of the profiles to allow estimation of a terminal half-life or AUC to infinity values. Peak serum concentrations (C_{max}) were noted from the individual profiles for each animal.

Statistical comparisons for the effect of the carrier on the C_{max} and AUC values obtained for hGH following oral administration were conducted using a t-test, testing for

significance at α=0.05. The natural log of data exhibiting unequal variance was used for the t-test analysis. Results are reported as mean ± standard deviation (SD).

The bioavailability of orally administered hGH relative to subcutaneously administered hGH was estimated from the ratio of the dose corrected oral AUC to the dose corrected subcutaneous AUC.

2.1.3 Results and Discussion

2.1.3.1 Validation of the hGH ELISA

Intra-assay precision and accuracy data for the hGH ELISA are presented in Table 2.1.1 and inter-assay data are presented in Table 2.1.2. Intra-assay precision (% CV) was less than 14% for all concentrations within the calibration curve and accuracy generally fell within ±15% of the stated concentration. Inter-assay precision was less than 8% for all concentrations studied. A representative calibration curve generated using the hGH ELISA is presented in Figure 2.1.1.

It is essential when employing an ELISA that the sample matrix is free of cross-reacting antigens so as not to produce a high background absorbance, which will reduce the sensitivity of the assay. Reference standards supplied with the hGH ELISA were comprised of hGH and preservatives in horse serum (lyophilised) and it was therefore necessary to validate the assay with rat serum which was utilised in these studies. Blank rat serum was analysed as a negative control and resulted in a background absorbance comparable to that produced by blank horse serum, confirming the absence of any cross-reacting species.

As standards and QCs were prepared in horse serum, the response of standards in rat serum was evaluated and compared to the response obtained in horse serum. The hGH concentration measured in 100% rat serum was approximately 50% lower than that measured in 100% horse serum, while both 25% and 50% rat serum samples gave rise to the same response

Table 2.1.1 Intra-assay precision and accuracy data for the hGH ELISA. The assay was conducted as described in Section 2.1.2.3.5.

Nominal Serum Conc. (ng/mL)	*Measured Serum Conc. (ng/mL)*[a]	*Accuracy*[b] *(%)*	*Precision*[c] *(CV%)*
1.5	1.7	13.3	12.7
1.5	1.4	-6.7	
1.5	1.8	20.0	
3	3.2	6.7	5.0
3	3.1	3.3	
3	2.9	-3.3	
6	5.5	-8.3	7.1
6	5.7	-5.0	
6	6.3	5.0	
15	16.5	10.0	13.6
15	16.5	10.0	
15	12.9	-14.0	
30	29.0	-3.3	2.2
30	30.3	1.0	
30	29.6	-1.3	

[a] determined by back calculation of the concentration using Equation 2.1.2 as described in Section 2.1.2.3.5

[b] deviation from nominal concentration

[c] coefficient of variation for mean of n=3 measurements

Table 2.1.2 Inter-assay precision and accuracy data for the hGH ELISA. The assay was conducted as described in Section 2.1.2.3.5.

Nominal Serum Conc. (ng/mL)	*Measured Serum Conc. (ng/mL)*[a]	*Accuracy*[b] *(%)*	*Precision*[c] *(CV%)*
1.5	1.5	0.0	7.1
1.5	1.7	13.3	
1.5	1.7	13.3	
3	2.9	-3.3	0.0
3	2.9	-3.3	
3	2.9	-3.3	
6	6.2	3.3	3.3
6	5.8	-3.3	
6	6.0	0.0	
15	14.6	-2.7	5.3
15	16.2	8.0	
15	15.1	0.7	
30	30.4	1.3	4.5
30	27.8	-7.3	
30	29.5	-1.7	

[a] determined by back calculation of the concentration using Equation 2.1.2 as described in Section 2.1.2.3.5

[b] deviation from nominal concentration

[c] coefficient of variation for mean of n=3 measurements

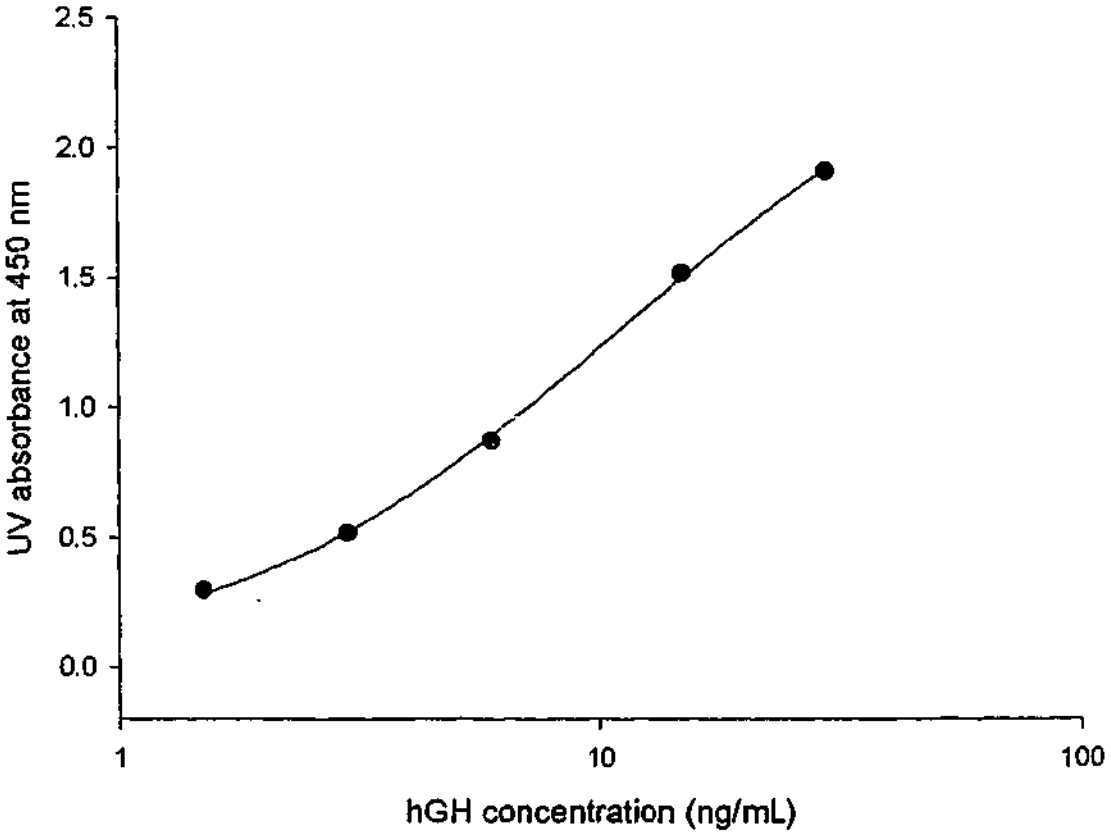

Figure 2.1.1 Representative calibration curve for hGH obtained using the ELISA. The solid line represents the line of best fit using Equation 2.1.1.

as samples in 100% horse serum. All rat serum samples were therefore diluted a minimum of 1:2 with horse serum prior to analysis.

Potential interference by SNAC-2 was investigated by assaying reference standards diluted with horse serum and spiked with SNAC-2. A 200 μg/mL final serum concentration of SNAC-2 was used as a nominally high concentration. SNAC-2 did not affect the ELISA as there was no increase in the background absorbance in blank serum and there was no effect of SNAC-2 on the measured concentration of a calibration standard.

QCs stored frozen with samples and standards at -20°C prior to analysis showed no trends or changes in concentration over time. QC concentrations generally varied by less than ± 20%, thus confirming hGH stability under these storage conditions.

In order to determine the short-term stability of hGH in the presence of freshly collected rat serum, hGH was spiked into serum and incubated at 37°C. Samples were collected at regular time intervals and analysed for hGH content using the ELISA. Figure 2.1.2 presents the concentration of hGH in rat serum over time, with no loss of hGH detected over the 6 h time period investigated. These results are consistent with previous studies conducted in sheep blood and lymph, which also demonstrated hGH stability in similar biological matrices (Charman et al., 2000).

2.1.3.2 hGH Serum Concentrations Following Oral and SC Administration to Rats

Figure 2.1.3 depicts serum hGH profiles for rats dosed orally with hGH (3 mg/kg) in buffer. hGH concentrations were negligible in the oral control group, with C_{max} values ranging from <1.5 ng/mL (LOQ) to 5.1 ng/mL.

Figure 2.1.4 presents the individual serum hGH profiles for rats dosed orally with hGH (3 mg/kg) and SNAC-2 (300 mg/kg). Despite the considerable variability in the hGH concentrations between rats, significant serum levels were obtained in rats dosed orally with the

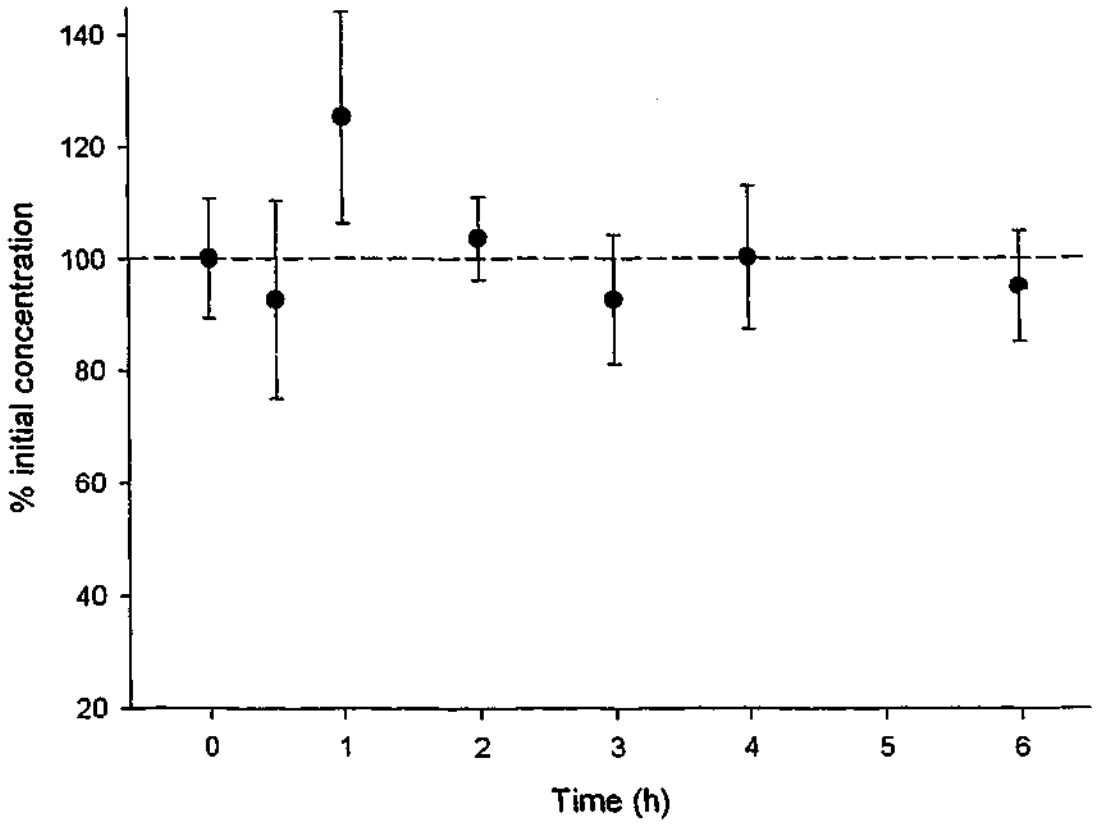

Figure 2.1.2 Stability of hGH in collected rat serum at 37°C over 6 h. Symbols represent the mean ± SD (n = 3-5 measurements) concentration as a percentage of the initial concentration and the dotted line represents 100% of the mean initial concentration. hGH concentrations were determined using the ELISA as described in Section 2.1.2.3.5.

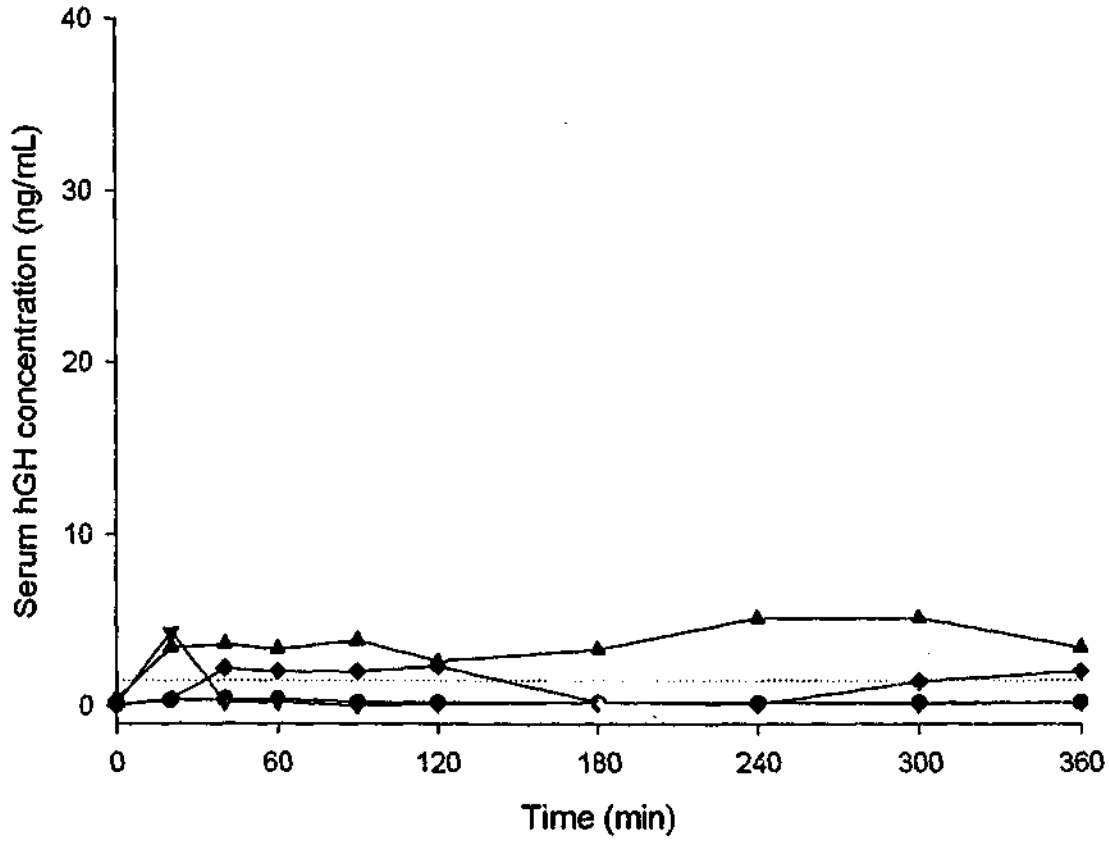

Figure 2.1.3 Individual hGH serum profiles for four rats following the oral administration of 3 mg/kg hGH in buffer. Serum concentrations were determined using the hGH ELISA. The LOQ of the assay was 1.5 ng/mL and is shown by the dotted line.

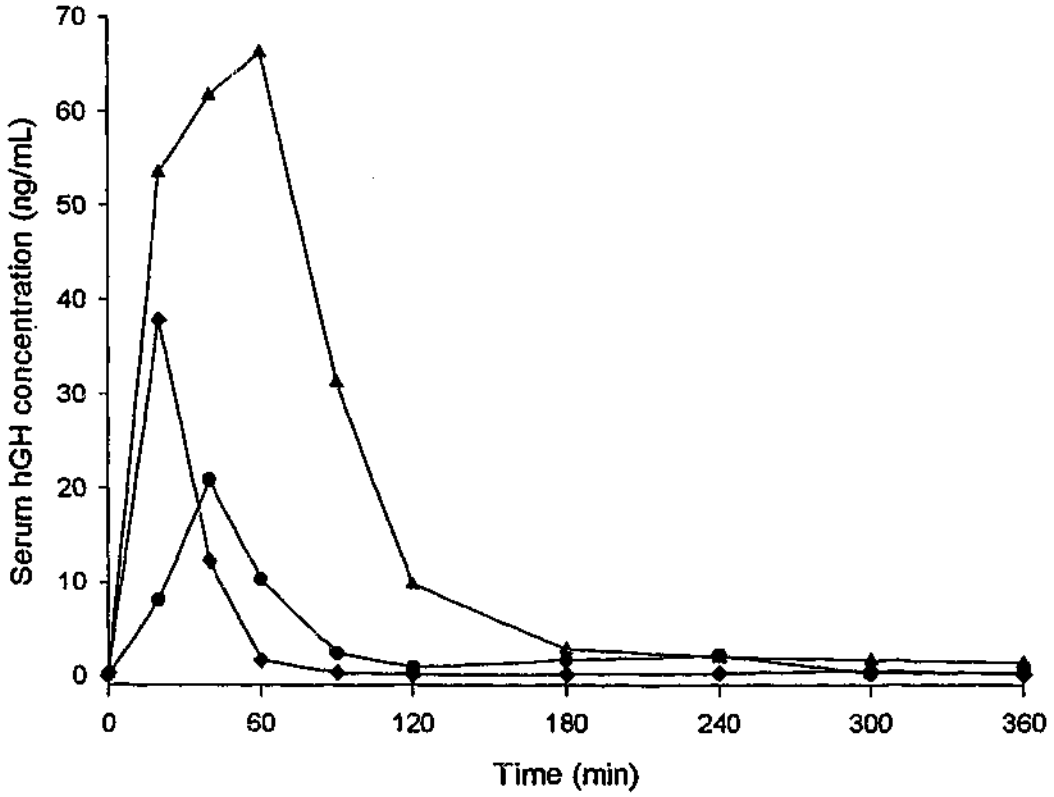

Figure 2.1.4 Individual hGH serum profiles for three rats following the oral administration of 3 mg/kg hGH and 300 mg/kg SNAC-2. Serum concentrations were determined using the hGH ELISA. The LOQ of the assay was 1.5 ng/mL.

combined hGH/SNAC-2 solution, with C_{max} values ranging from 20.8 to 66.2 ng/mL representing a statistically significant seven to twenty-three-fold increase over that seen with hGH in buffer (p=0.012). hGH concentrations measured in this group typically peaked within an hour or less and declined rapidly thereafter. While there was not a statistically significant difference between the AUC values obtained between rats orally administered the hGH/SNAC-2 solution compared to the rats orally administered hGH in buffer (p=0.160) most likely owing to variability, a trend toward higher (approximately six-fold) AUC values with the hGH/carrier animals was clearly evident. Differences in the amount of hGH absorbed between rats was attributed to normal biological variability, which was likely to be more evident with a protein drug and may have also reflected differences in the concentration of proteolytic enzymes present in the gastrointestinal tract of individual rats.

Serum profiles for animals administered hGH (0.125 mg/kg) by SC injection are presented in Figure 2.1.5. Serum levels were more consistent between animals within this group and C_{max} values were similar to those obtained following oral administration of hGH combined with SNAC-2 (dosed with 3 mg/kg and 300 mg/kg, respectively), although serum concentrations declined much more slowly in the SC group. Table 2.1.3 describes the AUC and C_{max} data obtained from the study. hGH serum concentrations measured following SC administration were of a similar order of magnitude to those reported by Jorgenson and co-workers in SPF Wistar rats following SC administration of 0.1 mg/kg hGH (Jorgensen et al., 1988). The C_{max} values for the SC control group and the oral hGH/SNAC-2 group were similar and both approximately 14-fold higher than the oral control group.

Figure 2.1.6 shows the average serum hGH concentrations (± SEM) for each of the three groups of animals. The addition of the carrier, SNAC-2, markedly increased the absorption of hGH following oral administration. Following correction of the AUCs for dose differences, a bioavailability (relative to the SC administration) of approximately 2% was estimated for the hGH/SNAC-2 group, which was at least five-fold higher than for oral hGH alone.

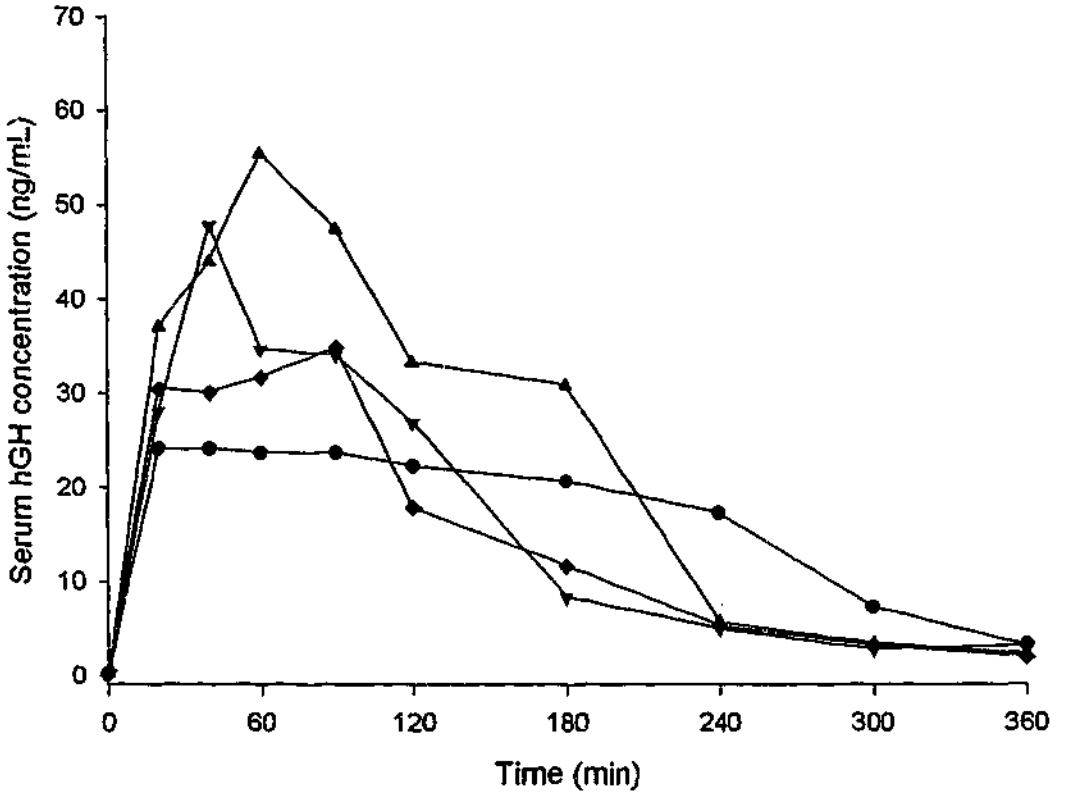

Figure 2.1.5 Individual hGH serum profiles for four rats following the subcutaneous administration of 0.125 mg/kg hGH. Serum concentrations were determined using the hGH ELISA. The LOQ of the assay was 1.5 ng/mL.

Table 2.1.3 Pharmacokinetic parameters for hGH in rats following oral and SC administration. The C_{max} and AUC values were obtained as described in Section 2.1.2.4. Serum concentrations were measured using the hGH ELISA as described in Section 2.1.2.3.5.

hGH dose	*SNAC-2 dose*	*Route of administration*	*No. of rats (n)*	*$C_{max} \pm SD$ (ng/mL)*	*$AUC \pm SD$ (ng min/mL)*
3 mg/kg	300 mg/kg	Oral	3	41.6 ± 22.9	2591 ± 2657
3 mg/kg	Nil	Oral	4	2.9 ± 2.3	400 ± 649
0.125 mg/kg	Nil	Subcutaneous	4	40.5 ± 13.9	6299 ± 1440

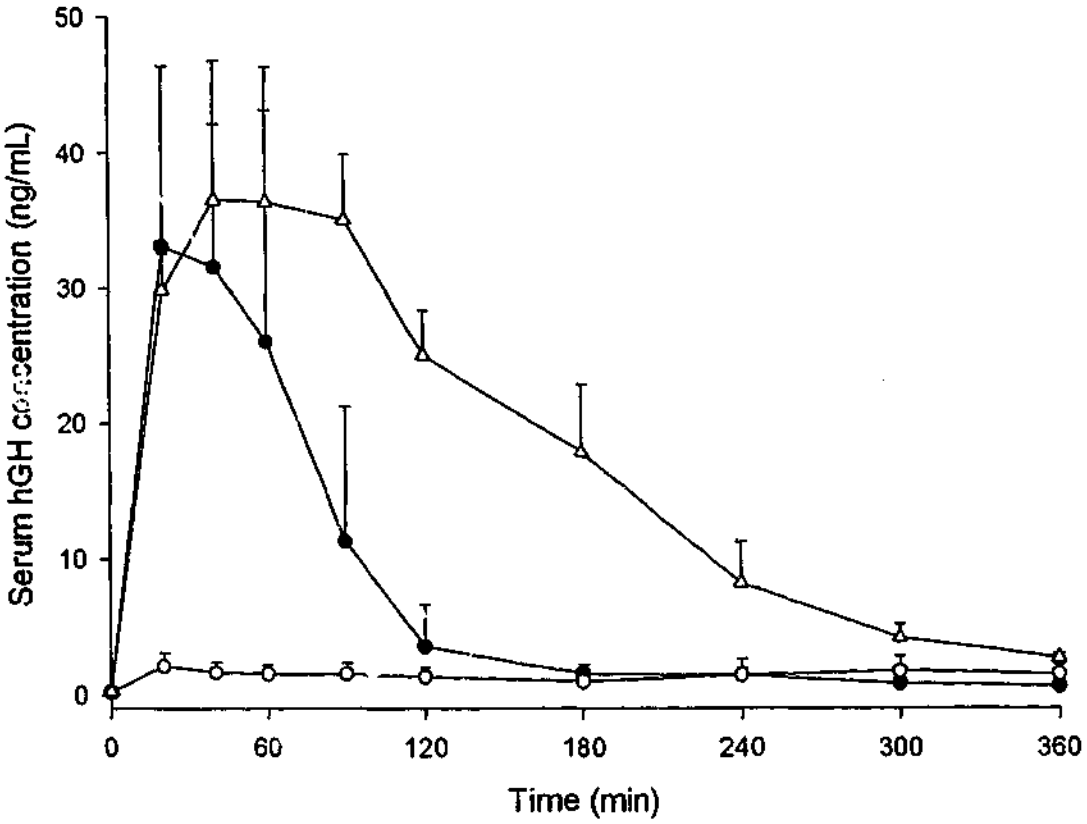

Figure 2.1.6 Average serum hGH concentrations (± SEM) in rats following oral administration of 3 mg/kg hGH and 300 mg/kg SNAC-2 (closed circles), 3 mg/kg hGH alone (open circles), or subcutaneous administration of 0.125 mg/kg hGH (open triangles). Serum concentrations were determined using the hGH ELISA with an LOQ of 1.5 ng/mL.

Previous studies have reported the absolute bioavailability of hGH in rats following SC administration to be approximately 50% (Jorgensen et al., 1988), while a figure of 75% in humans is quoted in the Lilly product information for Humatrope®. The absolute bioavailability of orally administered hGH (when combined with SNAC-2) determined in the rat study described in this chapter can therefore be estimated to be around 1%. Considering that these data were obtained using simple solutions of drug in buffer only, it is thought that the elucidation of the SNAC-2 mechanism and the subsequent optimisation of formulation (e.g. dose of drug and carrier, dose volume, addition of appropriate excipients) could further improve the oral bioavailability of hGH.

2.1.4 Summary

A brief *in vivo* study was conducted in rats to confirm the utility of the model carrier, SNAC-2, for enhancing the oral absorption of hGH. The inclusion of SNAC-2 in the oral dosing solution markedly enhanced the absorption of hGH, with the C_{max} and AUC values increasing more than fourteen-fold and six-fold, respectively, in comparison to those obtained in the absence of carrier. The bioavailability of hGH following oral administration with SNAC-2 was approximately 2% relative to the SC route of administration.

2.2 LYMPHATIC TRANSPORT OF hGH IN THE PRESENCE OF SNAC-2

2.2.1 Goals

It is well recognised that the absorption of intact proteins and peptides into the lymphatics occurs following oral administration (Rubas and Grass, 1991). Previous studies have examined the intestinal lymphatic transport of a number of proteins including horseradish peroxidase (Warshaw et al., 1971), albumin (Warshaw et al., 1974), elastase (Katayama and Fujita, 1972) and ovalbumin (Tsume et al., 1996).

As described in Section 1.5.4, one of the possible mechanisms responsible for the oral absorption enhancement of hGH by the model carrier, SNAC-2, is that of increased uptake by the intestinal lymphatics. The aim of the pilot study outlined in this section was to explore the potential role of intestinal lymphatic transport in the absorption of hGH, using an unconscious rat model. Surgery involved cannulation of the mesenteric lymph duct and carotid artery for lymph and blood sampling, respectively, and cannulation of the duodenum to facilitate dosing and rehydration. The concentration of hGH in blood and lymph was determined using the ELISA (Section 2.1.2.3.5), while the concentration of SNAC-2 in lymph was determined using a RP-HPLC assay (Section 2.2.2.5).

2.2.2 Experimental

2.2.2.1 Materials

The internal standard, E537, was kindly provided by Emisphere Technologies Inc. (Tarrytown, NY). Hypnorm® (0.315 mg/mL fentanyl citrate combined with 10 mg/mL fluanisone) was purchased from Janssen Animal Health (division of Janssen-Cilag, Berchem, Belgium), Hypnovel® (5 mg/mL midazolam) was purchased from Roche (Basel, Switzerland) and analytical grade EDTA was supplied by BDH Chemicals (Victoria, Australia). All other reagents were of analytical grade or higher. Water was obtained from a Milli-Q (Millipore,

Bedford, MA) water purification system and was used throughout.

2.2.2.2 Study Design – Pilot Experiment

The experimental procedure was approved and conducted in accordance with the guidelines from the Institutional Animal Experimentation Ethics Committee. The pilot experiment involved the intra-duodenal dosing of two triple-cannulated male Sprague-Dawley rats with a solution (1.5 mL) to provide a dose of 3 mg/kg hGH and 300 mg/kg SNAC-2 in a 5 mM phosphate buffer. Controls (i.e. rats dosed with hGH alone) were not included in the pilot study owing to the complexity of the surgical procedures. If lymphatic transport of hGH in the presence of SNAC-2 had proved significant, control experiments would have been subsequently performed. Doses of hGH and SNAC-2 were the same as those used in the oral absorption study described in the previous section (3 mg/kg and 300 mg/kg, respectively). Sampling of lymph and blood was conducted over a 5 h period in total, including the 1 h dosing time. The concentration of hGH in lymph and blood was determined using the ELISA (Section 2.1.2.3.5) and the analysis of SNAC-2 in lymph was conducted using the RP-HPLC assay (Section 2.2.2.5).

2.2.2.3 Surgical Method and Experimental Procedure

Surgery was performed as described in detail in a previous publication (Porter and Charman, 1996). Briefly, fasted male Sprague-Dawley rats (approximately 300 g) were anaesthetised by a 1 mL intraperitoneal injection of FFM (Hypnorm®:Hypnovel®:water in a 1:1:2 ratio), and subsequent top-up doses (0.2 mL) were administered when necessary throughout the procedure and the experimental period.

Rats were placed on a warming pad (37°C) to maintain body temperature and the throat and abdominal regions cleaned and shaved. A small incision was made just above the left clavicle and the exposed connective and adipose tissue pushed aside to isolate the carotid artery. The artery was clamped and ligated before inserting a pre-prepared polyethylene cannula (PE-50

tubing, Bioscience, Victoria, Australia). The cannula was sutured in place and its patency confirmed by flushing with heparinised saline (20 U/mL).

The abdominal incision was performed from a position approximately 0.5 cm caudal to the xiphoid process of the sternum and along the length of the ribs to the flank. The intestine was moved to one side with saline-soaked gauze to allow exposure of the mesenteric lymph duct and artery. Fine, membranous connective tissue was carefully removed from the area surrounding the lymph duct and a dissecting microscope (50x magnification) employed to facilitate insertion of the cannula (polyethylene, 0.50 mm i.d., 0.8 mm o.d., Bioscience, Victoria, Australia) into the duct. A drop of cyanoacrylate adhesive was used to secure the cannula (prefilled with 1% EDTA solution) in place and its patency confirmed by observing the flow of lymph.

The duodenum was cannulated by piercing the intestine approximately 1 cm below the pylorus with a 23G needle and following this with the cannula (PE-50) which was secured in place with cyanoacrylate adhesive. The abdominal wall was closed with 3-0 silk sutures and rehydration commenced. Normal saline was infused into the duodenum at a rate of 1.5 mL/h over a two hour recovery period.

Following the rehydration and recovery phase, the dose of hGH/SNAC-2 (3 mg/kg hGH and 300 mg/kg SNAC-2 in 1.5 mL) was administered via the duodenal cannula at a rate of 1.5 mL/h. Lymph was collected in 4 mL polypropylene tubes, to which 1% EDTA (50 µL) had been added to prevent clotting. The tubes were changed each hour. Lymph samples were immediately stored at 4°C after collection and were assayed for hGH content by ELISA (described in Section 2.1.2.3.5) within 24 h. Samples were pooled to determine cumulative hGH in lymph for each rat and were diluted 1 in 200 with horse serum prior to analysis. This dilution eliminated the effect of the anticoagulant EDTA on the ELISA (Section 2.2.2.4). SNAC-2 concentrations in lymph were determined by RP-HPLC (described in Section 2.2.2.5).

Blood samples (300 μL) were withdrawn from the carotid cannula at time 0, 30, 60, 90, 120, 150, 180, 240 and 300 min (where time 0 min represents the commencement of the dosing time). Samples were immediately transferred to an Eppendorf tube without anticoagulant, allowed to clot for 5-10 min, then centrifuged for 15 min in a microcentrifuge and the serum separated and stored at -20°C until analysis. Serum hGH concentrations were determined using the ELISA as described in Section 2.1.2.3.5.

The intraduodenal saline infusion was resumed following dosage administration and continued for the duration of the sampling period. At the completion of the experiment, rats were euthanased by an overdose of pentobarbitone administered via the carotid cannula.

2.2.2.4 Interference by the Anticoagulant EDTA with the hGH ELISA

It is reported in the literature accompanying the hGH ELISA kit that various anticoagulants, including EDTA, may interfere with the ELISA. It was necessary therefore to evaluate the effect of EDTA on the ELISA and determine the dilution factor required for the elimination of any interference. Dilutions of a hGH standard of known concentration with either blank horse serum (control) or various concentrations of EDTA in horse serum were performed such that the final dilution of the 1% EDTA solution ranged from 1:200 to 1:1000 and the resulting samples were then assayed using the ELISA.

2.2.2.5 SNAC-2 Chromatography

A RP-HPLC assay was developed and used for determining the concentration of SNAC-2 in aqueous solutions and biological matrices including rat plasma and lymph. The assay development was conducted, in part, in preparation for later studies (described in Chapter Four) involving a rat intestinal perfusion model where the determination of SNAC-2 concentrations in both aqueous perfusate and rat plasma was required.

The HPLC instrument was a Beckman 116 pump and 167 UV detector (Beckman Coulter, Inc., Fullerton, CA), with integration conducted using Beckman System Gold software version V810. The column was an Ultrasphere C18 (5 μm particle size, 250 x 2 mm i.d., Beckman Coulter, Inc., Fullerton, CA) in conjunction with a Brownlee RP-18 cartridge guard column (7 μm particle size, 15 x 3.2 mm i.d., PE Biosystems, Foster City, CA). The mobile phase consisted of 35% v/v acetonitrile, 65% v/v water and 0.1% w/w orthophosphoric acid. Separations were conducted at ambient temperature using a flow rate of 0.3 mL/min and an injection volume of 30 μL. The detection wavelength was 244 nm and the run time was 20 min.

The concentration of SNAC-2 in rat plasma and lymph was determined by performing an extraction followed by RP-HPLC analysis. For the purposes of the pilot lymph study (n=2 rats), it was assumed that the extraction and analysis of SNAC-2 in plasma and lymph were the same and hence validation studies were conducted in rat plasma only. The internal standard employed for the assay was E537, a compound which is structurally similar to SNAC-2.

Standards were prepared by adding 30 μL of a 50 μg/mL E537 solution in 5 mM phosphate buffer and 70 μL of a suitable concentration of SNAC-2 solution in 5 mM phosphate buffer to 100 μL of blank rat plasma in a polypropylene tube. Samples were prepared by the addition of 30 μL of 50 μg/mL E537 solution and 70 μL of blank buffer to 100 μL of rat plasma. Acetonitrile (80 μL) and 10% w/v trichloroacetic acid (20 μL) were added, vortexed for 30 s and allowed to stand for 15 min. Tertiary-butyl methyl ether (1 mL) was added and the mixture vortexed for 60 s and centrifuged at approximately 1200 *g* for 15 min. The supernatant was subsequently pipetted into a clean polypropylene tube and evaporated to dryness under N_2. Mobile phase (100 μL) was used to reconstitute the samples/standards, which were then centrifuged for a further 10 min prior to injection onto the column.

Recovery was calculated by comparing the peak areas of SNAC-2 and the internal standard, E537, extracted from spiked plasma samples with the peak areas obtained from standard solutions prepared in sample buffer (not extracted). Inter-assay precision was assessed by the analysis of standards across the calibration range on three different days. Intra-assay precision was determined by analysis of replicate injections (n = 5) of spiked plasma standards at SNAC-2 concentrations of 1, 20 and 60 μg/mL, while back calculated concentrations of these standards gave a measure of the accuracy of the assay.

2.2.2.6 Data Analysis

The area under the serum concentration versus time curve (AUC) was calculated by the linear trapezoidal method between 0 and 150 min and peak serum concentrations (C_{max}) were noted from the individual profiles from each animal. A statistical comparison of the serum profiles obtained from the pilot lymphatic transport study was not conducted due to the small number of animals (n=2).

2.2.3 Results and Discussion

2.2.3.1 Choice of Unconscious Rat Model

The choice of the unconscious rat model in the current study was based on greater experimental control and the improved likelihood of success coupled with a shorter experimental time (no overnight recovery necessary). If significant lymphatic uptake of hGH had been achieved in this pilot experiment, it would have been pertinent to consider the use of the conscious model for a more definitive assessment.

2.2.3.2 *hGH Stability in Lymph and Serum*

The stability of hGH in freshly collected rat serum was demonstrated to be satisfactory in an earlier study (described in Section 2.1.3.1), with no loss of immunoreactive hGH in rat serum incubated at 37°C for up to 6 h. In addition, it has been previously reported that hGH is stable in whole sheep blood and lymph at ambient temperature for at least 2.5 h, and in freshly collected sheep lymph at 37°C for at least 6 h when concentrations are assayed by ELISA (Charman et al., 2000). On the basis of these studies, it was assumed that hGH stability in rat lymph would be sufficient for the purpose of this pilot investigation.

2.2.3.3 *Interference by EDTA with the hGH ELISA*

Interference with the ELISA by EDTA was assessed by diluting a hGH standard of known concentration with either blank horse serum (control) or various concentrations of EDTA in horse serum such that the final dilution of the 1% EDTA solution ranged from 1:200 to 1:1000. The concentration of hGH in a control solution was determined to be 20.9 ± 2.9 ng/mL (mean ± SD, n=3), while the concentration of hGH in the sample containing 1% EDTA diluted 1:200 was determined to be 19.7 ± 3.3 ng/mL (mean ± SD, n=3). At a dilution of 1:200 or greater of solutions containing 1% EDTA, there was no interference observed with the ELISA. Given an hGH dose of 0.9 mg (3 mg/kg in a 300 g rat), an expected lymph flow in the unconscious rat of approximately 300 µL/h (1.5 mL in 5 h, including the 1 h dosing period), and the LOQ of the ELISA of 1.5 ng/mL, the 200-fold dilution factor would have allowed the detection of 0.05% of the hGH dose in lymph. This detection was considered sufficient to assess whether lymph was a major contributor to hGH absorption in the presence of SNAC-2.

2.2.3.4 *Validation of the RP-HPLC Assay for SNAC-2*

The performance of the RP-HPLC assay for SNAC-2 in aqueous solution was assessed. The retention time of SNAC-2 varied less than 0.3% within day and less than 0.5% between days. Intra-assay precision was assessed by comparing peak areas obtained from three replicate analyses of standard solutions (1-60 μg/mL) and was found to be less than 1.3%, while inter-assay precision was determined over three separate days and was less than 2.4% for all concentrations studied. The linearity of the assay was confirmed over the range of 1 to 60 μg/mL ($r^2 > 0.99$).

The assay was also evaluated for the analysis of SNAC-2 in rat plasma and lymph. Figure 2.2.1 presents a typical chromatogram of SNAC-2 with the internal standard, E537, following extraction from rat plasma. The peak eluting at approximately 11 min represents the internal standard and the peak eluting at 16.5 min represents SNAC-2. Linearity was confirmed over the concentration range of the calibration curve (1 to 60 μg/mL, $r^2 > 0.99$). Recovery, intra-assay precision and accuracy are detailed in Table 2.2.1. Recovery of SNAC-2 was greater than 95% for all concentrations assessed and the mean (± SD, n = 19) recovery of E537 was 98 ± 3%. Intra-assay precision was less than 4% and accuracy was within ± 8% at each concentration studied. Inter-assay precision was less than 7% for all concentrations within the calibration curve.

2.2.3.5 *hGH Serum Levels Following Duodenal Administration to Rats*

Figure 2.2.2 presents the serum profiles obtained for individual rats after intraduodenal administration of hGH (3 mg/kg) in the presence of SNAC-2 (300 mg/kg), with both rats achieving similar serum concentrations of hGH. A C_{max} of approximately 5 ng/mL was reached at the completion of the 60 min dosing period and no hGH was detected in serum following the 120 min timepoint. The AUCs were determined to be 351 and 219 ng min/mL for

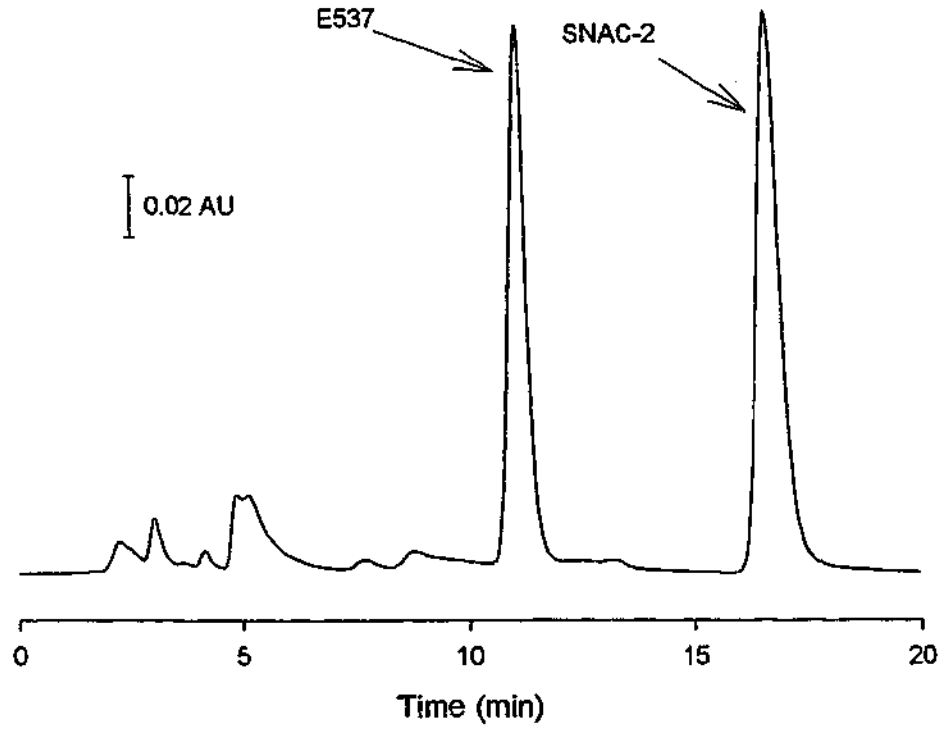

Figure 2.2.1 A typical chromatogram of 20 μg/mL SNAC-2 and 7.5 μg/mL E537 following extraction from rat plasma. The extraction procedure and chromatographic conditions were as described in Section 2.2.2.5.

Table 2.2.1 Validation of the SNAC-2 RP-HPLC plasma assay. The recoveries of SNAC-2 and the internal standard, E537, following extraction were calculated using peak areas obtained from spiked plasma standards relative to those acquired from unextracted standard solutions. The extraction procedure and chromatographic conditions were as described in Section 2.2.2.5.

Spiked Plasma Conc. (μg/mL)	*Recovery of SNAC-2 (%)*	*Recovery of E537 (%)*	*Accuracy (% deviation from nominal conc.)*	*Precision (% CV for mean peak area)*
1	107.0	95.9	6.1	3.0
1	103.0	91.9	6.1	
1	102.6	96.2	1.0	
1	107.2	95.4	7.0	
1	104.6	99.0	1.0	
2	98.9	96.8	-4.0	
5	98.1	98.3	-3.4	
10	97.7	91.1	3.6	
20	96.1	99.4	-3.0	1.4
20	99.2	100.3	-0.7	
20	101.7	101.8	0.4	
20	99.9	100.0	0.4	
20	99.0	99.3	0.2	
40	96.9	97.7	0.9	
60	95.5	103.0	-5.7	3.5
60	96.4	97.1	0.9	
60	98.0	96.0	3.9	
60	96.4	97.2	0.8	
60	97.1	98.6	0.2	

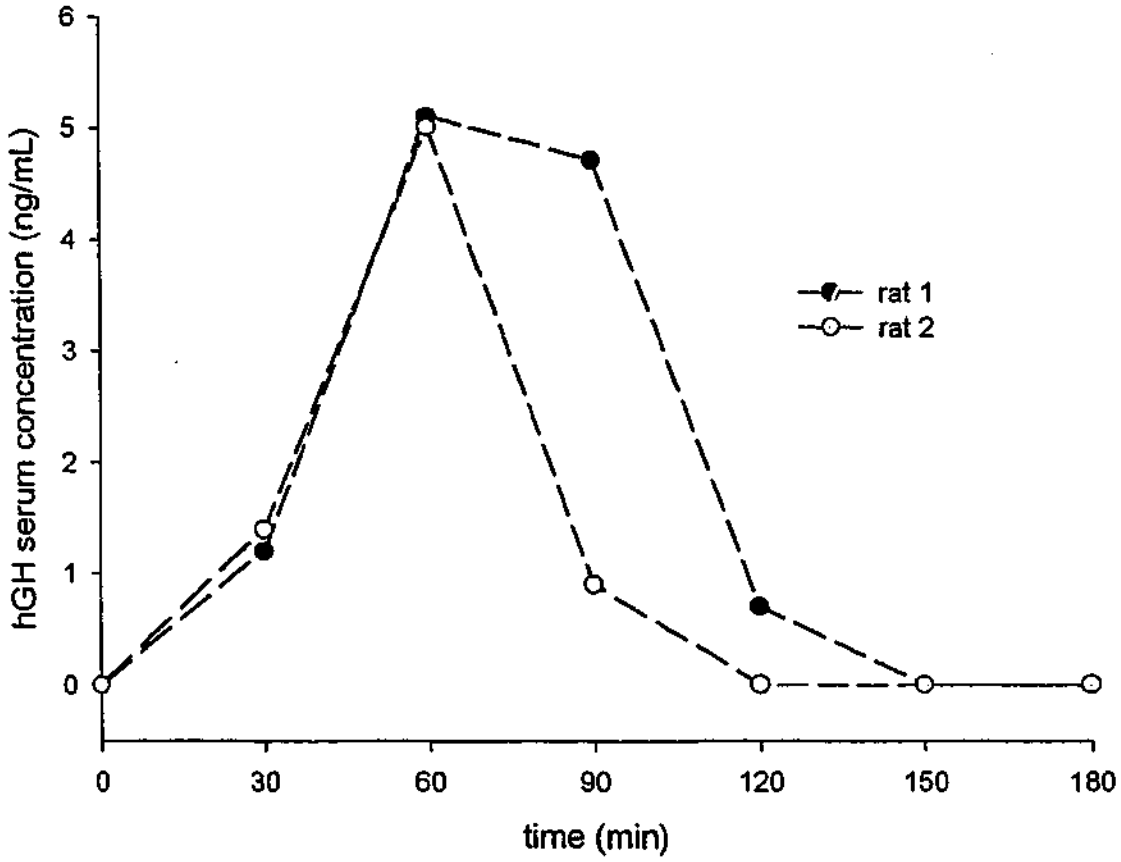

Figure 2.2.2 Individual hGH serum profiles for rats 1 and 2 following the intraduodenal administration of a dose of 3 mg/kg hGH and 300 mg/kg SNAC-2. Serum concentrations were determined using the hGH ELISA as described in Section 2.1.2.3.5.

rats 1 and 2, respectively. The more prolonged hGH serum concentrations and resulting higher AUC observed in rat 1 were likely due in part to a slightly higher dose (i.e. longer infusion time) of hGH inadvertently administered to this rat.

The maximum hGH serum levels measured in the pilot lymph experiment were considerably lower than those measured in the *in vivo* rat study described in Section 2.1. This discrepancy was most likely due to the increased duodenal dose volume employed in the lymph study (double the oral dose volume), since it has been reported that increasing the volume of hGH/carrier dosing solutions leads to a significant reduction in hGH serum levels in rats, relative to the administration of the same dose in a smaller volume (Leone-Bay et al., 1996a). The larger dose volume and slow administration rate used in the lymph study were selected based upon a recommended general experimental protocol for studying the intestinal lymphatic transport of drugs using the triple-cannulated anaesthetised rat model (Porter and Charman, 1996). The influence of anaesthesia (conscious versus unconscious) on hGH absorption is difficult to assess, since the anaesthetised lymph-cannulated rats were dosed intraduodenally, but it is possible that this factor may also have contributed to the observed differences in hGH maximum serum concentrations. Furthermore, as discussed in Section 2.2.3.1, if significant lymphatic uptake of hGH had been achieved in this pilot experiment, the use of a conscious model, which entails oral dosing, would have been considered. It is likely that the conscious model would provide more comparable hGH serum data to that obtained in the studies described in Section 2.1.

2.2.3.6 hGH Lymph Levels Following Duodenal Administration to Rats

The average lymph flow was determined to be 287 µL/h and 131 µL/h for rats 1 and 2, respectively. Samples of lymph collected from each rat were pooled, such that the total amount of drug appearing in the lymph over the experimental period could be assessed. Following a 1 in 200 dilution in horse serum, the pooled lymph samples for each rat were analysed for hGH by ELISA. As discussed in Section 2.2.3.3, it was estimated that as low as

0.05% of the dose could have been detected by the ELISA, however no hGH was detected in the lymph of either rat. It was therefore considered unlikely that the improved oral absorption of hGH in the presence of SNAC-2 was the result of significantly enhanced lymphatic transport.

2.2.3.7 SNAC-2 Lymph Levels Following Duodenal Administration to Rats

The pooled lymph samples were analysed undiluted for SNAC-2 content using the RP-HPLC assay (described in 2.2.2.5). The lymph sample from rat 1 was determined to contain a total of 13.3 μg of SNAC-2, while the lymph sample from rat 2 contained 5.4 μg, both equating to the appearance of approximately 0.01% of the administered dose of SNAC-2 in the lymph. In view of the relatively large dose of SNAC-2 administered (300 mg/kg), the lymphatic transport of the carrier itself was considered insignificant under these experimental conditions.

2.2.4 Summary

The use of an unconscious triple-cannulated rat model was employed in this pilot study to evaluate the potential for intestinal lymphatic transport of hGH in the presence of the model carrier, SNAC-2. While it would have been possible to detect the appearance of as little as 0.05% of the administered dose of hGH in lymph, no hGH was detected following the intraduodenal dosing of rats with 3 mg/kg hGH combined with 300 mg/kg SNAC-2. Negligible levels of SNAC-2 were measured in lymph, representing around 0.01% of the administered dose. It was therefore considered unlikely that the oral absorption enhancement of hGH by the carrier, SNAC-2, was the result of significantly increased lymphatic transport.

2.3 SUMMARY OF *IN VIVO* STUDIES

The bioavailability of hGH following oral administration with SNAC-2 in rats was determined to be approximately 2% relative to the SC route of administration which represents at least a five-fold increase over that obtained with hGH alone. Although the increase in hGH oral absorption afforded by the model carrier represents a significant improvement in the oral delivery of hGH, the mechanism of absorption enhancement, and therefore the potential for maximising the observed enhancement, was still unknown.

Having verified that the carrier improves the oral absorption of hGH *in vivo*, a pilot study was conducted in rats to assess the potential contribution of intestinal lymphatic transport to the increased uptake of hGH in the presence of SNAC-2. No hGH was detected in lymph following the intraduodenal dosing of rats with hGH combined with SNAC-2, and hence it was unlikely that the increased oral absorption of hGH in the presence of the carrier was a result of enhanced lymphatic transport.

The next series of studies (described in Chapter Three) was conducted to examine the physical interaction between hGH and the model carrier, SNAC-2, with a view to further probing the basis for the observed oral absorption enhancement of hGH by SNAC-2.

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CHAPTER THREE

INVESTIGATION OF hGH CONFORMATIONAL CHANGES IN THE PRESENCE OF SNAC-2

3.1 INVESTIGATION OF hGH CONFORMATION BY CIRCULAR DICHROISM

3.1.1 Goals

One hypothesis for the oral absorption enhancement of hGH by the model carrier, SNAC-2, is the presence of a physical interaction between the two molecules. DSC studies conducted by Milstein et al. demonstrated that a number of carrier compounds (structurally similar to SNAC-2) gave rise to destabilisation of the native conformation of hGH in solution (Milstein et al., 1998). The greater the destabilisation of hGH *in vitro*, the more efficient was the carrier for promoting the oral absorption of hGH *in vivo*. The authors suggested that the increased permeation was possibly due to an ability of the altered hGH conformation to translocate lipid membranes.

If an altered conformation of hGH in the presence of SNAC-2 was responsible for the observed oral absorption enhancement, the addition of selected excipients may either stabilise these conformational changes or possibly induce greater destabilisation and further improve lipid membrane translocation of hGH. Using an *in situ* rat model, Brayden and co-workers determined that propylene glycol modulated the absorption of the anticoagulant, heparin, in the presence of a similar carrier compound (Brayden et al., 1997). They also observed that instillations of heparin alone, or heparin in combination with propylene glycol without carriers, failed to increase the blood clotting time, indicating that the carrier was necessary for the absorption enhancement of heparin. Propylene glycol has also been demonstrated to potentiate the effect of carriers on hGH absorption (Emisphere communications). It was possible that the incorporation of excipients such as propylene glycol, could lead to further improvements in the oral absorption of hGH in the presence of SNAC-2. The addition of excipients may also aid in reducing the substantial dose of SNAC-2 currently required for hGH absorption enhancement (300 mg/kg in rats, see Section 2.1.2.2). Carrier induced conformational changes of hGH and either further conformational alterations or stabilisation of intermediate forms by propylene glycol and other

selected excipients were explored and the results are presented in this section of Chapter Three (3.1).

The primary objective of the studies presented in this section was to characterise the conformation of hGH in the absence and presence of SNAC-2, utilising circular dichroism (CD). CD is a spectroscopic technique which is highly sensitive to protein conformation due to the detection of differences in the absorption of left- and right-handed circularly polarised light by these chiral macromolecules. Importantly, for the purposes of this study, CD allows evaluation of excipient effects on both the secondary and tertiary structure of proteins. Disruption of these ordered structures induced by temperature (thermal unfolding) and/or the addition of destabilising excipients weakens the spectra (i.e. reduces the CD signal) as a direct consequence of the unfolding process. The signal reduction is a reflection of the exposure of hydrophobic domains that are normally buried within the interior of the protein.

The measurement of protein conformational changes as a function of temperature (thermal denaturation) provides valuable information regarding the nature of the transition and allows the determination of the transition temperature (T_m), which is the temperature at which the concentrations of folded and unfolded protein are equivalent. Calculation of additional thermodynamic parameters, such as the conformational stability of the protein at 25°C ($\Delta G(25°C)$), from the thermal denaturation profile depends on the assumption of a two-state transition for the unfolding process. This assumption is verified by several criteria including a monophasic transition, coincidence of the observed transition when monitored by different methods and reversibility of the unfolding. Although T_m is not primarily dependent on the unfolding mechanism, the false assumption of a two-state transition can introduce significant errors to the calculation of $\Delta G(25°C)$ (Pace et al., 1989). The alternative assumption of unfolding occurring via a multi-state transition involves a considerably more complex process of analysis, which will not be further addressed in this thesis.

In the current study, thermal unfolding profiles for hGH were established by CD in both the near (350-250 nm) and far (250-200 nm) UV regions in the absence of additives at concentrations of 0.3-10 mg/mL and the reversibility of the thermal unfolding was investigated at 1 mg/mL. A range of excipients including propylene glycol, polyethylene glycol 1000 (PEG 1000), polyethylene glycol 400 (PEG 400), Tween 80 and Pluronic® F127 were evaluated for their effects on the CD spectra of hGH. The aim of these studies was to identify excipients capable of altering hGH conformation and/or reducing the T_m to possibly potentiate the action of SNAC-2. The effects of up to 2 mg/mL SNAC-2 and SNAC-2/excipient combinations were also assessed. Comparison of protein stability under the different solution conditions was limited to the use of T_m and the change in T_m (ΔT_m) owing to the possibility of the unfolding mechanism deviating from a simple two-state transition. Furthermore, complete reversibility of unfolding could not be demonstrated in these studies.

3.1.2 Experimental

3.1.2.1 Materials

Poloxamers (Pluronic® L64, F68 and F127), povidone (PVP-C30, molecular weight 50,000 Da) and Cremophor® EL (PEG-35 castor oil) were supplied by BASF (Parsippany, NJ) and low molecular weight (30,000 – 70,000 Da) polyvinyl alcohol (PVA), high molecular weight (4×10^6 Da) hyaluronic acid and dimyristoyl phosphatidyl choline (DMPC) were purchased from Sigma Chemical Co. (St. Louis, MO). Tween 80 (polyoxyethylene sorbitan monooleate), PEG 400 and PEG 1000 were supplied by BDH Chemicals (Poole, England). HPMC E4M (hydroxypropyl methylcellulose) and low molecular weight hyaluronic acid (Hyalastine, 165,000 Da) were provided by the Dow Chemical Company (Midland, MI) and Fidia, S.p.A. (Abano Terme, Italy), respectively. Propylene glycol was obtained from Ajax Chemicals (New South Wales, Australia). All other reagents were of at least analytical grade.

Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA) and was used throughout.

3.1.2.2 Methods

3.1.2.2.1 hGH and SNAC-2 Solution Preparation

Stock solutions of hGH (3-4 mg/mL) were prepared in 5 mM phosphate buffer, pH 7.0, and the concentration determined by UV absorbance as described in Section 2.1.2.3.1. SNAC-2 stock solutions (50-100 mg/mL) were also prepared in 5 mM phosphate buffer (described in Section 2.1.2.3.2).

3.1.2.2.2 Excipient Stock Solution Preparation

Excipient stock solutions were prepared in 5 mM phosphate buffer, pH 7.0, at concentrations at least double that required for use in experiments, with appropriate dilutions performed on the day of each experiment. Propylene glycol was used at concentrations of 20 and 30% w/v, PEG 1000 at concentrations of 10, 20 and 30% w/v, while PEG 400 was evaluated at 20% w/v only. The poloxamers F127, F68 and L64, and Tween 80 were each used at concentrations of 0.5% w/v. PVP and PVA were employed at 5% w/v and HPMC and high molecular weight hyaluronic acid (HA) at 0.1% w/v concentrations. Low molecular weight HA and Cremophor® EL were utilised at concentrations of 1% w/v.

3.1.2.2.3 Circular Dichroism

The circular dichroism (CD) studies were conducted using a JASCO model J-710 spectropolarimeter equipped with a Neslab RTE111 recirculating water bath (NESLAB Instruments, Inc., Newington, N.H.) containing 50:50 ethylene glycol:water. The instrument was calibrated with δ-10-camphorsulfonic acid (Katayama Chemicals, Japan) as recommended by JASCO.

All spectra were recorded after a 10 min thermal equilibration period at the desired temperature. The far UV spectra for hGH in buffer and in the presence of excipients were recorded in the wavelength range of 250 nm to 200 nm using jacketed fused quartz cells of 0.2 mm pathlength, while the near UV spectra were recorded from 350 nm to 250 nm using cells of a 10 mm pathlength. Spectra represented the average of four accumulations obtained with 0.1 nm resolution and a 20 nm/min scan speed. A response time of 1 s was used with a sensitivity of 50 mdeg (for 1 mg/mL hGH) and a band width of 1 nm. Experimental conditions were varied slightly for CD measurements of hGH concentrations other than 1 mg/mL, with the 0.2 mg/mL concentration requiring the use of a 1 mm cell for the far UV scans and a 50 mm cell for the near UV scans. Sensitivity settings were increased appropriately for the far UV measurements conducted using higher hGH concentrations (100 mdeg for 2 mg/mL and 200 mdeg for 5 mg/mL). A temperature correction was made using a previously determined temperature calibration for the instrument, accounting for heat loss between the water bath and the CD sample cell. A blank spectrum for buffer containing the excipient of interest was subtracted from each protein spectrum and noise reduction was conducted using the instrument software. The CD values were converted to molar ellipticity values ([θ], deg cm^2/dmole) using a value of 115.18 for the mean residue weight of hGH (MW divided by number of residues).

Thermal unfolding experiments were conducted by continuously monitoring the CD signal at 222 nm (far UV) or 294 nm (near UV) as a function of temperature. The temperature was increased linearly at a rate of 30°C/h from 20°C to 92°C and data were collected in 0.1°C increments. Other parameters were as described above for the acquisition of spectra. The CD signal (at 222 nm or 294 nm) for buffer containing the excipient of interest was subtracted from the thermal profile, assuming no temperature dependence of the signal for buffer containing excipient. Noise reduction was conducted and mean residue ellipticity values were obtained as described above.

3.1.2.3 Analysis of CD Data

The transition temperature (T_m) of hGH was determined using a method previously described by Pace et al. (Pace et al., 1989). A two-state folding mechanism was assumed and hence

$$f_F + f_U = 1 \qquad (3.1.1)$$

where f_F is the fraction of folded protein and f_U is the fraction of unfolded protein in solution. f_U was calculated using:

$$f_U = (y_F - y)/(y_F - y_U) \qquad (3.1.2)$$

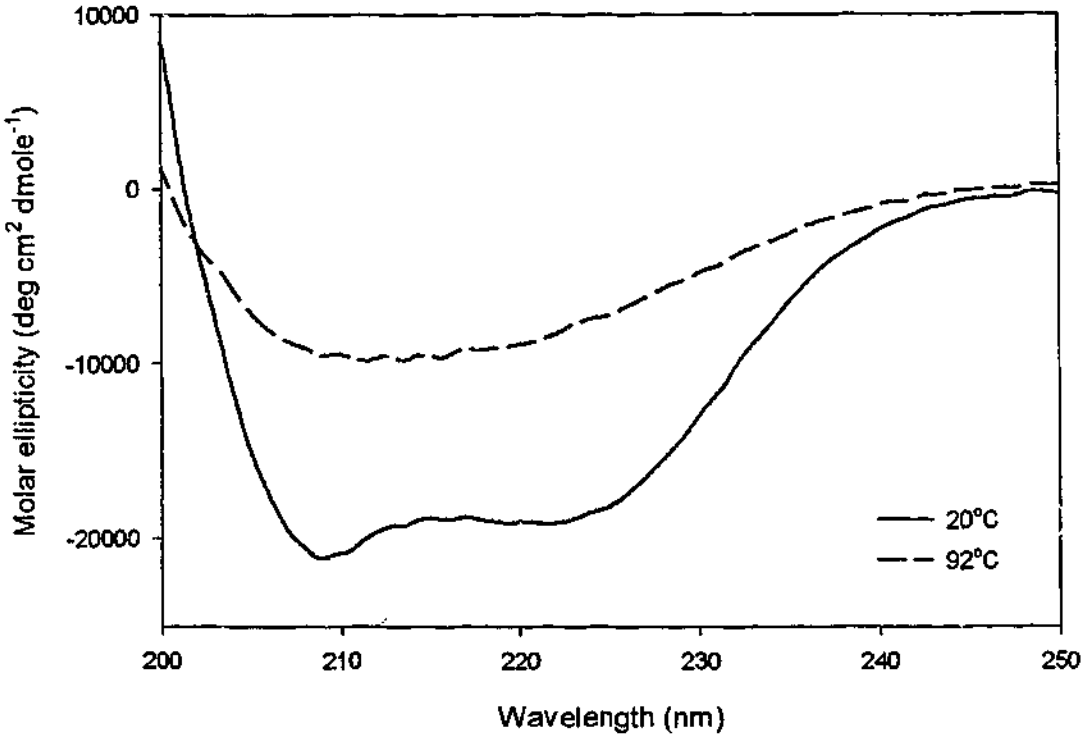
where y is the molar ellipticity at a given temperature and y_F and y_U are ellipticities characteristic of the folded (F) and unfolded (U) protein conformations, respectively. y_F and y_U were obtained by performing a linear regression on data in the pre- and post-transition regions. The transition temperature, T_m, was determined as the temperature at which 50% of the molecules were in the unfolded state (f_u=0.5). Extraction of further thermodynamic parameters was not attempted due to the absence of complete reversibility (Section 3.1.3.1) and the observed concentration dependency (Section 3.1.3.2).

The effect of excipients on the T_m of hGH was reported as ΔT_m, which is the T_m obtained for 1 mg/mL hGH in buffer subtracted from the T_m obtained for 1 mg/mL hGH in a solution of the excipient of interest. Negative values for ΔT_m therefore indicated destabilisation whereas positive values for ΔT_m indicated stabilisation relative to buffer alone.

3.1.3 Results and Discussion

3.1.3.1 Thermal Unfolding of hGH

The far and near UV CD spectra of 1 mg/mL hGH are presented in Figure 3.1.1 prior to unfolding (20°C) and following unfolding/denaturation (92°C). In both cases there was a



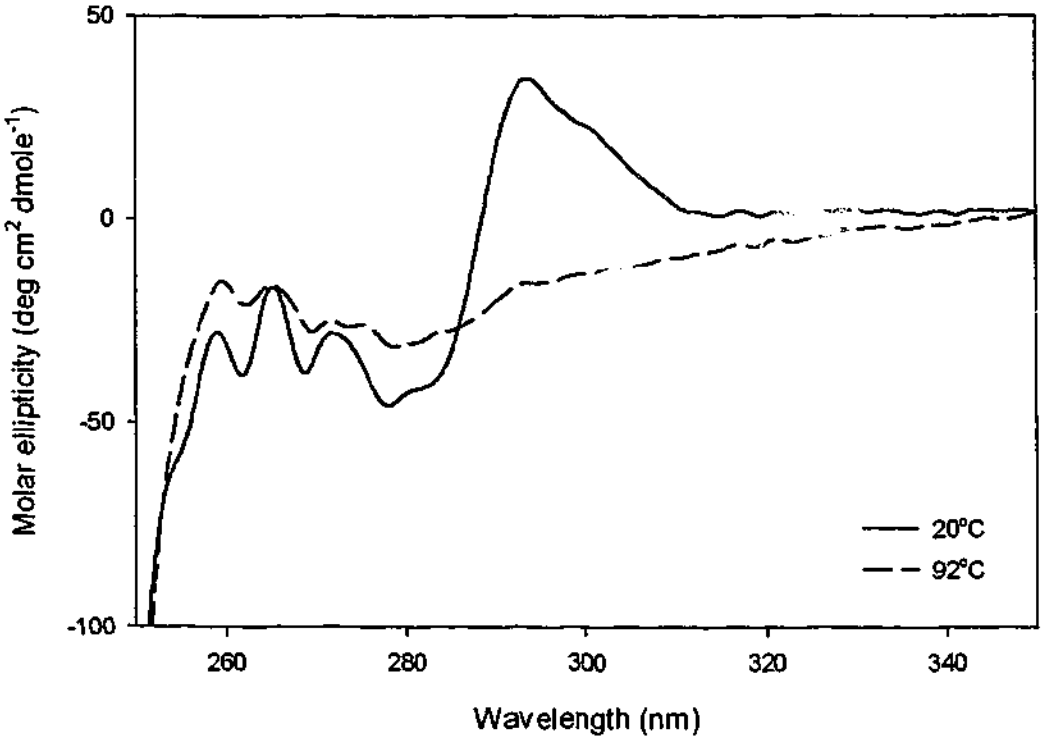


Figure 3.1.1 Representative far (top) and near (bottom) UV CD spectra for hGH (1 mg/mL) in pH 7.0 buffer in the folded (20°C) and unfolded (92°C) states. Spectra were obtained as described in Section 3.1.2.2.3.

substantial decrease in the CD signal upon heating, reflecting thermally induced changes to both the secondary and tertiary structure of the protein.

The CD spectrum of hGH has been well characterised (Aloj and Edelhoch, 1972, Bewley and Li, 1972, Holladay et al., 1974, Aubert et al., 1986), with an excellent description written by Pearlman and Bewley (Pearlman and Bewley, 1993). The secondary structure of hGH is predominantly comprised of α-helix (50-60%), which gives rise to two strong negative minima at around 209 and 221 nm. Contributing to the near UV spectrum are two disulfide bonds, which produce two wide negative bands from 300-250 nm, and aromatic residues including phenylalanine, tyrosine and tryptophan. Phenylalanine is responsible for two negative minima at 261 and 268 nm, while tyrosine produces two negative minima at 283 and 277 nm. The positive band between 320 and 288 nm has been attributed to the tryptophan-86 residue which is buried within the core of the protein.

Figure 3.1.2 depicts the thermal unfolding profiles for 1 mg/mL hGH determined in the far (222 nm) and near (294 nm) regions, with the resulting transition temperatures (T_m) determined to be 79.3°C and 80.5°C, respectively. The reason for the marginally higher transition observed in the near region was not readily apparent, but it is possible that if the unfolding deviated from a simple two-state transition (most likely due to aggregation), that an altered sensitivity to the self-association of hGH in solution may account for this discrepancy.

The reversibility of the thermal denaturation of 1 mg/mL hGH (pH 7.0) was evaluated by cooling the sample solution following the thermal unfolding experiment and rescanning the solution. Figure 3.1.3 presents the far UV scan of hGH prior to and immediately following the thermal transition and also following an 8 and 20 h re-equilibration period. The molar ellipticity values failed to return to those recorded in the pre-transition region, although partial reversibility was observed. Since there was no further change in the scan between 8 and 20 h, it is possible that the irreversibility was not simply a function of time and was most likely due to aggregation of hGH (covalent and/or non-covalent) with heat. These data are consistent with previous

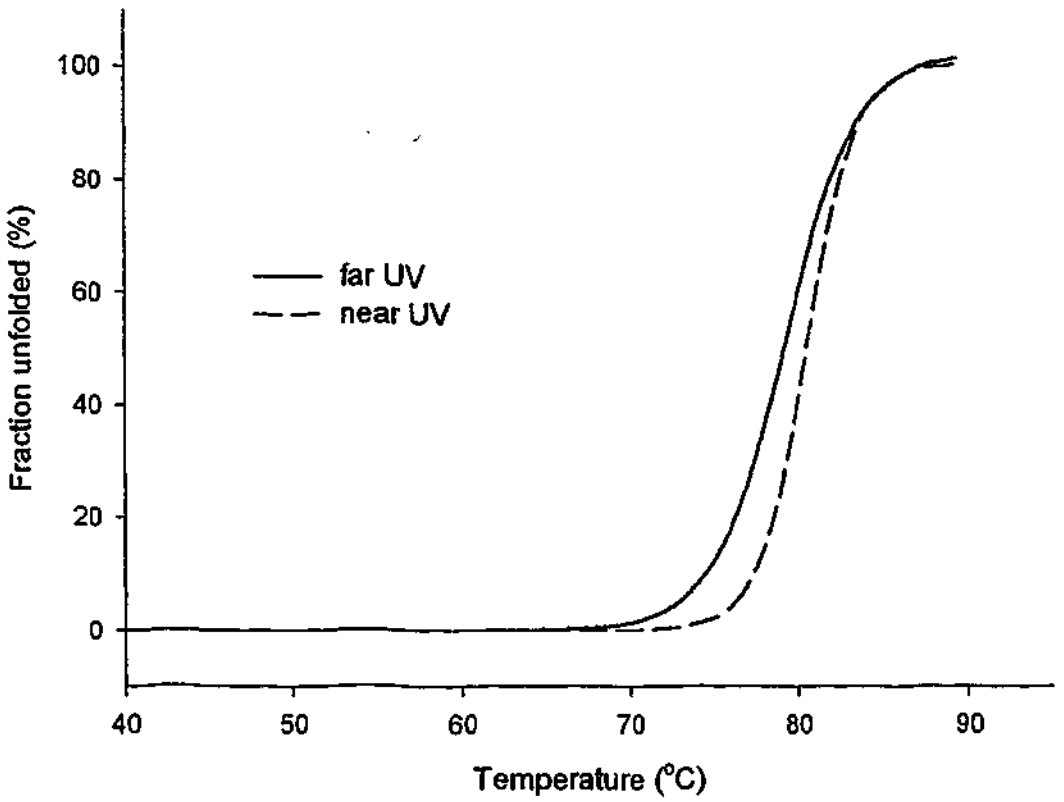


Figure 3.1.2 Fraction unfolded (%) versus temperature profiles for 1 mg/mL hGH in the far and near UV regions. CD thermal unfolding profiles were obtained as described in Section 3.1.2.2.3 and data were analysed according to the method outlined in Section 3.1.2.3.

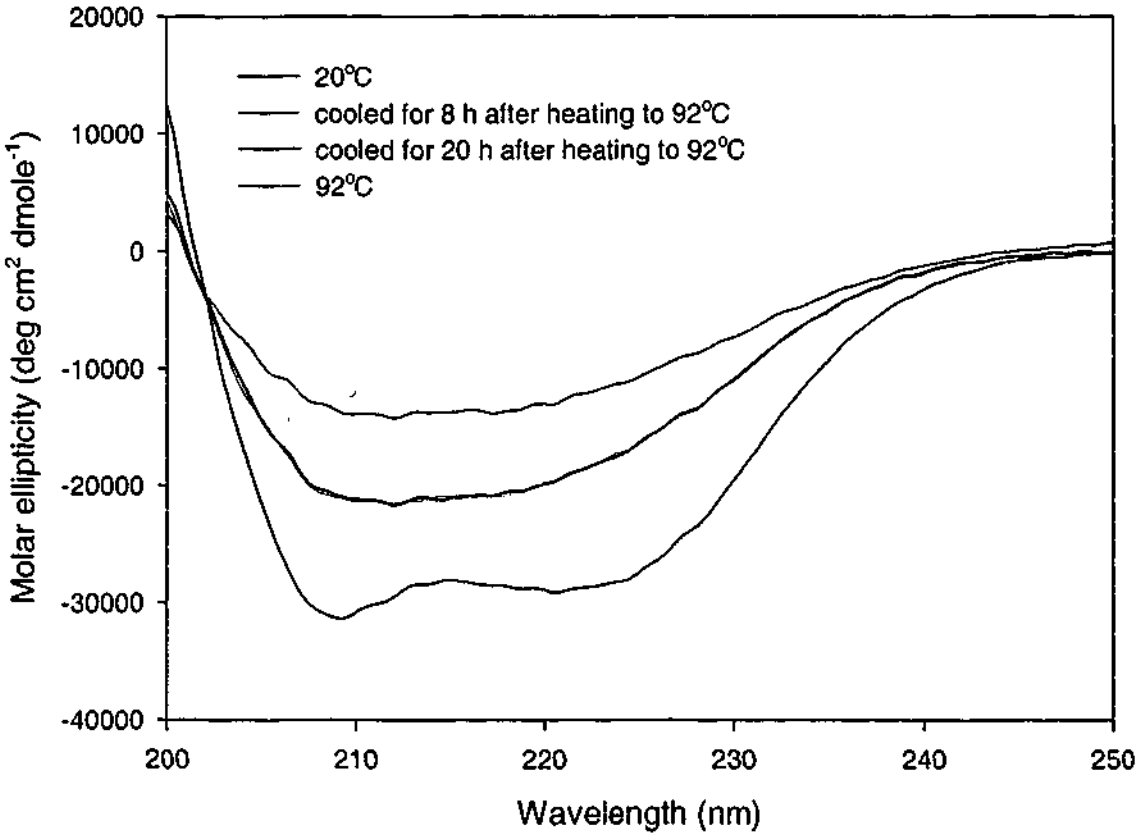


Figure 3.1.3 Far UV CD spectra of hGH at ambient temperature, at 92°C and following 8 h and 20 h re-equilibration periods. Spectra were obtained as described in Section 3.1.2.2.3.

observations by Gomez-Orellana and co-workers, who examined the thermal unfolding of hGH by DSC and CD, with reversibility of unfolding only demonstrated below pH 3.5. The irreversibility observed above this pH (pH 3.5-8.0) was attributed to aggregation upon unfolding (Gomez-Orellana et al., 1998).

The equilibrium denaturation of hGH has been the subject of a number of studies, with analysis conducted assuming two-state behaviour (Brems et al., 1990). There is some evidence to suggest that under certain conditions, hGH deviates from a simple two-state transition (DeFelippis et al., 1993, Bam et al., 1996, Gomez-Orellana et al., 1998). In the current investigation, a monophasic transition was observed under all solution conditions and T_m values were generally consistent amongst data obtained for both near and far UV CD thermal unfolding experiments, thus supporting the proposed two-state hypothesis. As mentioned earlier however, it is important to interpret data cautiously where there may be doubt regarding the nature of the unfolding mechanism and the lack of complete reversibility.

3.1.3.2 hGH Concentration Effects on CD Spectra and Thermal Unfolding Profiles

Evaluation of the concentration dependency of the thermal unfolding of hGH provided a further indication of the nature of the unfolding transition of hGH under the solution conditions employed in these studies. Concentrations of 0.3, 1, 2, 5 and 10 mg/mL hGH were utilised in the thermal unfolding studies. Table 3.1.1 describes the effect of hGH concentration on T_m, with a trend toward lower T_m values observed as the concentration was increased. The T_m at 10 mg/mL could not be measured due to protein precipitation, while the T_m at the lowest concentration, 0.3 mg/mL, was an estimate only as the post- transition region was not readily apparent prior to reaching the upper temperature limit (92°C). The T_m for 15.5 mg/mL hGH (5 mM phosphate buffer, pH 7.4) has previously been measured by DSC and reported to be 74°C (Maa and Hsu, 1996), a value which is broadly in agreement with the trends emerging from the data obtained at pH 7.0 in the current study. DeFelippis and co-workers described the guanidine

Table 3.1.1 Effect of hGH concentration on the transition temperature (T_m) at pH 7.0 monitored by near and far UV CD. The T_m values were calculated as described in Section 3.1.2.3.

hGH conc. (mg/mL)	*Far UV CD (222 nm)* T_m (℃)	*Near UV CD (294 nm)* T_m (℃)
0.3	82.2[a]	83.2[a]
1	79.3	80.5
2	77.7	78.8
5	74.3	75.7
10	---	ppt[b]

[a] values are estimates only
[b] precipitation of hGH precluded measurement of T_m

HCl (GuHCl) mediated denaturation of hGH, noting that there was no apparent effect of concentration (0.1 to 8 mg/mL) on the midpoint of the transition, which was approximately 4.5 M GuHCl for all hGH concentrations studied (DeFelippis et al., 1993). It is important to remember that the characteristics of the unfolded protein may vary depending on the method of denaturation and the solution conditions employed (Shirley, 1992). Furthermore, GuHCl unfolding is generally more reversible than thermal unfolding of proteins (Pace et al., 1989).

Gomez-Orellana described the far UV CD spectra for hGH unfolded by both temperature (pH 3, 95°C) and 6 M GuHCl (pH 7.5 at 15°C and pH 3 and 7.5 at 90°C) (Gomez-Orellana et al., 1998). In these studies, a reduction in the T_m of hGH with increasing concentration was reported. The thermally denatured protein (pH 3) appeared to retain some secondary structure whereas the spectra obtained for the GuHCl denatured protein showed almost complete loss of secondary structure, with molar ellipticity values approaching zero. The concentration dependency and lack of reversibility of unfolding at neutral pH observed by Gomez-Orellana indicated that the unfolding of hGH under these conditions did not conform to a two-state transition.

The concentration dependent thermal transition and lack of reversibility of hGH observed in the current study are consistent with published data and also indicative of an unfolding process which deviates from that of a simple two-state transition.

3.1.3.3 Excipient Effects on the CD Spectra and Thermal Unfolding of hGH

A number of excipients were screened for their effects on the conformation and thermal transition of hGH. Table 3.1.2 presents the effects of the excipients investigated on the transition temperature (T_m) of 1 mg/mL hGH at pH 7.0. Propylene glycol was evaluated at two concentrations (20 and 30% w/v). Since the most significant effect on the thermal transition of hGH was observed with PEG 1000, this excipient was assessed at three concentrations (10, 20 and 30% w/v).

Table 3.1.2 Excipient effects on the transition temperature (T_m) of hGH (1 mg/mL) at pH 7.0 monitored by near and far UV CD. The T_m values were calculated as described in Section 3.1.2.3.

Excipient (% w/v)	*Far UV CD (222 nm)*		*Near UV CD (294 nm)*	
	T_m (°C)	*ΔT_m (°C)[a]*	*T_m (°C)*	*ΔT_m (°C)[a]*
Buffer only	**79.3**	---	**80.5**	---
PEG 400 (20%)	76.2	-3.1	76.5	-4.0
PEG 1000 (10%)	76.5	-2.8	76.4	-4.1
PEG 1000 (20%)	74.8	-4.5	75.1	-5.4
PEG 1000 (30%)	70.9	-8.4	(b)	---
Propylene glycol (20%)	78.9	-0.4	80.2	-0.3
Propylene glycol (30%)	76.0	-3.3	78.7	-1.8
Pluronic F127 (0.5%)	77.9	-1.4	78.2	-2.3
Pluronic F68 (0.5%)	77.9	-1.4	78.6	-1.9
Pluronic L64 (0.5%)	78.9	-0.4	(c)	---
Tween 80 (0.5%)	78.3	-1.0	(b)	---
HPMC E4M (0.1%)	(c)	---	(c)	---
PVP (5%)	(d)	---	78.1	-2.4
PVA (5%)	78.3	-1.0	75.9*	-4.6*
Low MW HA (0.1%)	(d)	---	76.3*	-4.2*
High MW HA (1.0%)	79.0	-0.3	80.5	0.0
Cremophor EL (1%)	78.1	-1.2	(b)	---

(a) $\Delta T_m = T_m$ (+ excipient) – T_m (buffer); a negative value is indicative of destabilisation
(b) light scattering was evident at high temperatures
(c) not measurable due to precipitation/gelation of excipient at high temperatures
(d) not measurable due to high background absorbance of excipient
* estimate only, as precipitation was evident following post-heating scan

Figure 3.1.4 shows the effect of PEG 1000 on the thermal unfolding of hGH monitored in the far UV region. A significant concentration-dependent decrease was observed in the thermal transition, with 30% w/v PEG 1000 reducing the T_m by 8.4°C in the far UV region. These results are in agreement with literature reports, which describe the PEG-induced destabilisation of proteins. PEGs have been used to crystallize or precipitate proteins (McPherson, 1985) and have previously been shown to reduce the T_m of the model proteins ribonuclease, lysozyme, chymotrypsinogen and β-lactoglobulin by preferentially interacting with hydrophobic side chains exposed in the unfolding process (Lee and Lee, 1987). This mechanism was proposed to be similar to that of the well- characterised protein crystallising solvent, 2-methyl-2,4-pentane-diol. Lee's work also demonstrated a size dependent effect of PEG on the thermal denaturation of proteins, with a larger decrease in T_m produced by PEG 1000 than by PEGs 400 and 200. The data obtained in the current study were also consistent with this observation, with a greater effect of 20% w/v PEG 1000 on the T_m of hGH than that observed with 20% w/v PEG 400.

The dihydric alcohol, propylene glycol, produced a minimal effect on the thermal unfolding of hGH at a concentration of 20% w/v (ΔT_m -0.4°C, far UV and 1.8°C, near UV), while the higher concentration of 30% w/v produced a more significant effect on the T_m (ΔT_m - 3.3°C, far UV). The unfolding transitions of hGH measured in the presence of propylene glycol compare favourably to those observed by Gomez-Orellana, whose graphical illustration of the dependence of hGH T_m (pH 7.5) on propylene glycol concentration showed very similar unfolding temperatures for hGH at solvent concentrations of 20 and 30%, respectively (Gomez-Orellana et al., 1998). The destabilising effect of propylene glycol on protein conformation has also been demonstrated in earlier studies examining the thermal stability of the protein, tropocollagen (Harrap, 1969). Propylene glycol was shown to decrease the T_m of tropocollagen via hydrophobic interactions, except at very high solvent concentrations (> 76% w/v) where the

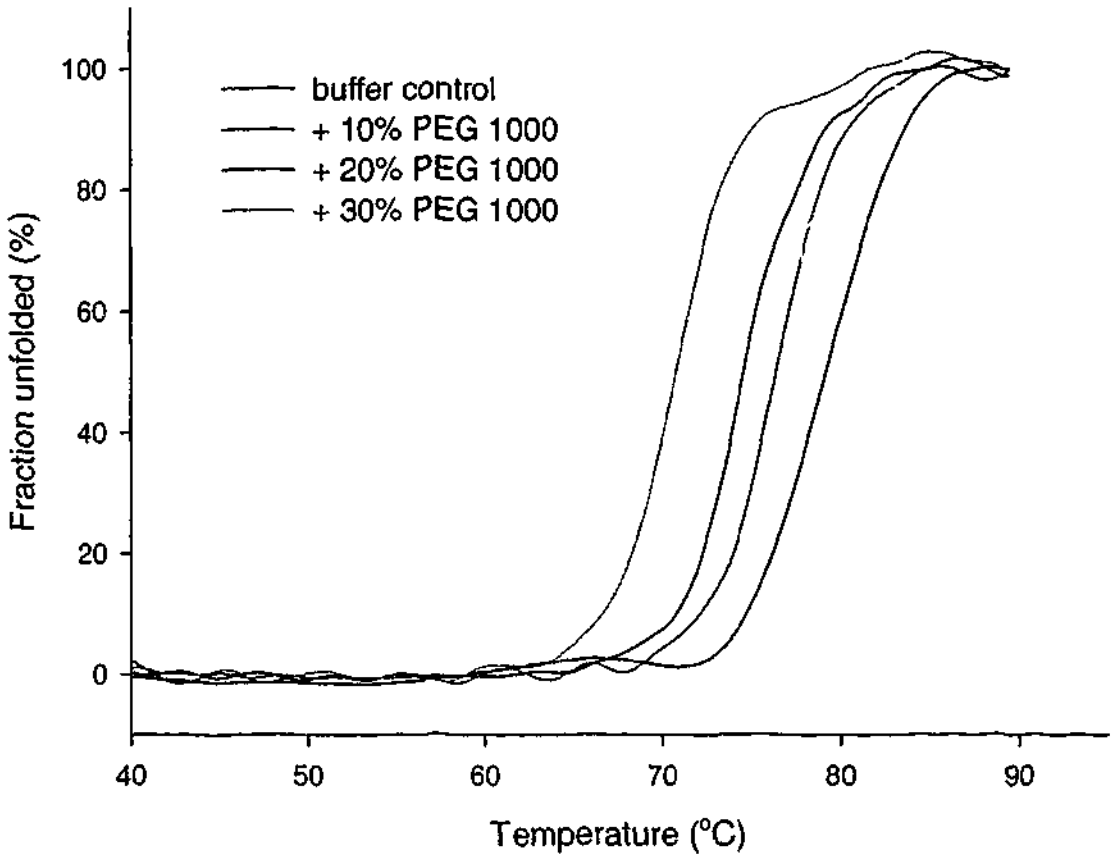
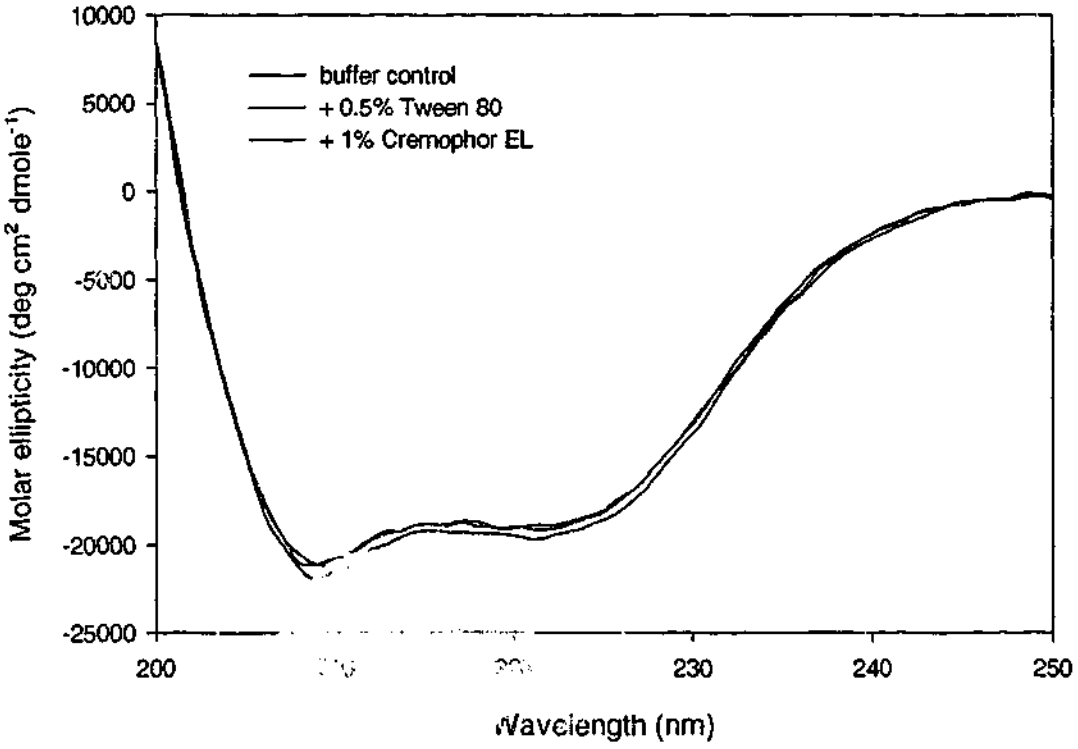


Figure 3.1.4 Fraction unfolded (%) versus temperature profiles for 1 mg/mL hGH in the presence and absence of 10, 20 and 30% PEG 1000 in the far UV region. CD thermal unfolding profiles were obtained as described in Section 3.1.2.2.3 and data were analysed according to the method outlined in Section 3.1.2.3.

predominant effect was one of stabilisation. This effect was ascribed to the accompanying reduction in the dielectric constant.

Propylene glycol was expected to have a greater effect on the T_m of hGH than was measured, given that it has been shown to potentiate the *in vivo* absorption enhancement of hGH by carriers (Emisphere communications). An alternative explanation for the *in vivo* effects of propylene glycol is that of solubility enhancement of SNAC-2 rather than an effect on hGH conformation. Studies conducted by Emisphere with the carrier compound, SNAC (referred to as P414 by the authors), have determined that propylene glycol can significantly increase the solubility of the carrier (Agarwal et al., 1996). Considering that the weak acid, SNAC-2, is poorly soluble at acid pH, it is possible that propylene glycol increases the solubility of the carrier in the acidic environment of the upper gastrointestinal tract. With SNAC-2 remaining in solution, it is then better able to exert its absorption enhancing effect on hGH, whether it be by facilitating conformational changes or otherwise.

Several surfactants were evaluated in the study including Tween 80, Cremophor EL and the poloxamers Pluronic® F127, F68 and L64. Figure 3.1.6 presents the far and near UV spectra for 1 mg/mL hGH in the absence and presence of Tween 80 and Cremophor EL at ambient temperature, with no changes detected in spectra acquired in the presence of Tween 80, while some minor changes were evident in the near UV spectrum obtained in the presence of Cremophor EL, at approximately 250-265 nm. Only marginal changes were noted in the T_m values for hGH in the presence of these surfactants (Table 2.1.2). hGH conformational changes were not detected in the presence of the poloxamers and again, these surfactants caused only minor alterations to the thermal transition. The use of surfactants with proteins has produced varying effects on protein stability characteristics, with both stabilising and destabilising effects reported. Katakam and co-workers described the use of Pluronic® F68 and Tween 80 with hGH; both surfactants prevented hGH aggregation, but failed to protect the protein against thermal stress (Katakam et al., 1995). Higher concentrations of F68 and Tween 80 (5-10%) were shown



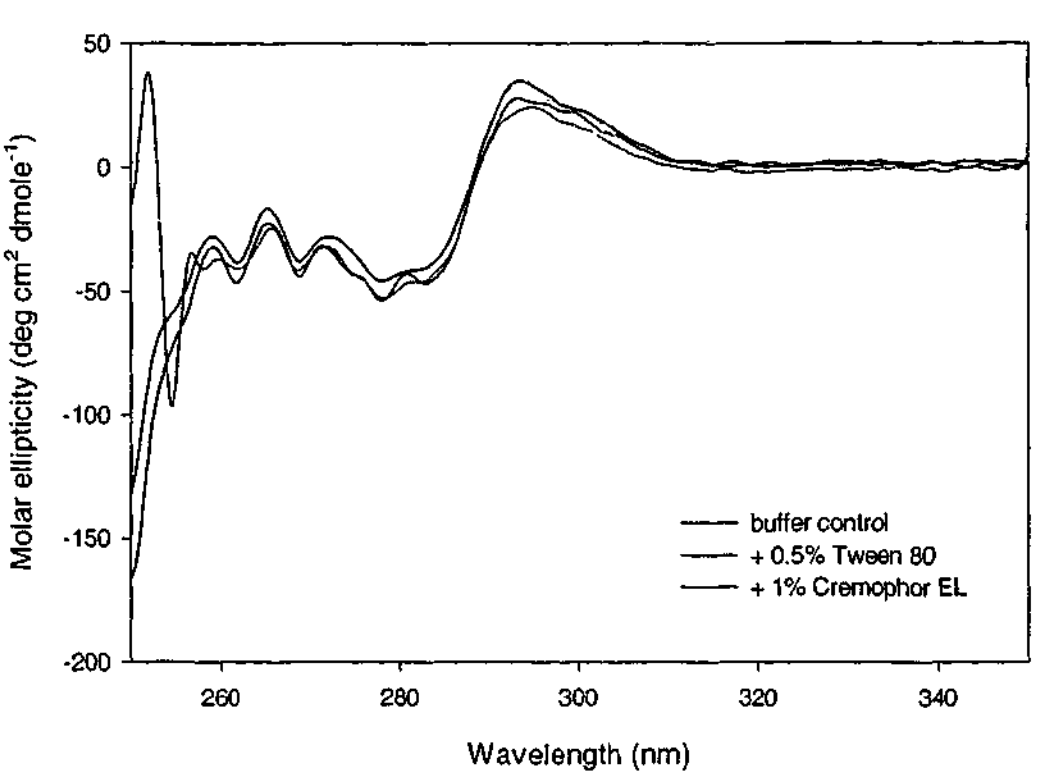


Figure 3.1.6 Near and far UV CD spectra of 1 mg/mL hGH in the presence and absence of 0.5% Tween 80 and 1% Cremophor EL at ambient temperature. Spectra were acquired as described in Section 3.1.2.2.3.

to cause destabilisation of hGH in response to high temperature, most likely as a result of hydrophobic interactions with the unfolded protein. Earlier studies by Hageman et al. found that the thermal stability of bovine growth hormone was diminished in the presence of Tween 80 (Hageman et al., 1994). More recently, Bam et al established the existence of a molten globule conformation of hGH induced by 4.5 M GuHCl and stabilised by Tweens 20, 40 and 80 (Bam et al., 1996). The intermediate conformation was characterised by CD, which indicated a significant loss of the native tertiary structure, while the secondary structure remained largely unaltered.

Of the remaining excipients investigated, either there was a negligible effect on the thermal transition of hGH or precipitation/gelation of the excipient at higher temperatures precluded their use in the study. No changes were detected in the near or far UV CD spectra of hGH in the presence of any of the excipients at ambient temperature indicating that the conformation of hGH was unaffected by the excipients under these solution conditions.

3.1.3.4 SNAC-2 Effects on the CD Spectra and Thermal Unfolding of hGH

Communications with Emisphere Technologies Inc. indicated that DSC studies revealed pronounced destabilisation of hGH by the model carrier, SNAC-2, at concentrations of 25 mg/mL and above. Even at the relatively low concentration of 1 mg/mL SNAC-2, significant background noise was evident in the CD spectra, suggesting that it would not be feasible to monitor hGH conformational changes by CD in the presence of higher SNAC-2 concentrations. A limited study was conducted at a SNAC-2 concentration of 1 mg/mL in combination with a small number of excipients, in order to explore the possibility that the excipients may reduce the concentration of SNAC-2 required to alter hGH conformation.

The effect of SNAC-2 (2 mg/mL, highest SNAC-2 concentration investigated) on hGH far UV spectra is shown in Figure 3.1.7, with spectra acquired in the presence of SNAC-2 confined to a wavelength range of 250-210 nm owing to increasing signal noise. There was

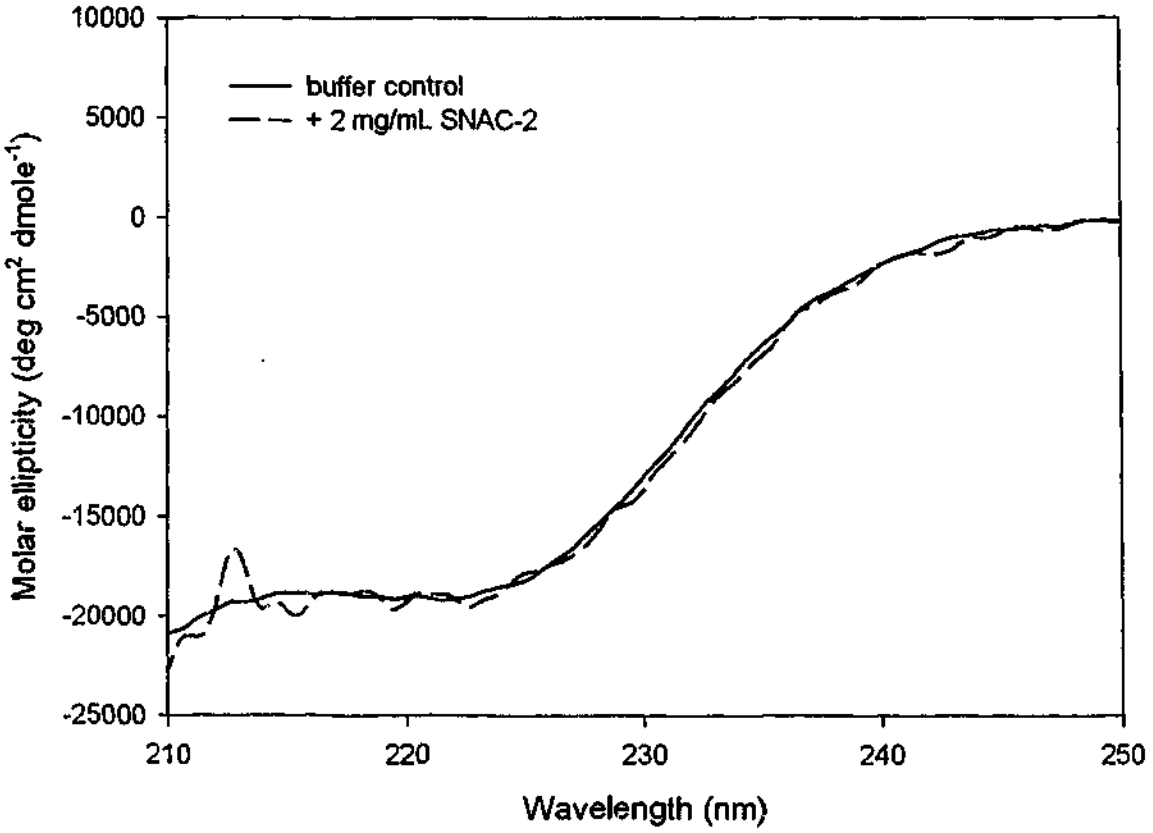


Figure 3.1.7 Far UV CD spectra of 1 mg/mL hGH in the absence and presence of 2 mg/mL SNAC-2 at ambient temperature. Spectra were obtained as described in Section 3.1.2.2.3.

negligible difference between spectra obtained with and without SNAC-2, indicating that under these solution conditions SNAC-2 had no effect on hGH secondary structure. The effect of carrier concentrations greater than 2 mg/mL was not investigated further (far UV) and no measurements were conducted in the near UV region owing to the background absorbance of the carrier. Therefore, the effect of SNAC-2 on the tertiary structure of hGH could not be defined. If SNAC-2 were to induce an altered conformation of hGH at the carrier concentrations employed, the most significant effect would be alteration/loss of tertiary structure. It is expected that this would be accompanied by a detectable (although smaller) change in the secondary structure. CD spectra of the molten globule intermediate of hGH (induced by 4.5 M GuHCl and Tween 40) observed by Bam and co-workers showed significant loss of tertiary structure (near UV); and while the secondary structure (far UV) was largely unchanged, a reduction in molar ellipticity was still observed (Bam et al., 1996).

Table 3.1.3 describes the effect of SNAC-2 and the combined effects of SNAC-2 and selected excipients on the T_m of hGH measured in the far UV region. Only minor decreases in T_m were observed in the presence of 1 and 2 mg/mL SNAC-2 alone (ΔT_m −1.2°C and −1.0°C). The combination of 1 mg/mL SNAC-2 and excipients only marginally increased ΔT_m from that measured with the excipients in the absence of SNAC-2. The thermal unfolding profiles for hGH with and without SNAC-2, and with SNAC-2 combined with each of PEG 1000, propylene glycol and Pluronic® F127 are presented in Figure 3.1.8. PEG 1000 was responsible for the most significant shift in the transition, with effects similar to those in the absence of SNAC-2 (Table 3.1.2). Spectra obtained for hGH in the presence of SNAC-2 combined with excipients was unaltered relative to those obtained both in the presence of SNAC-2 alone and in blank buffer, an indication that hGH conformation remained unaffected by the model carrier under the solution conditions assessed.

An important consideration in the current study is that of whether the changes observed in the T_m of hGH in the presence of excipients and SNAC-2 would be likely to

Table 3.1.3 SNAC-2 effects on hGH (1 mg/mL) transition temperature (T_m) at pH 7.0 with and without excipients determined using far UV CD. The T_m values were calculated as described in Section 3.1.2.3.

SNAC-2 (mg/mL)	*Excipient (% w/v)*	*Far UV CD (222 nm)*	
		T_m (°C)	*ΔT_m (°C)*[a]
0	**Buffer**	**79.3**	---
1	Buffer	78.1	-1.2
2	Buffer	78.3	-1.0
1	Propylene glycol (20%)	78.4	-0.9
1	PEG 1000 (20%)	74.0	-5.3
1	Pluronic F127 (0.5%)	77.3	-2.0
1	Pluronic L64 (0.5%)	77.5	-1.8
1	Pluronic F68 (0.5%)	78.4	-0.9

[a] $\Delta T_m = T_m$ (+ excipient) – T_m (buffer); a negative value is indicative of destabilisation

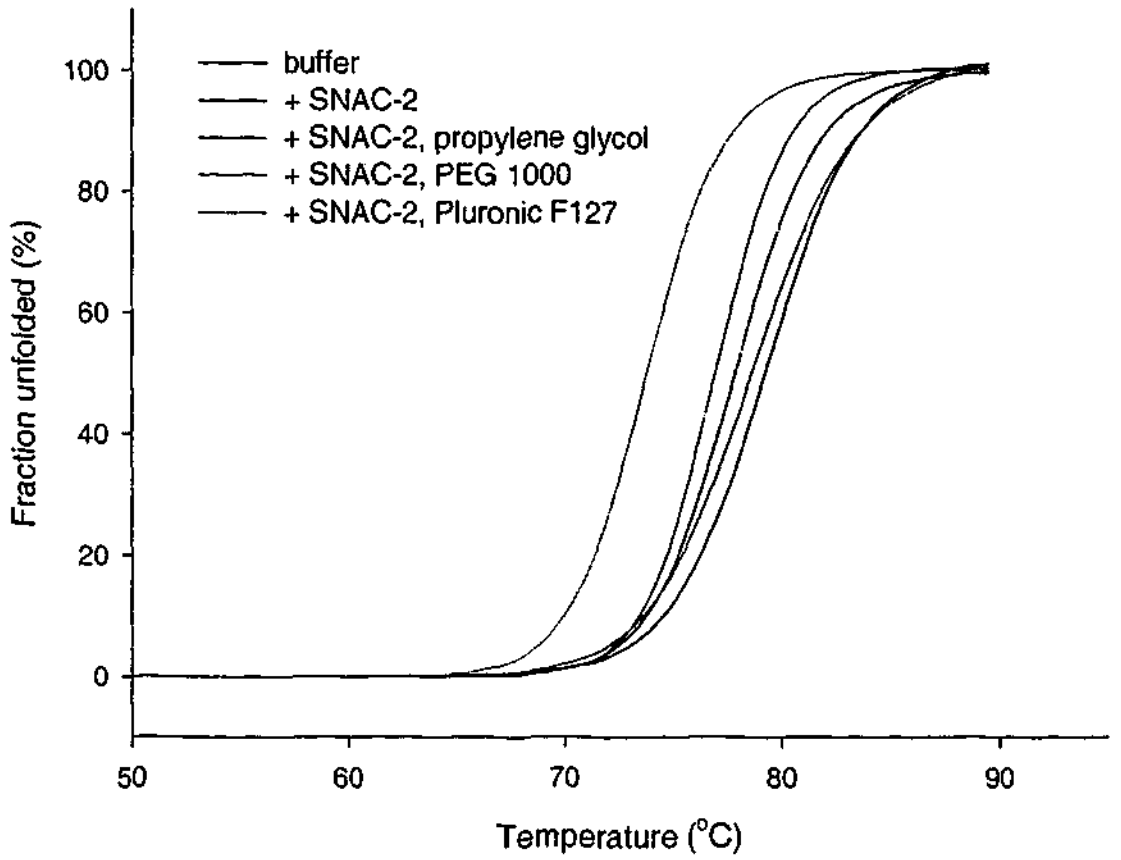


Figure 3.1.8 Fraction unfolded (%) versus temperature profiles for 1 mg/mL hGH in the presence and absence of 1 mg/mL SNAC-2 combined with 20% propylene glycol, 0.5% Pluronic® F127 or 20% PEG 1000 in the far UV region. CD thermal unfolding profiles were obtained as described in Section 3.1.2.2.3 and data were analysed according to the method outlined in Section 3.1.2.3.

influence the *in vivo* absorption characteristics of hGH. Microcalorimetry unfolding experiments with hGH in combination with each of three different Emisphere carriers were conducted by Leone-Bay et al. (Leone-Bay et al., 1996). The carrier described as a "good" delivery agent for hGH in rats was found to lower the transition temperature of hGH by 2°C, with a carrier concentration of 5 mg/mL employed in the unfolding experiment. The magnitude of the changes in the T_m of hGH in the presence of excipients observed in the current study suggest that these may, in combination with appropriate carrier concentrations, impact on the *in vivo* absorption enhancing effects of SNAC-2.

Given the limitations of CD in monitoring hGH conformation in the presence of SNAC-2, it is likely that similar background noise would also preclude the use of other spectroscopic methods of analysis (e.g. fluorescence spectroscopy) that may be used to monitor excipient effects on proteins. Other possible techniques for monitoring the effect of the carrier on hGH include microcalorimetry (facility unavailable in our laboratory), which has previously been employed by Emisphere for selected carrier studies (Leone-Bay et al., 1996, Milstein et al., 1998) and light scattering, although sensitivity issues may present difficulties with the latter method. Capillary electrophoresis (CE) is another direct method which may be used to monitor excipient effects on the thermal unfolding of proteins (McIntosh et al., 1998). Thermal studies with CE are however generally limited to temperatures ranging from approximately 15-60°C, which is well below the T_m of hGH, thus ruling out the utility of this form of analysis for monitoring hGH thermal stability under the neutral pH conditions employed in the current study. Investigation of the interaction of hGH/SNAC-2 with a model membrane (liposomes) utilising an ultracentrifugation method was explored as an alternative means of probing carrier induced conformational changes of hGH. These studies are described in Section 3.2.

3.1.4 Summary

The conformation of hGH in the absence and presence of the model carrier, SNAC-2, was investigated utilising CD spectroscopy. The thermal unfolding of hGH alone was assessed as a function of concentration to evaluate the nature of the transition. A trend toward lower T_m was observed as the concentration of hGH was increased, a result most likely due to aggregation of the protein, suggesting that the unfolding process was not a simple two-state transition. Furthermore, the reversibility of the unfolding could not be demonstrated in these studies, also indicative of an unfolding process which deviates from a two-state transition. The effect of the addition of a range of excipients including propylene glycol, PEG 1000, Pluronic® F127, PVP, Tween 80 and Cremophor EL on the T_m of hGH was explored, with several excipients producing significant reductions in T_m.

Significant background absorbance of SNAC-2 did not allow measurements to be conducted in the near UV region and restricted the carrier concentration to ≤ 2 mg/mL in the far UV region. Minimal destabilisation of hGH resulted from the inclusion of these low concentrations of carrier. The addition of a limited selection of excipients in combination with 1 mg/mL SNAC-2 did not appear to further reduce the T_m of hGH relative to that seen without SNAC-2. No changes were detected in hGH spectra at ambient temperature in the presence of SNAC-2 with or without excipients, indicating that hGH conformation was unaltered under these solution conditions. Consequently, no conclusions could be made from these studies regarding the effect of higher SNAC-2 concentrations (i.e. ≥ 25 mg/mL) on hGH conformation.

3.2 INTERACTION OF hGH WITH LIPOSOMES

3.2.1 Goals

Partially folded proteins exhibiting significant secondary structure but little or no tertiary structure (commonly referred to as "molten globules") are thought to possess the capability to cross lipid membranes (Bychkova et al., 1988). As mentioned in Section 3.1.1, it has been suggested that the Emisphere carriers may induce an altered conformation of hGH in solution and thus facilitate its passage across the gastrointestinal membrane. A study by Shin and co-workers successfully employed liposomes to explore the interaction of partially folded acetylcholinesterase with lipid membranes (Shin et al., 1996). Both folded and partially folded protein were each incubated with liposomes, added to a sucrose density gradient and then centrifuged in an ultracentrifuge. The partially folded acetylcholinesterase associated with the liposomes in the lighter (top) fraction, while the native (folded) protein remained in the heavy fraction (bottom), thereby demonstrating the capability of a partially folded protein to associate with lipid membranes.

In order to explore the interaction of hGH with lipid membranes in the presence of the model carrier, SNAC-2, a brief feasibility study was conducted to assess the use of liposomes in defining the interaction of hGH/SNAC-2 with a model lipid membrane. Liposomes were prepared from dimyristoyl phosphatidyl choline (DMPC) and characterised by particle size measurements in the absence and presence of SNAC-2 to firstly determine whether the carrier was likely to interact with and/or disrupt the liposomes in the absence of hGH.

3.2.2 Experimental – Liposome Preparation

Liposomes were prepared by a method similar to that reported by Shin and co-workers (Shin et al., 1996). Dry DMPC (20 mg) was weighed into a polypropylene tube and dissolved in 1 mL of chloroform:methanol (2:1). The solution was evaporated to dryness under

N_2 and placed under vacuum for 1 h. Buffer (2 mL of 5 mM phosphate containing 179 mM NaCl, pH 7.0) was added and vortexed to resuspend the DMPC. Following sonication with a Misonix XL-2020 Ultra sonicator (Misonix Inc., Farmingdale, NY) for approximately 60 min at 20% power, liposomes were extruded ten times through two polycarbonate membranes with a 0.05 μm pore diameter (Nuclepore Corp., Pleasanton, CA).

Particle size measurements were conducted using a Malvern Zetasizer 3000 (Malvern Instruments Ltd, Malvern, UK). The desired parameters for liposomes were a diameter of approximately 50 nm accompanied by a polydispersity of less than 0.3. This choice of parameters was based on studies by Shin, who utilised liposomes of 30-50 nm in diameter.

In order to evaluate the effect of SNAC-2 on the integrity of the liposomes, freshly prepared liposomes were gently mixed with a 50 mg/mL SNAC-2 solution and the size characteristics of the liposomes subsequently monitored over time using the Zetasizer. All liposome experiments were conducted at ambient temperature.

3.2.3 Results and Discussion – Characterisation of Liposomes

Prior to the development of an ultracentrifugation technique for separating liposomes and protein in solution, it was necessary to prepare liposomes displaying appropriate size and size distribution characteristics and which remained unaffected by the presence of SNAC-2. Although liposomes were prepared based on the method of Shin et al. (Shin et al., 1996), an extrusion step was included to reduce the size and polydispersity of the liposomes produced. The diameter and polydispersity of liposomes over a 7 h time period following extrusion was found to be satisfactory, with liposomes demonstrating minimal changes to size and size distribution under these conditions. The apparent diameter of liposomes was 54.9 nm and polydispersity remained marginally below 0.3. It was necessary for preparations to remain stable for a

minimum of 7 h as this was the time required for the ultracentrifugation step, based on a previously published method (Futerman et al., 1985).

The feasibility of this series of experiments was tested by the addition of a 50 mg/mL solution of SNAC-2 to freshly prepared liposomes, with the resulting solution containing 25 mg/mL SNAC-2, the concentration at which hGH destabilisation is known to occur (DSC data, Emisphere communications). Particle size measurements for SNAC-2 solutions (up to a concentration of 50 mg/mL) in the absence of liposomes gave rise to signals that were below the limit of detection of the instrument. SNAC-2 was observed to exert a significant effect on the diameter of the DMPC liposomes. The initial diameter of liposomes in solution with SNAC-2 was 218.3 nm compared to the control of 54.9 nm. Liposomes increased further in diameter to over 300 nm in under 1 h. Polydispersity remained low for liposomes in the presence of the carrier, indicating a uniform effect of SNAC-2 on the liposome size.

The addition of SNAC-2 produced a rapid and dramatic effect on liposome integrity, with the initial diameter increasing by over 400% of control and then further increasing within an hour. The likely mechanism of the observed effect may have been either the incorporation of the surface active SNAC-2 into the lipid membrane, or the negatively charged SNAC-2 associating with the zwitterionic DMPC liposomes leading to a loss of electrostatic repulsion on the liposome surface and subsequent lipid membrane fusion. It is also possible that curvature stress in the small diameter liposomes may have contributed to the observed increase in liposome diameter in the presence of SNAC-2 since such phenomena have been reported to increase the likelihood of membrane fusion (Cevc and Richardsen, 1999). The consequence of the detrimental effects of SNAC-2 on liposomes in terms of the experimental goals for this part of the study was that the system was deemed unsuitable for examining the effect of SNAC-2 on the incorporation of hGH into liposomes under these conditions. Alternative liposome compositions and diameters could be explored with a view to finding one that does not interact with the carrier, but owing to time constraints, this option was not pursued in the current study.

3.2.4 Summary

The use of DMPC liposomes was evaluated for examining the interaction of hGH and SNAC-2 with a model membrane. The addition of SNAC-2 at a final concentration of 25 mg/mL was demonstrated to markedly affect liposome integrity, with liposome diameter increasing up to four to six-fold higher than that of the control. Increased liposome diameter was thought to result from fusion of the model lipid membrane vesicles due to a loss of electrostatic repulsion between the zwitterionic liposomes in the presence of the anionic SNAC-2, with the curvature stress associated with small diameter vesicles also a possible contributing factor. Consequently, further experiments were not conducted utilising the DMPC liposomes.

3.3 SUMMARY OF hGH CONFORMATIONAL STUDIES

CD spectroscopy was employed to investigate hGH conformation and thermal transitions in the absence and presence of the model carrier, SNAC-2, and selected excipients. Irreversibility of unfolding and a concentration dependent thermal transition were observed for hGH in the absence of carrier, indicating that the unfolding process did not occur via a simple two-state transition such that the use of thermodynamic data from these experiments was limited to the transition temperature, T_m. The addition of several excipients including PEG 1000 and propylene glycol were shown to reduce the T_m of hGH, although no changes were observed in spectra acquired at ambient temperature in the presence of any of the excipients investigated indicating that hGH conformation was unaffected.

The use of SNAC-2 in these studies was restricted to a maximum carrier concentration of 2 mg/mL in the far UV region only, owing to significant background absorbance in the presence of SNAC-2. Minimal changes were observed in the T_m of hGH in the presence of the SNAC-2 concentrations evaluated and no changes were detected in hGH spectra at ambient temperature, indicating that hGH conformation remained unaltered under these conditions. The use of an alternative non-spectroscopic analytical technique, such as microcalorimetry, would be required to facilitate further studies of hGH thermal stability in the presence of higher carrier concentrations (e.g. 25-50 mg/mL).

The feasibility of using liposomes for studying the interaction of hGH in the absence and presence of SNAC-2 with lipid membranes was briefly explored using DMPC. Significant disruption of the liposomes in the presence of the carrier, SNAC-2, precluded additional investigations in this area.

The interaction of hGH with biological lipid membranes and absorption through lipid membrane barriers was examined further using an *in situ* rat intestinal perfusion model for investigating hGH intestinal permeability in the presence of SNAC-2 (Chapter Four).

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CHAPTER FOUR

DETERMINATION OF hGH PERMEABILITY USING AN *IN SITU* RAT INTESTINAL PERFUSION MODEL

4.1 GOALS

The aim of this series of studies was to develop an *in situ* rat intestinal perfusion model to examine the permeability of hGH in the presence of the carrier compound, SNAC-2. A major difficulty encountered in the determination of the intestinal permeability of protein drugs using *in situ* experimental techniques is the potential for enzymatic breakdown. It was therefore necessary to establish the suitability of the model for evaluating hGH permeability by assessing drug loss due to proteolytic degradation and exploring various approaches, including the use of protease inhibitors, to prevent this loss.

Preliminary studies were conducted using the *in situ* intestinal perfusion model to assess the potential effects of formulation variables, including the concentration of hGH and SNAC-2, and the use of the Protease Inhibitor Cocktail (PIC). A segment of rat jejunum was isolated and perfused with solutions containing hGH in the absence and presence of SNAC-2 and PIC. Blood sampling from the jugular vein allowed the determination of hGH and SNAC-2 blood concentrations throughout the perfusion experiments and hGH and SNAC-2 concentrations were measured in perfusate for the purpose of calculating the intestinal permeability of the two compounds.

4.2 EXPERIMENTAL

4.2.1 Materials

PIC and urethane were obtained from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid and orthophosphoric acid (85% w/w) were obtained from Ajax Chemicals (New South Wales, Australia). Acetonitrile (Mallinckrodt Baker, Inc., Paris, KY) and tertiary-butyl methyl ether (Fluka Chemicals, Sigma Aldrich, St. Louis, MO) were HPLC grade. All other reagents were of analytical grade or higher. Water was obtained from a Milli-Q (Millipore, Bedford, MA) water purification system and was used throughout.

4.2.2 Study Design

All experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Experimentation Ethics Committee. The study initially involved an investigation into the feasibility of the rat *in situ* intestinal perfusion model for the determination of hGH permeability, with a focus on the stability of hGH in the presence of gut proteolytic enzymes. The stability of hGH was assessed in rat serum, in collected perfusate samples and within the *in situ* model. In addition, the potential for hGH loss through adsorption to the silicone tubing was also evaluated.

A brief series of studies was conducted using perfusate concentrations of hGH and SNAC-2 that produced measurable blood levels of hGH. A total of five different perfusate compositions was assessed, with each tested in 2-3 animals: (i) 1 mg/mL hGH and 30 mg/mL SNAC-2, (ii) 1 mg/mL hGH and 60 mg/mL SNAC-2, (iii) 2 mg/mL hGH and 30 mg/mL SNAC-2 and (iv) 1 mg/mL hGH, 30 mg/mL SNAC-2 and 40 µL/mL PIC. Blood and perfusate levels of hGH were determined as described in Sections 2.1.2.3.5 and 4.2.3.4, respectively, while blood and perfusate concentrations of SNAC-2 were analysed as described in Section 2.2.2.5.

4.2.3 Methods

4.2.3.1 Perfusate Preparation

Stock solutions of hGH (4-5 mg/mL) were prepared in 5 mM phosphate buffer and the concentration determined by UV absorbance (as described in Section 2.1.2.3.1). SNAC-2 (50-100 mg/mL, see Section 2.1.2.3.2) stock solutions were also prepared in 5 mM phosphate buffer. Perfusate solutions containing hGH, with or without SNAC-2 and other excipients, were prepared fresh on the day of each experiment. An appropriate volume of each stock

sclution and 5 mM phosphate buffer pH 7.0 were gently mixed together and the pH of the final solution adjusted to approximately 7.1. Perfusate solution osmolality was measured by freezing point depression utilising a Fiske One-Ten Osmometer (Fiske Associates, Needham Heights, MA) and was adjusted to isotonicity (290 mOsm/kg) through the dropwise addition of 2 M NaCl.

4.2.3.2 Surgical Methods

Male Sprague-Dawley rats (250-350 g) were fasted overnight prior to surgery, with water available *ad libitum*. Rats were anaesthetised by two 1 mL subcutaneous injections of a 20% w/v urethane solution administered 15 minutes apart and then left for one hour before the procedure. Surgical anaesthesia was confirmed by a loss of response to a painful stimulus (paw pinch).

Rats were placed ventral side up on a heating pad (37°C) to maintain body temperature and limbs and tail were held down with surgical tape. The throat and clavicle region and a patch approximately 4 cm wide along the abdominal midline were shaved and cleaned. A 1 cm incision was made directly above the trachea, which was then opened with a scalpel, and a plastic tube inserted and secured with a silk suture to ensure a clear airway throughout the experiment. Next, a small incision was made to the left of the trachea in the sagittal plane and the underlying subcutaneous tissue parted using smooth curved dissecting forceps in order to expose the jugular vein. Mesentery surrounding approximately 1 cm of the vein was carefully removed and the vessel ligated at one end of its exposed length with a silk suture. The vein was then nicked using iridectomy scissors and a bevelled polyethylene cannula (PE-50 tubing, Bioscience, Victoria, Australia) pre-filled with heparinised saline was inserted into the vein to a length of around 2.5 cm. The cannula was sutured firmly in place and its patency confirmed by withdrawing blood into an attached syringe.

Laparotomy was performed by making a longitudinal midline incision through the skin and the abdominal wall to expose the small intestine. The ligament of Treitz was located using moistened cotton tips and a small incision made 2 cm distal to this region, with care being taken to minimally disrupt blood flow. The glass inlet cannula was inserted and securely sutured in place. The outlet cannula was inserted in a similar manner 10 cm distal to the inlet, with the length measured by placing a 10 cm piece of thread along the intestinal segment of interest. Silicone tubing (5/32″ i.d., Cole-Parmer, Niles, IL) was fitted to the inlet and outlet cannulae, with the inlet tubing attached to a Harvard peristaltic syringe pump (Harvard Apparatus, Inc., Holliston, MA), and the outlet tubing located above a 4 mL polypropylene sample collection tube. The cannulated segment of intestine was positioned such that the kinks in it were minimised to prevent the occlusion of perfusate flow. On completion of the surgery, the exposed abdominal area was moistened with saline and covered with plastic wrap to avoid water loss and tissue deterioration throughout the experiment.

4.2.3.3 Experimental Procedure

Following surgery, the intestine was flushed with warmed (37°C) normal saline at a flow rate of 1 mL/min for 20 min to remove any residual contents and the saline was then pumped out with air. Drug solution (37°C) was perfused through the intestine at 1 mL/min until it reached the end of the outlet tubing, at which point the flow rate was reduced to 0.2 mL/min and the experiment commenced.

The duration of each perfusion was typically 60 min, with perfusate collected in pre-weighed polypropylene tubes every 10 min for hGH and SNAC-2 analysis. Weighing the sample tubes prior to and following the experiment allowed the gravimetric analysis of perfusate mass, enabling the correction of drug concentration in perfusate outflow (C_{out}) for water flux into or out of the intestinal segment. PIC (20 µL) was added to each collection tube prior to the addition of sample (2 mL), but was not routinely added to the in-going perfusion

solution. Collected perfusate samples were centrifuged at 1200 *g* for 15 min and two 100 µL aliquots were removed for hGH and SNAC-2 analyses.

Blood samples (500 µL) were collected at time zero (pre-dose), and then at 10, 20, 30, 45 and 60 min from the jugular vein cannula. Half of the blood sample volume (250 µL) was added to a polypropylene tube without anticoagulant, allowed to clot for 5-10 min, centrifuged for 15 min in a microcentrifuge and the serum separated and stored at -20°C prior to analysis for hGH by ELISA (as described in Section 2.1.2.3.5). The remaining 250 µL of blood was added to a polypropylene tube containing heparin (1.25 U), centrifuged and the plasma separated and stored at -20°C prior to analysis for SNAC-2 by RP-HPLC (as described in Section 2.2.2.5).

Following collection of the final sample, animals were sacrificed by a lethal injection of pentobarbitone (approximately 1 g/kg) into the jugular vein. The intestinal segment utilised was subsequently excised and measured to confirm the length of intestine perfused.

4.2.3.4 Size Exclusion Chromatography for Determination of hGH in Perfusate

The concentration and stability of hGH in intestinal perfusate solutions was determined using size exclusion chromatography (SEC). The SEC system consisted of a Beckman 116 pump and 167 UV detector (Beckman Coulter, Inc., Fullerton, CA) with the detection wavelength set to 220 nm. Integration was conducted using Beckman System Gold software version V810. The analytical column was a Pharmacia Superose 12 HR 10/30 (Amersham Pharmacia Biotech, Uppsala, Sweden) and was used at ambient temperature. The mobile phase was 100 mM phosphate buffer, pH 7.5, pumped at a flow rate of 0.7 mL/min and the injection volume was 20 µL. The run time was set to 80 min to allow the elution of the SNAC-2 peak at approximately 68 min. hGH standards were prepared in the concentration range of 50 to 400 µg/mL in the sample buffer and were included in each analytical run.

Calibration curves were constructed by plotting peak area versus hGH concentration and performing a linear regression on the data. The concentration of samples was subsequently calculated from the regression line. Intra-assay precision was assessed by comparing peak areas obtained from three replicate analyses of hGH standard solutions, while inter-assay precision was determined over three separate days. The limit of quantitation was defined as the lowest standard used in the calibration curve for which acceptable accuracy (within ± 20%) and precision (< 15%) was obtained.

Prior to injection, perfusate samples were diluted such that the hGH concentration was within the range of the calibration curve. Samples were centrifuged at approximately 1200 *g* for 15 min and analysed on the same day as the perfusion experiment.

4.2.3.5 Investigation of hGH and SNAC-2 Adsorption to Silicone Tubing

Control experiments were conducted to determine the suitability of the silicone tubing for these studies by performing a mock intestinal perfusion. Perfusate containing 1 mg/mL hGH and 30 mg/mL SNAC-2 (37°C) was pumped through an appropriate length of silicone tubing at a rate of 0.2 mL/min for 60 min and samples were collected every 10 min. Solutions were analysed for hGH content using the SEC assay (Section 4.2.3.4) and SNAC-2 concentration was determined using the RP-HPLC assay described in Section 2.2.2.5.

4.2.3.6 Evaluation of hGH Stability in Serum and Perfusate

hGH stability in rat serum was assessed in earlier studies, as described in Section 2.1.2.3.5. The extent of enzymatic degradation after perfusate sample collection was investigated by spiking hGH into freshly collected blank intestinal perfusate at ambient temperature and monitoring the concentration over a 12 h time period. Blank perfusate was obtained from an intestinal perfusion in which buffer only (i.e. no hGH present in solution) was used as the perfusate. The effect of more extensive cleaning of the intestine (60 min saline

rinse) and the inclusion of PIC (10-100 µL per mL of perfusate) on the stability of hGH in collected perfusate were each also assessed. The concentration of hGH in the spiked, collected perfusate solution was determined using the SEC assay described in Section 4.2.3.4.

4.2.4 Data Analysis

The apparent permeability coefficient (P_{app}) was calculated using Equation 4.1 as follows:

$$P_{app} = \left(- Q/2\pi rl\right) \times \ln\left(C_{out}/C_{in}\right) \qquad (4.1)$$

where Q is the flow rate of the perfusion ($cm^3\ s^{-1}$), r is the effective radius of the lumen (cm), l is the length of the perfused intestinal segment (cm), C_{out} is the concentration of drug leaving the intestine at steady state and C_{in} is the concentration entering the intestine. A literature value for r (0.18 cm) was used to calculate the P_{app} (Komiya et al., 1980).

The area under the serum or plasma concentration versus time curve (AUC) for hGH or SNAC-2, respectively, was calculated by the linear trapezoidal method between 0 and 60 min and was used to compare the effect of the different perfusate compositions on hGH and SNAC-2 uptake from the perfused intestinal segment, particularly where degradation of hGH in perfusate did not allow for calculation of P_{app}. Peak serum and plasma concentrations (C_{max}) of hGH and SNAC-2, respectively, were noted from the individual profiles from each animal. Steady state concentrations were not achieved over the time period of the perfusions and hence this was not utilised as a comparator. A statistical comparison of the permeability coefficients and serum and plasma profiles obtained from the perfusion studies was not conducted due to highly variable data and a small number of experiments, although general trends with the changes in perfusate compositions were assessed from these data.

4.3 RESULTS AND DISCUSSION

4.3.1 Size Exclusion Chromatography

The size exclusion chromatography (SEC) assay provided a means of quantitatively determining the hGH concentration in intestinal perfusate samples and required only minimal sample preparation. The assay demonstrated linearity over the concentration range employed (50 to 400 μg/mL, $r^2 > 0.99$), with 50 μg/mL taken as the limit of quantitation. hGH retention times varied less than 0.1% within day and less than 0.6% between days. Inter-assay precision ranged from 2.8% at 400 μg/mL to 6.4% at 50 μg/mL, while intra-assay precision was less than 3% for all concentrations. A representative chromatogram of hGH in aqueous solution with the carrier SNAC-2, is depicted in Figure 4.1. The peak eluting at 22 min represents hGH, while the large peak eluting at approximately 68 min represents SNAC-2.

4.3.2 Choice of Silicone Tubing

Tubing employed in rat intestinal perfusion studies has included polyethylene (Zimmerman and Johnson, 1991), Tygon (Sinko and Balimane, 1998), and silicone (Langguth et al., 1994a, Crowe and Lemaire, 1998). The primary requirement for tubing used in perfusion experiments is that drug being perfused through it should not adsorb to the tubing. An additional requirement of the tubing is flexibility, such that it may be easily connected to the pump and the inlet/outlet glass cannulae with minimal trauma to the isolated intestinal segment.

Control experiments were conducted to determine the suitability of the silicone tubing for these studies by performing a mock intestinal perfusion. Figure 4.2 presents the results of the hGH and SNAC-2 control experiments, both of which showed no detectable loss of drug from perfusate over the time period investigated. Solutions were analysed for hGH

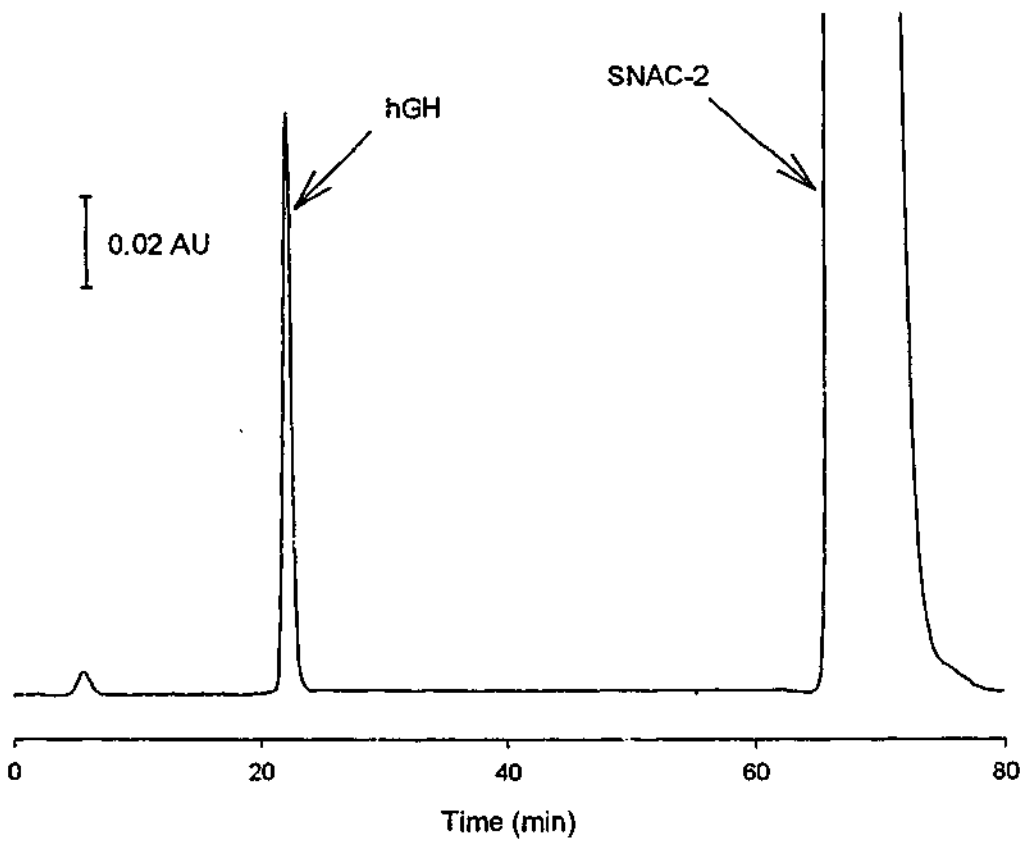

Figure 4.1 Representative SEC chromatogram of 250 μg/mL hGH and 5 mg/mL SNAC-2 in 5 mM phosphate buffer, pH 8.0. Chromatographic conditions were as described in Section 4.2.3.4.

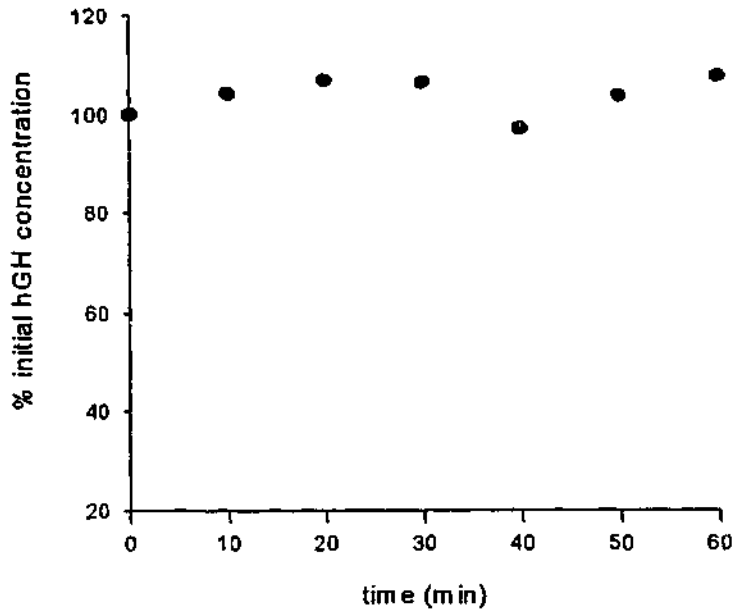

Figure 4.2a

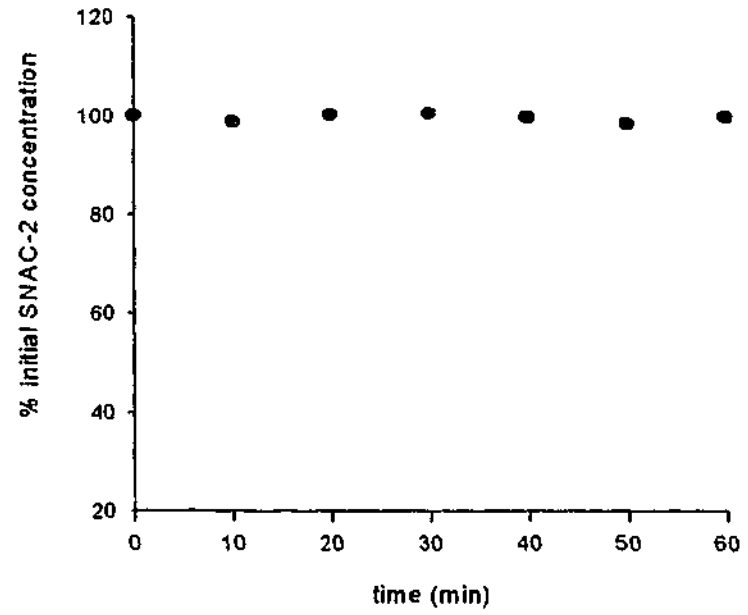

Figure 4.2b

Figure 4.2a Percentage of initial hGH concentration remaining after perfusion through the silicone tubing as a function of time. hGH concentrations were analysed by SEC as described in Section 4.2.3.4.

Figure 4.2b Percentage of initial SNAC-2 concentration remaining after perfusion through the silicone tubing as a function of time. SNAC-2 concentrations were analysed by RP-HPLC as described in Section 2.2.2.5.

content using the SEC assay (Section 4.2.3.4) and SNAC-2 concentration was determined using the RP-HPLC assay described in Section 2.2.2.5.

4.3.3 Choice of Anaesthetic Agent

Selection of an appropriate anaesthetic regimen for intestinal surgery is an important consideration as most, if not all, of these agents have the potential to affect intestinal blood flow and both absorption and secretion processes occurring in the intestine (Coupar, 1985). The most commonly used anaesthetic agents for intestinal absorption experiments are urethane and pentobarbitone (Yuasa et al., 1993). While pentobarbitone has been shown to provide good surgical anaesthesia within a reasonable time period after subcutaneous administration to rats (Coupar, 1985), it has the disadvantage of a high mortality rate and the potential for severe respiratory depression. Urethane was the agent selected for the current study and by way of comparison, produces a stable long lasting anaesthesia with little respiratory depression and low cardiac toxicity, although its carcinogenic properties necessitate suitable precautionary measures when preparing or using solutions (Remie, 1990).

4.3.4 hGH Stability Issues

4.3.4.1 Stability in Serum

The *in vitro* stability of hGH in the presence of freshly collected rat serum enzymes was evaluated and discussed in Chapter Two. No significant loss of hGH in rat serum was detected at a temperature of 37°C over a 6 h period.

4.3.4.2 Stability of hGH in Collected Perfusate

The extent of enzymatic degradation after sample collection was investigated by

spiking hGH into freshly collected blank intestinal perfusate at ambient temperature and monitoring the concentration over a 12 h time period by SEC. In the absence of protease inhibitor, there was a rapid decline in hGH concentration with time, with less than 50% of initial hGH remaining after 3 h incubation, indicating the presence of proteolytic enzymes in the collected perfusate. Modification of the intestinal rinsing procedure by increasing the time from 20 to 60 min (equivalent to 60 mL of saline) made no appreciable difference to the observed loss of hGH. More extensive rinsing of the intestinal segment was therefore unlikely to significantly reduce the degradation by luminal proteases and may have resulted in unwanted damage to the intestinal tissue. The addition of SNAC-2 to the perfusion solution was also observed not to decrease the proteolytic degradation of hGH, suggesting that enzyme inhibition is an unlikely explanation for the mechanism of absorption enhancement of hGH in the presence of the carrier (discussed further in Section 4.3.5).

The proteolytic enzymes present in the jejunum are the luminally secreted pancreatic proteases trypsin, chymotrysin, elastase and carboxypeptidases A and B (Lee, 1988). The addition of a combination of various protease inhibitors was examined as a means of providing the best protection against proteolysis of hGH. The proprietary Protease Inhibitor Cocktail (PIC) was selected which contains a mixture of inhibitors with a broad specificity for the inhibition of a range of proteases. The mixture includes 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), bestatin, leupeptin and aprotinin in DMSO. Aprotinin is reported in be an effective inhibitor of trypsin and chymotrypsin, while leupeptin and AEBSF also inhibit trypsin (Bernkop-Schnurch, 1998). The concentrations of the inhibitors present in PIC were not disclosed by the manufacturer, although a recommendation of 100 µL of the cocktail to a suspension of 10^7 mammalian cells per mL is made in the accompanying literature.

The presence of PIC in hGH solutions was determined not to interfere with the analysis of hGH by the SEC assay, with two large peaks eluting at approximately 25 and 34

min attributed to components present in PIC. Both PIC peaks were sufficiently well separated from hGH, which elutes at approximately 21.5 min.

A nominal volume of 100 µL of PIC was included per 1 mL of freshly collected blank perfusate prior to the addition of hGH. Figure 4.3 depicts the effect of PIC on the *in vitro* stability of hGH in collected perfusate over time, with PIC shown to completely inhibit the proteolytic degradation of hGH under these conditions. A 24 h sample was also taken from the incubation mixture and demonstrated no loss of hGH over this time period, confirming the stability of hGH in the presence of PIC between collection and analysis by SEC.

In an effort to reduce the consumption of PIC, further experiments were conducted to determine whether a concentration of 10 µL of PIC per 1 mL of perfusate was equally effective in preventing the proteolysis of hGH. This lower concentration also provided complete protection against proteolysis and hence a 20 µL aliquot of PIC (for a 2 mL sample) was included in perfusate sample collection tubes for all intestinal perfusion experiments. The dilution of perfusate samples prior to SEC analysis (to reduce the hGH concentration to be within the range of the standard curve) was also performed using an appropriate concentration of PIC in buffer such that the necessary concentration of PIC was maintained in the sample.

4.3.4.3 In Situ Enzymatic Stability of hGH

The accurate determination of P_{app} relies on the difference between the concentration of drug entering the intestine (C_{in}) and the concentration of drug measured leaving the intestine at steady state (C_{out}) being due to intestinal absorption only, not loss of drug due to degradation in the intestinal environment. It was essential therefore that hGH stability also be investigated within the *in situ* model.

The determination of hGH in the first collected perfusate sample following the commencement of the intestinal perfusion experiment (10 min time point) was enormously variable when compared to the known concentration (measured in initial perfusate solution

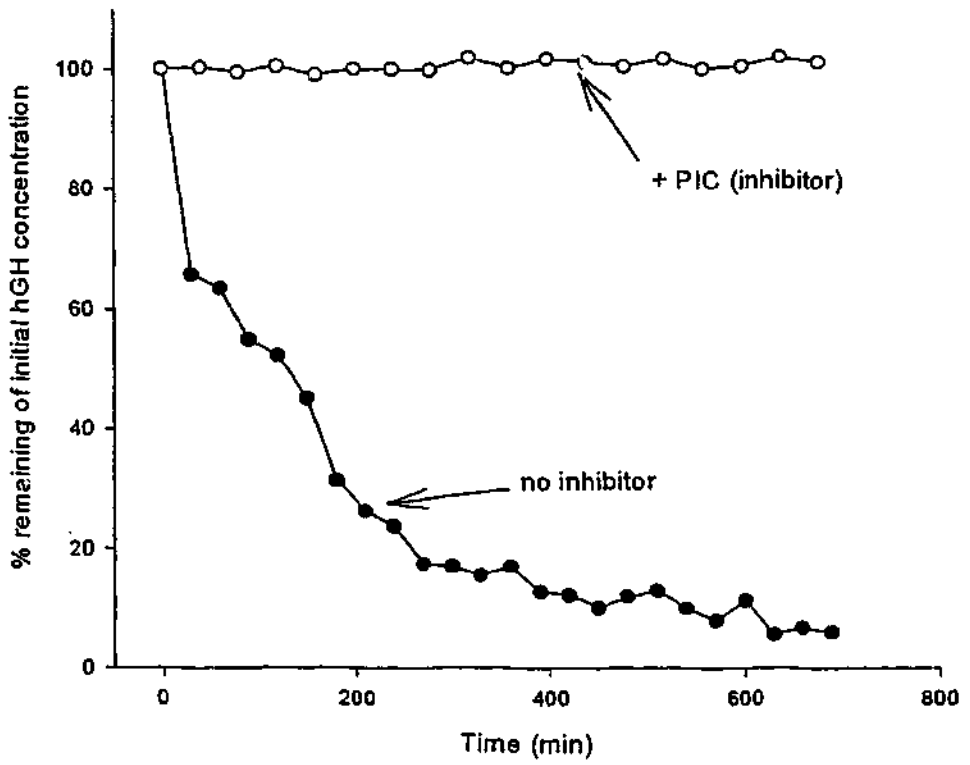

Figure 4.3 hGH concentration (as a percentage of initial concentration) with and without PIC (open and closed circles, respectively) versus time after spiking into collected perfusate. hGH concentrations were analysed by SEC as described in Section 4.2.3.4.

prior to perfusion). From one perfusion to the next, the percentage of hGH remaining in the collected perfusate at 10 min ranged from 14 to 81% of that known to be present in the initial perfusing solution. The significant loss of drug observed in these experiments was thought to most likely reflect varying degrees of proteolytic degradation within the intestinal segment.

In addition to luminal enzymes within the intestine (described in Section 4.3.4.2), there are a substantial number of other proteolytic enzymes present in the brush border membrane and the cytosol of the enterocyte. These include aminopeptidases, carboxypeptidases, endopeptidases, γ-glutamyl transpeptidase, dipeptidyl peptidase IV, enteropeptidase and angiotensin converting enzyme (Lee, 1988, Bernkop-Schnurch, 1998). The optimum pH range at which these enzymes function is between 7.5 and 8.5. Friedman and Amidon (Friedman and Amidon, 1991) attempted to overcome enzymatic degradation of the pentapeptide, leu-enkephalin, in the rat intestinal perfusion model by decreasing jejunal pH with the purpose of reducing enzyme activity. They observed that at pH 5 the activity of aminopeptidases was markedly reduced and to a smaller extent, endopeptidase activity was also reduced. Reduction of perfusate pH to 5 or below to improve the stability of hGH in the current studies may have reduced the *in situ* proteolytic degradation, although the high concentration of SNAC-2 thought necessary to promote hGH absorption was likely to precipitate out of the acidified perfusate (SNAC-2 is a weak acid with a pKa of approximately 5). Friedman also observed that proteolysis of leu-enkephalin occurred predominantly as a result of brush border enzyme activity, rather than luminal enzymes. Having already determined in the present study that more extensive "cleaning" of the intestinal segment had a negligible effect on proteolytic degradation (Section 4.3.4.2), it was likely that the loss of hGH was also due to the brush border enzymes.

Prior to conducting further studies on the use of protease inhibitors for the prevention of hGH degradation *in situ* however, a brief series of experiments was conducted using the rat intestinal perfusion model in order to evaluate the effect of formulation variables

on the resulting hGH and SNAC-2 blood profiles and to measure the permeability of the carrier compound itself.

4.3.5 hGH Serum and Perfusate Concentrations in *In Situ* Intestinal Perfusion Experiments

A series of experiments was conducted to determine suitable perfusate concentrations of hGH and SNAC-2, with the objective being to utilise concentrations which would produce measurable serum levels of hGH in the intestinal perfusion model. In the absence of carrier, an hGH perfusate concentration of up to 1 mg/mL pumped through the intestinal segment at a flow rate of 0.2 mL/min failed to produce detectable serum levels (i.e. the resulting concentrations were below the limit of quantitation of the ELISA of 1.5 ng/mL). Only minimal serum concentrations of hGH were found using hGH concentrations in the range of 14 to 240 µg/mL combined with SNAC-2 concentrations of up to 12 mg/mL. An hGH concentration of 1 mg/mL together with a SNAC-2 concentration of 30 mg/mL or greater however, was found to produce significant and measurable serum hGH concentrations.

Figure 4.4 presents the hGH serum profile obtained using 1 mg/mL hGH and 30 or 60 mg/mL SNAC-2 perfusate concentrations, while Figure 4.5 presents the effect of doubling the hGH perfusate concentration to 2 mg/mL using 30 mg/mL SNAC-2. Increasing the SNAC-2 perfusate concentration increased the hGH serum concentrations to a greater extent than by increasing the hGH perfusate concentration. The effect of the addition of PIC (40 µL/mL) to the perfusate is shown in Figure 4.6, with no significant difference apparent in the hGH serum profiles. Possible effects of DMSO (PIC solvent, present in perfusate at a concentration of approximately 4% v/v), as distinct from inhibitor effects, were not assessed in these experiments. These trends are reflected in the data described in Table 4.2, with both the AUC and the C_{max} for hGH being increased in the presence of 60 mg/mL SNAC-2, while no changes were observed upon the addition of PIC to the perfusate. AUC values were used to

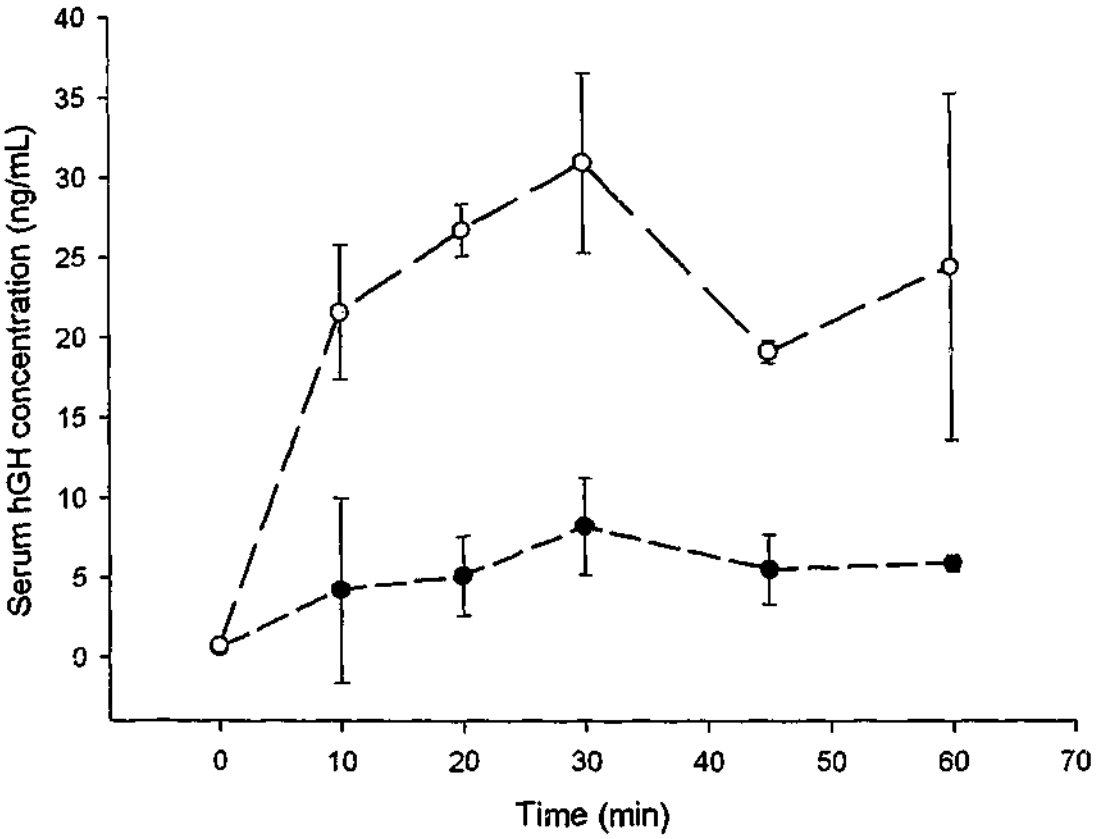

Figure 4.4 hGH serum profiles obtained using 1 mg/mL hGH and 30 mg/mL SNAC-2 (closed circles) and 1 mg/mL hGH and 60 mg/mL SNAC-2 (open circles) perfusate concentrations. For these experiments, PIC was not included in the perfusate solution but was added to the collected exiting perfusate samples. Symbols represent mean values and error bars represent standard deviations for n=3 measurements. hGH serum concentrations were determined using the ELISA as described in Section 2.1.2.3.5.

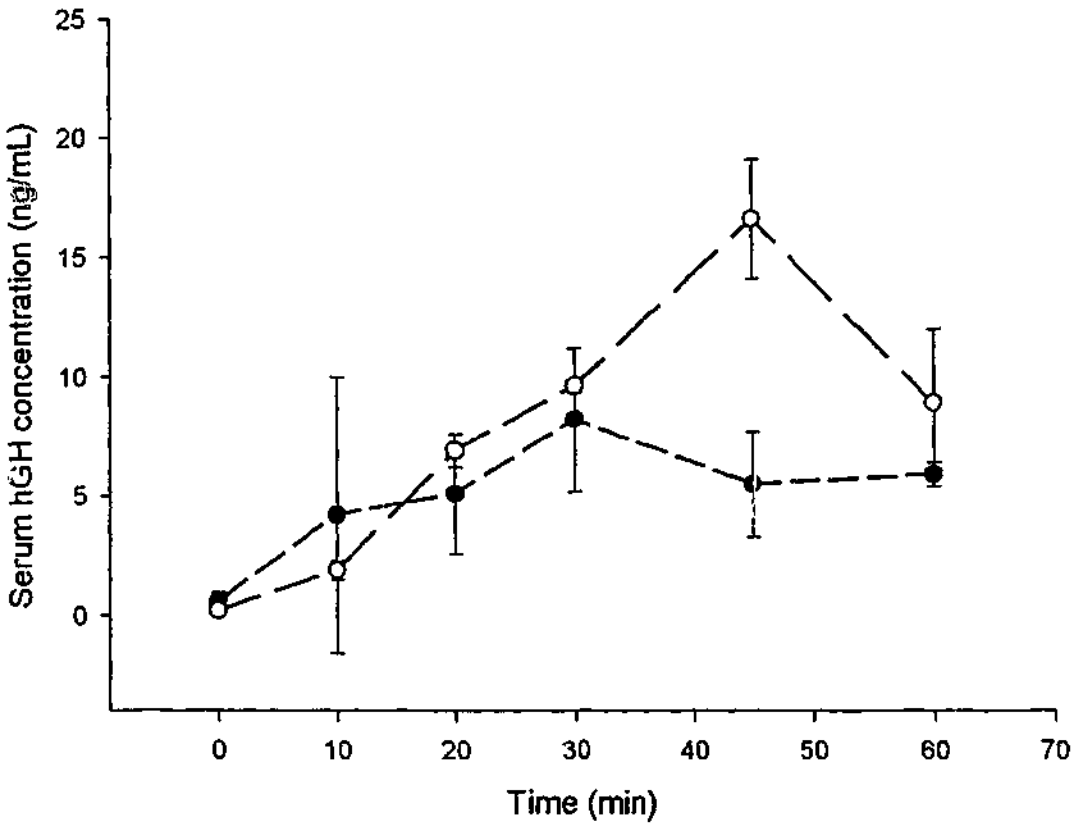

Figure 4.5 hGH serum profiles obtained using 1 mg/mL hGH and 30 mg/mL SNAC-2 (closed circles) and 2 mg/mL hGH and 30 mg/mL SNAC-2 (open circles) perfusate concentrations. For these experiments, PIC was not included in the perfusate solution but was added to the collected exiting perfusate samples. Symbols represent mean values and error bars represent standard deviations for n=3 measurements. hGH serum concentrations were determined using the ELISA as described in Section 2.1.2.3.5.

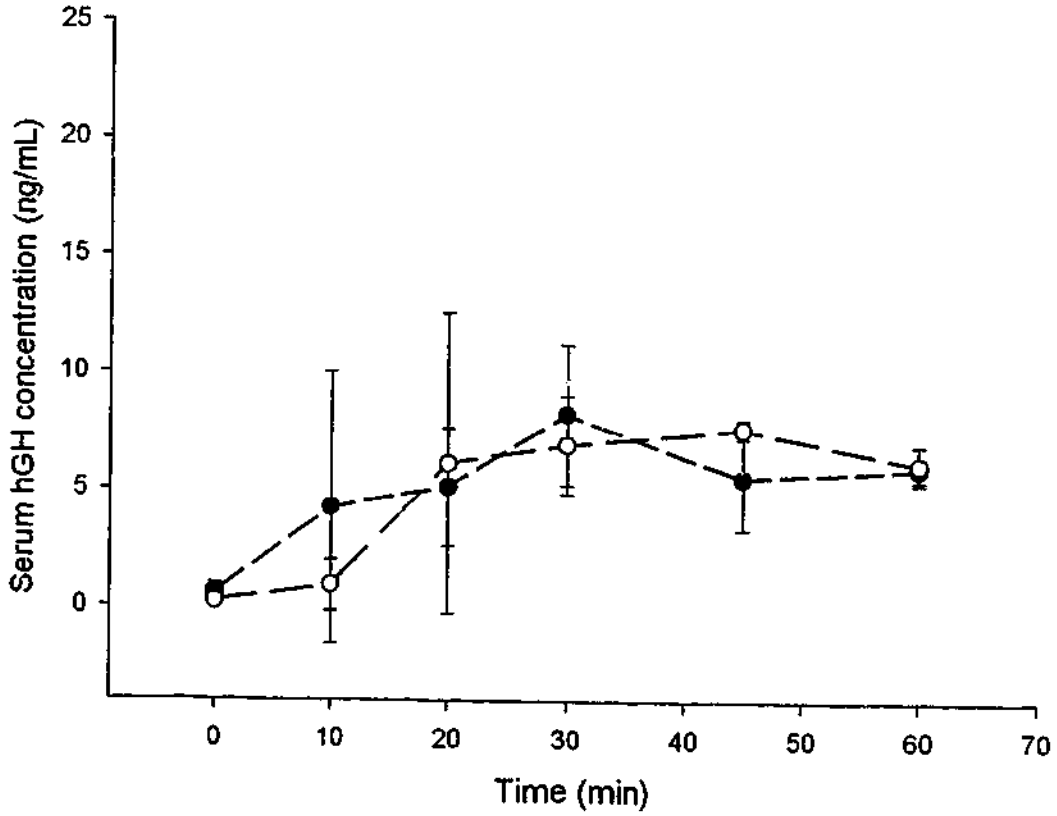

Figure 4.6 hGH serum profiles obtained using 1 mg/mL hGH and 30 mg/mL SNAC-2 (closed circles) and 1 mg/mL hGH, 30 mg/mL SNAC-2 and 40 μL/mL PIC (open circles) perfusate concentrations. In both cases, PIC was added to the exiting, collected perfusate samples. Symbols represent mean values and error bars represent standard deviations for n=2-3 measurements. hGH serum concentrations were determined using the ELISA as described in Section 2.1.2.3.5.

Table 4.2 hGH serum data following intestinal perfusion studies. The C_{max} and AUC were calculated as described in Section 4.2.4. Serum concentrations were measured using the hGH ELISA as described in Section 2.1.2.3.5.

hGH perfusate concentration	*SNAC-2 perfusate concentration*	*Flow rate (mL/min)*	*Total time of perfusion (min)*	*No. of rats (n)*	*$C_{max} \pm SD$ (ng/mL)*	*AUC ± SD (ng min/mL)*
14 µg/mL	340 µg/mL	0.2	90	1	none detected	-
16 µg/mL	403 µg/mL	0.2	90	1	none detected	-
20 µg/mL	490 µg/mL	0.2	60	1	none detected	-
100 µg/mL	836 µg/mL	0.2	90	1	< 3.0	-
210 µg/mL	3.5 mg/mL	0.2	45	1	< 4.0	-
240 µg/mL[a]	12 mg/mL	0.2	45	1	< 5.0	-
1 mg/mL	0 mg/mL	0.2	60	2	none detected	-
1 mg/mL	30 mg/mL	0.2	60	3	9.9 ± 2.0	324 ± 76
1 mg/mL	60 mg/mL	0.2	60	3	34.2 ± 3.0	1209 ± 249
2 mg/mL	30 mg/mL	0.2	60	2	16.6[b]	524[b]
1 mg/mL	30 mg/mL[c]	0.2	60	2	9.0[b]	316[b]

[a] jugular vein cannula blocked – not all blood samples collected
[b] average of n=2
[c] including 40 µL/mL PIC in perfusion solution

provide a means by which a general comparison of the serum profiles following the intestinal perfusions could be made.

The concentration of hGH in perfusate was determined using the SEC assay as described in Section 4.2.3.4. While the use of PIC was demonstrated to effectively inhibit the degradation of hGH in collected perfusate (Section 4.3.4.2), the prevention of hGH proteolysis within the *in situ* model remained a problem. Significant and variable loss of hGH from perfusate was observed to lead to equally variable perfusate profiles. Figure 4.7 presents selected exiting perfusate profiles obtained for intestinal perfusion experiments conducted using perfusates containing 1 mg/mL hGH only, 1 mg/mL hGH and 30 mg/mL SNAC-2, and 1 mg/mL hGH and 30 mg/mL SNAC-2 combined with 40 µL/mL PIC (the use of PIC in the perfusion solution is discussed further in Section 4.3.7). Similar profiles were obtained for hGH in the absence and presence of SNAC-2, with the addition of PIC to the actual perfusion solution also failing to make any difference to the perfusate profiles. These results suggest that it was unlikely that SNAC-2 was acting as an enzyme inhibitor. This finding is consistent with studies conducted by Emisphere Technologies, Inc. on structurally related carrier compounds which demonstrated only minimal enzyme inhibitory activity (Leone-Bay et al., 1995). Further, owing to the persistent and variable loss of hGH from perfusate and the failure of hGH absorption to reach steady state, the apparent permeability coefficient (P_{app}) of hGH could not be calculated from these data.

4.3.6 SNAC-2 Plasma and Perfusate Concentrations

Figures 4.8 and 4.9 present selected plasma profiles for SNAC-2 obtained under various conditions. A general trend toward rapid absorption over the first 10 min was observed for all the SNAC-2 plasma profiles, followed by a gradual decline or plateau in concentration. The average of the last two plasma concentrations for each profile was reported (C_{avg}) rather than C_{max}. Doubling the SNAC-2 perfusate concentration to 60 mg/mL resulted

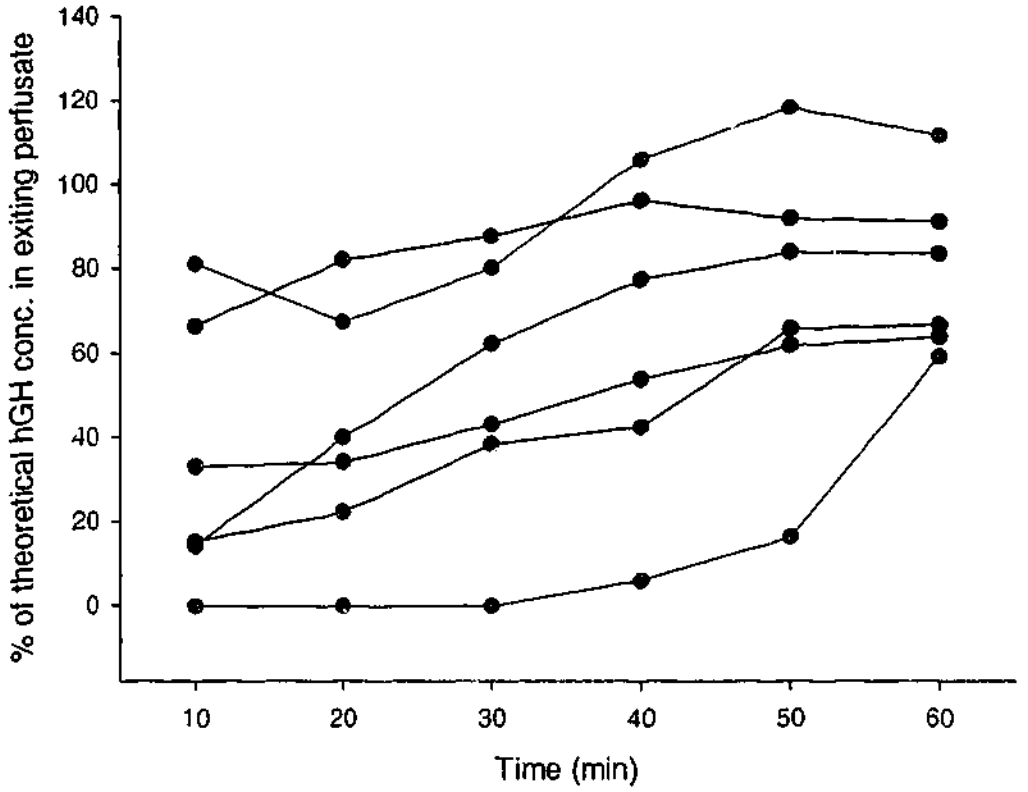

Figure 4.7 Representative perfusate profiles for hGH obtained from six individual perfusion experiments employing 1 mg/mL hGH only (black symbols), 1 mg/mL hGH and 30 mg/mL SNAC-2 (blue symbols) and 1 mg/mL hGH, 30 mg/mL SNAC-2 and PIC (red symbols).

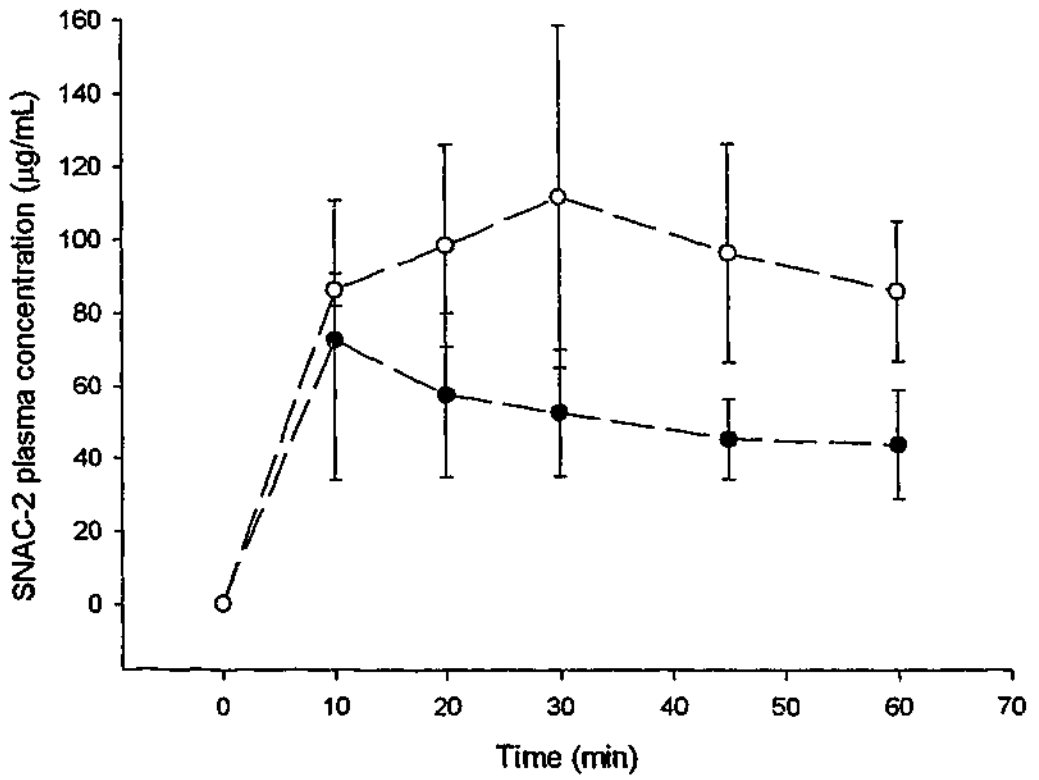

Figure 4.8 SNAC-2 plasma profiles obtained using 1 mg/mL hGH and 30 mg/mL SNAC-2 (closed circles) and 1 mg/mL hGH and 60 mg/mL SNAC-2 (open circles) perfusate concentrations. Symbols represent mean values and error bars represent standard deviations for n=3 measurements. SNAC-2 plasma concentrations were determined using the RP-HPLC assay as described in Section 2.2.2.5.

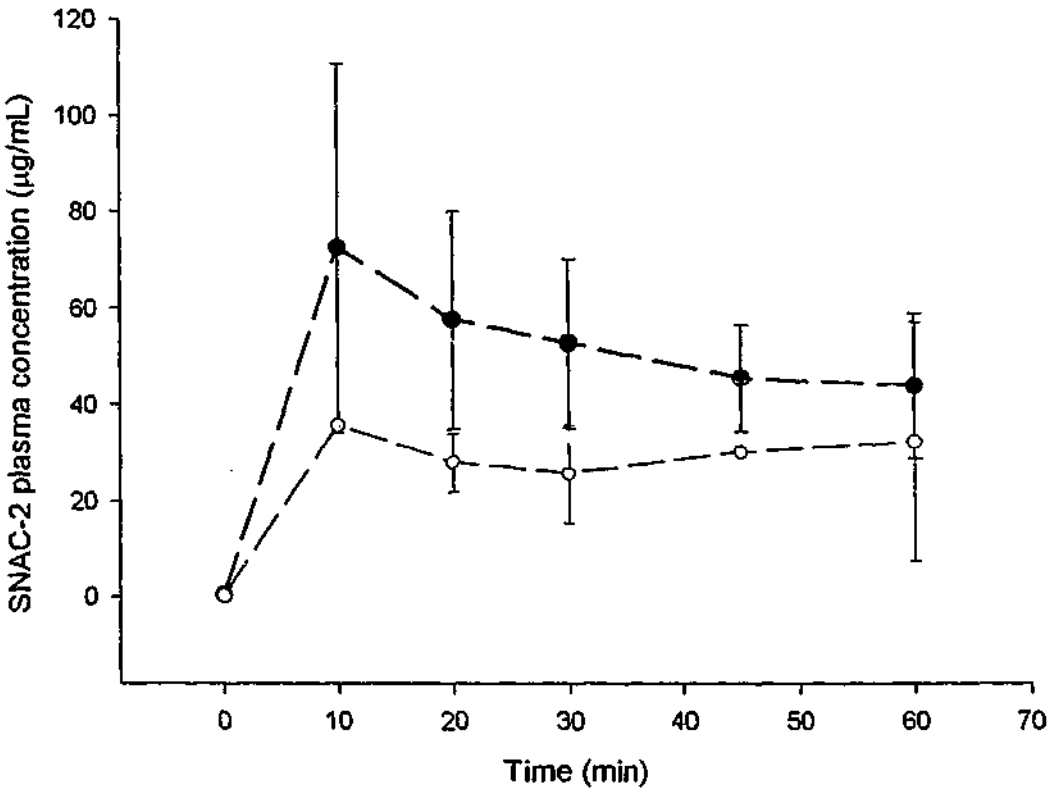

Figure 4.9 SNAC-2 plasma profiles obtained using 1 mg/mL hGH and 30 mg/mL SNAC-2 (closed circles) and 2 mg/mL hGH and 30 mg/mL SNAC-2 (open circles) perfusate concentrations. Symbols represent mean values and error bars represent standard deviations for n=2-3 measurements. SNAC-2 plasma concentrations were determined using the RP-HPLC assay as described in Section 2.2.2.5.

in a marked increase in plasma concentrations (Figure 4.8), while doubling the hGH concentration to 2 mg/mL lead to a slight reduction in SNAC-2 plasma levels (Figure 4.9).

AUC and C_{avg} data for SNAC-2 are detailed in Table 4.3, with the AUC increasing with the use of the 60 mg/mL SNAC-2 perfusate concentration. Addition of 2 mg/mL hGH to the perfusate reduced the SNAC-2 AUC to approximately half that determined using 1 mg/mL hGH and 30 mg/mL SNAC-2. The basis for this reduction in the AUC is unknown, and further experiments were not conducted due to the evidence of hGH degradation in the intestinal perfusion studies.

A typical perfusate profile for SNAC-2 is depicted in Figure 4.10, with the data obtained using a perfusate containing 1 mg/mL hGH and 30 mg/mL SNAC-2. The SNAC-2 perfusate profiles were consistent with that expected from an intestinal perfusion experiment and as such, were used to determine apparent permeability coefficients (P_{app}). Table 4.4 presents the P_{app}s calculated for SNAC-2 under the various conditions investigated. The influx/efflux of water was found to be minimal for all formulations/perfusates since sample weights were within ± 5% of theoretical based on perfusion flow rate and collection times. The higher SNAC-2 perfusate concentration (60 mg/mL) produced an apparent increase in the P_{app}, while increasing the hGH concentration to 2 mg/mL lead to a decrease in the P_{app}. The P_{app}s determined for SNAC-2 using the *in situ* technique are of the same order of magnitude to those obtained using compounds of a similar molecular weight range. Fagerholm and co-workers reported the intestinal permeability coefficients for a series of passively transported compounds (MW 188-331) with varying physicochemical properties (pKa, log D) obtained using the *in situ* rat intestinal perfusion model to range from 0.1-2.4 x 10^{-4} cm/s in jejunum (Fagerholm et al., 1996).

4.3.7 Enzyme Inhibitors for the Prevention of hGH Degradation *In Situ*

The occurrence of proteolytic enzymes and the rate of degradation effected by these

Table 4.3 SNAC-2 plasma data acquired from preliminary intestinal perfusion studies. The AUC was calculated as described in Section 4.2.4. Perfusion flow rate for these experiments was 0.2 mL/min and total time of perfusions was 60 min. Plasma SNAC-2 concentrations were determined using the RP-HPLC assay as described in Section 2.2.2.5. Data are reported as mean ± SD (n=3).

hGH Perfusate concentration	*SNAC-2 Perfusate Concentration*	C_{avg}[a] *(μg/mL)*	*AUC (μg min/mL)*
1 mg/mL	30 mg/mL	44.5 ± 12.9	2963 ± 1098
1 mg/mL	60 mg/mL	104.2 ±38.8	4788 ± 440
2 mg/mL	30 mg/mL	28.2[b]	1426[b]

[a] average of the last two measured concentrations
[b] average for n=2

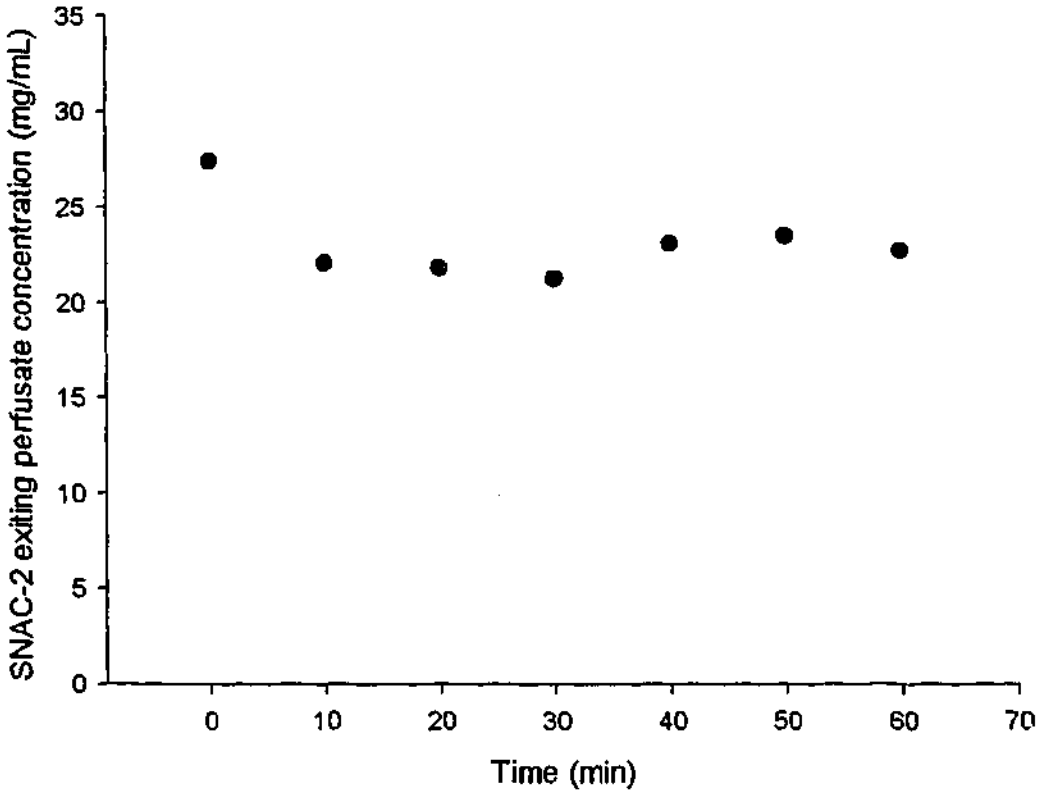

Figure 4.10 Representative SNAC-2 perfusate profile obtained from an experiment employing 1 mg/mL hGH and 30 mg/mL SNAC-2 in the perfusion solution. The concentration of SNAC-2 was determined using the RP-HPLC assay as described in Section 2.2.2.5.

Table 4.4 SNAC-2 permeability data obtained from preliminary intestinal perfusion studies employing a perfusion flow rate of 0.2 mL/min for 60 min. Permeability coefficients (P_{app}) were calculated as described in Section 4.2.4.

SNAC-2 perfusate conc. (mg/mL)	*hGH perfusate conc. (mg/mL)*	P_{app} *(x 10^4 cm/s)*
30	1	0.50[a]
60	1	1.74 ± 0.95[b]
30	2	0.14[a]

[a] average for n=2
[b] mean ± SD (n=3)

enzymes varies along the gastrointestinal tract, with pancreatic enzymes such as trypsin and chymotrypsin showing greatest activity in the duodenum and proximal jejunum, while much lower activity is observed in the distal small intestine (Langguth et al., 1997). An examination of the degradation and absorption of the synthetic peptide metkephamid in the rat by Langguth and co-workers demonstrated negligible enzymatic metabolism in the colon, a phenomenon attributed to the lower levels of aminopeptidases in this region (Langguth et al., 1994b). It is possible that the use of isolated colon segments in the current rat intestinal perfusion studies would significantly reduce the rate and extent of proteolytic degradation of hGH compared to that observed in the jejunum. However, while the study of regional differences of hGH permeability would be of interest, the absorption of hGH following oral administration is most likely to occur from the proximal small intestine and therefore mechanistic studies conducted only in colonic tissue may not be representative of *in vivo* absorption mechanisms.

The use of protease inhibitors to enhance the stability of peptide and protein drugs in the intestine has been extensively reviewed (Lee, 1988, Zhou, 1994, Langguth et al., 1997, Bernkop-Schnurch, 1998) and appears to be best approached on a case by case basis. Studies investigating the oral bioavailability of hGH have generally focussed on the use of absorption enhancing compounds which act by modifying intestinal membrane permeability characteristics rather than by enzyme inhibition. For example, the use of sodium salicylate and mineral oil for enhancing the intestinal absorption of methionyl hGH in rats has been described (Moore et al., 1986). In these studies, the mechanism of absorption enhancement of hGH was thought to be due to an alteration in membrane permeability, although the authors suggested that the oil vehicle may have also protected the hGH from enzymatic degradation prior to absorption. The aminopeptidase inhibitors amastatin and bestatin were employed by O'Hagan and co-workers (O'Hagan et al., 1990) to promote the nasal absorption of hGH in rats, with amastatin shown to increase the bioavailability of hGH from 7.4% (no enhancer) to 28.9%, while bestatin did not improve hGH bioavailability. It is unlikely however, that the effect of amastatin on hGH absorption observed in rat nasal tissue by O'Hagan et al would

also be observed in the intestine.

With adequate stability in collected perfusate samples having been achieved using PIC, an attempt was made to inhibit the proteolytic degradation of hGH in the *in situ* model by the inclusion of PIC in the intestinal perfusate. Results of preliminary studies employing four times the concentration of PIC used to prevent degradation in the collected perfusate samples made no detectable difference to the *in situ* degradation, with the concentration of hGH at the first time point of the perfusion still being significantly variable (0 to 66%).

A thorough search for an appropriate enzyme inhibitor or combination of inhibitors for use with hGH in the *in situ* model would entail considerable time and effort with no guarantee of success. The search would necessarily involve relatively complex and time-consuming studies regarding hGH degradation mechanisms and kinetics. The focus of the present studies was the investigation of the interaction of hGH with the carrier SNAC-2 and to use the *in situ* rat intestinal perfusion model to quantify and define the mechanism of intestinal absorption enhancement for hGH in the presence of SNAC-2. With the intestinal perfusion data obtained thus far, it appeared unlikely that the permeability of hGH could be accurately determined using this technique and it was decided therefore to consider the use of an *in vitro* model for examining hGH permeability, where experimental variables may be more readily controlled.

4.4 SUMMARY

The feasibility of the single pass *in situ* rat intestinal perfusion model for examining the permeability of hGH in the presence of the model carrier compound, SNAC-2, was investigated in these studies. Significant proteolytic degradation of hGH gave rise to reduced and variable concentrations of drug in perfusate throughout the experiment and instability of the drug in collected perfusate. While the addition of PIC to perfusate samples successfully prevented further proteolysis of hGH following sample collection, attempts to provide adequate protection of hGH against enzymatic degradation within the *in situ* model,

firstly, by more extensive rinsing of the intestinal segment, and secondly, through the addition of PIC to the actual perfusion solution, failed to inhibit enzymatic degradation.

A series of experiments conducted using the model demonstrated the effects of altering perfusate composition on the resulting blood levels of hGH and SNAC-2. Increasing the perfusate SNAC-2 concentration from 30 to 60 mg/mL lead to an increase in both hGH and SNAC-2 blood levels, while increasing perfusate hGH concentration from 1 to 2 mg/mL produced a small increase in hGH serum concentration and a reduction in SNAC-2 plasma concentrations. The presence of PIC in the perfusate resulted in no detectable difference in the hGH serum concentrations.

The intestinal permeability (P_{app}) of SNAC-2 was determined in a small number of animals and was comparable to that reported for compounds of a similar molecular weight. Changes to both hGH and SNAC-2 perfusate concentrations impacted on the P_{app} of the carrier, with higher SNAC-2 perfusate concentrations resulting in an increased P_{app}, while higher hGH perfusate concentrations resulted in a reduced P_{app}.

The problematic nature of the *in situ* rat intestinal perfusion model for application in these studies led to its abandonment in favour of an *in vitro* permeability model in which experimental variables may be more readily controlled and enzymatic degradation less likely to occur. The development and use of an *in vitro* modified Ussing chamber model for examining the intestinal permeability of hGH in the presence of SNAC-2 is described in Chapters Five and Six.

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CHAPTER FIVE

A MODIFIED USSING CHAMBER MODEL FOR ASSESSING hGH INTESTINAL PERMEATION

5.1 GOALS

The objective of the studies described in this chapter was to establish a modified Ussing chamber model for determining the intestinal permeability of hGH. Advantages of this commonly used approach include greater experimental control (e.g. compared to *in situ* or *in vivo* models), the potential to elucidate absorption mechanisms and the capability to obtain more data from fewer animals using relatively simple surgical techniques.

Despite the widespread use of such *in vitro* models for examining intestinal permeability, the incorporation of a protein into this system was unlikely to be straightforward. Having already attempted to measure the permeability of hGH using a rat *in situ* intestinal perfusion model (see Chapter 4) and observing the difficulty presented by enzymatic degradation of hGH within the intestine, these aspects were again considered within the *in vitro* model. In addition to the potential for enzymatic degradation, he significant tendency of hGH to aggregate in the presence of a high air water interface, such as that induced by mixing/bubbling, was also likely to be problematic in the modified Ussing chamber.

This study involved the establishment and validation of a modified Ussing chamber model incorporating isolated rabbit ileum for the measurement of hGH permeability. The use of radiolabelled marker compounds (^{14}C-mannitol, ^{14}C-D-glucose and ^{3}H-diazepam) was employed to confirm tissue permeability characteristics and allow comparisons to published data. Tissue samples were also evaluated by standard histological techniques in order to confirm viability and integrity. Analysis of hGH in the Ussing chamber was accomplished by SEC for donor chamber samples (approximately 0.5 mg/mL) and the ELISA for receiver chamber samples (ng/mL concentrations). Initial hGH studies involved an investigation into the stability of hGH in the Ussing chamber and the use of various approaches including excipients (Pluronic® F127, Tween 20 and sodium taurocholate) and the enzyme inhibitor, aprotinin, for preventing hGH loss. Furthermore, the utilisation of excipients within the model necessitated an evaluation of possible

effects on the solution conformation of hGH, potential interference with the ELISA and the effects of excipients on the permeability of mannitol and diazepam, with any changes being indicative of altered tissue permeability characteristics.

5.2 EXPERIMENTAL

5.2.1 Materials

Calcium chloride dihydrate, sodium taurocholate and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium chloride, potassium chloride and magnesium chloride hexahydrate were obtained from Ajax Chemicals (New South Wales, Australia). Mannitol (Merck, Darmstadt, Germany), D-glucose and sodium bicarbonate (BDH Chemicals Australia Pty. Ltd., Australia) were of analytical grade. Nembutal® (pentobarbitone sodium, 60 mg/mL) was purchased from Rhone Merieux Australia Pty Ltd (Queensland, Australia) and Starscint® scintillation fluid was obtained from Packard Bioscience B.V. (Groningen, The Netherlands). The marker compounds ^{14}C-mannitol, ^{3}H-diazepam and ^{14}C-D-glucose were obtained from NEN™ Life Science Products, Inc. (Boston, MA). All other reagents were of analytical grade or higher. Water was obtained from a Milli-Q (Millipore, Bedford, MA) water purification system and was used throughout.

5.2.2 Study Design

All experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Experimentation Ethics Committee. Male New Zealand White rabbits of 2-3.5 kg (Nanowie Small Animal Production Unit, Victoria, Australia) were employed. Rabbits were housed in standard cages with free access to food and water.

Establishment of the *in vitro* model initially involved the determination of the intestinal permeability of marker compounds under standard conditions, i.e. in standard diffusion buffer at 37°C, in order to assess tissue permeability characteristics and allow comparisons, where possible, to previously published data obtained using similar models. Mannitol and diazepam were employed as passive paracellular and transcellular transport markers, respectively, and D-glucose was utilised as a marker of active transport. A histological evaluation was used to confirm tissue integrity and viability in the modified Ussing chamber.

The introduction of hGH into the *in vitro* model necessitated a stability study, both for an examination of the susceptibility of hGH to enzymatic degradation and also potential loss due to mixing-induced aggregation and precipitation. The effect of the inclusion of the enzyme inhibitor, aprotinin, and the surface active agents, Pluronic® F127, Tween 20 and sodium taurocholate, on the concentration of hGH in the diffusion chamber over time was studied.

Addition of excipients to the diffusion buffer for the purpose of enhancing hGH stability in turn required supportive studies to investigate the effect of excipients on hGH and its analysis, in addition to possible effects on the permeability of marker compounds. The conformation of hGH in the presence of excipients was followed by circular dichroism and potential interference of excipients with both the SEC assay and the ELISA was assessed. The permeability of mannitol and diazepam was determined in the presence of excipients in an attempt to monitor any changes in tissue transport characteristics.

5.2.3 Methods

5.2.3.1 Diffusion Buffer Preparation

The standard intestinal bicarbonate diffusion buffer was prepared by a previously described method (Smith, 1996). Briefly, four stock solutions, A, B, C and D, were prepared as outlined in Table 5.1. A 25 mL aliquot was withdrawn from each stock solution and added to

Table 5.1 Stock solutions utilised for preparing the standard diffusion buffer used in the Ussing chamber model. Buffer preparation is described in Section 5.2.3.1.

Components of stock solutions	*Grams added to 250 mL*
Stock A	
Sodium chloride	32.71
Potassium chloride	1.87
Stock B	
Calcium chloride dihydrate	0.88
Magnesium chloride hexahydrate	1.22
Stock C	
Sodium dihydrogen orthophosphate monohydrate	0.28
Disodium hydrogen orthophosphate anhydrous	1.14
Stock D	
Sodium bicarbonate	10.5

approximately 350 mL of water in a 500 mL volumetric flask after which the volume was adjusted to 500 mL. If the resulting solution appeared cloudy, carbogen was bubbled through the buffer until it became clear, giving a final pH of approximately 7.4. The final composition of the diffusion buffer was (in mM): Na^+, 141; K^+, 5; Ca^{2+}, 1.2; Mg^{2+}, 1.2; Cl^-, 122; HCO_3^-, 25; $H_2PO_4^-$, 0.4; and HPO_4^{2-}, 1.6.

The serosal bathing buffer comprised standard diffusion buffer to which D-glucose (10 mM) was added to help maintain tissue viability, while the mucosal bathing buffer comprised standard diffusion buffer to which mannitol (10 mM) was added to balance the osmotic load between mucosal and serosal solutions. For D-glucose permeability experiments, 10 mM D-glucose was placed on the mucosal side instead of mannitol. Excipients were examined for their ability to inhibit hGH aggregation and were added to both mucosal and serosal bathing buffers. Pluronic® F127 was employed at concentrations of 0.1% w/v and 0.01% w/v, Tween 20 at concentrations of 0.1% w/v and 0.25% w/v and taurocholate at concentrations of 2 mM and 5 mM. Lyophilised hGH was dissolved in diffusion buffer with or without excipients to give a final concentration of 0.5 mg/mL. The solution preparation of hGH and concentration determination by UV spectroscopy were conducted as described in Section 2.1.2.3.1.

5.2.3.2 Surgical Methods

Male New Zealand white rabbits, weighing between 2-3.5 kg and maintained on standard rabbit chow and water *ad libitum*, were fasted overnight prior to experiments. Rabbits were sacrificed by an injection of a lethal dose of pentobarbitone sodium into the marginal ear vein. The abdomen was shaved and a longitudinal midline incision through the skin and abdominal wall performed such that the small intestine was exposed. A 20 cm segment of ileum was quickly removed from the intestinal region around 10 cm proximal to the *sacculus rotundus*. The intestinal segment was rinsed clean with ice-cold diffusion buffer and threaded onto a

smooth glass rod (diameter approximately 12 mm), and the serosal muscle layers stripped away in a manner similar to that described previously (Sutton et al., 1992). The blunt edge of a scalpel blade was employed to lightly score the intestinal segment and, using curved jewellers forceps, gently peel the muscle layers away from the tissue starting at the score line.

Following the stripping procedure, the intestine was opened longitudinally along the mesenteric border using the sharp edge of the scalpel blade, and laid flat, mucosal side up, on filter paper soaked in cold diffusion buffer. Sections of tissue (approximately 1.5 cm in length) were cut and mounted onto the pins of the acrylic half chamber, the filter paper carefully removed and the intestinal segment adjusted between the pins. The other half chamber was attached and the tissue bathed in physiological diffusion buffer warmed to 37°C. The exposed tissue surface area was 0.64 cm^2 and the buffer volume in each half chamber was 1.5 mL. Care was taken to add buffer to both chambers simultaneously to prevent hydrostatic pressure effects. Carbogen (95% O_2-5% CO_2) was bubbled through the chambers to facilitate both oxygenation of tissues and mixing, and temperature control was maintained at 37°C by a heating block. Photographs of the modified Ussing chamber experimental setup employed in these studies are presented in Figure 5.1. Tissues were allowed to equilibrate for approximately 45 min, with diffusion buffer changed once throughout the equilibration period. Immediately prior to the experiment, hGH (0.5 mg/mL) and/or radiolabelled markers (mannitol, D-glucose or diazepam) were added to the mucosal (donor) chamber and sampling conducted for up to 3 h.

In each animal, six to twelve tissue segments were studied. For experiments involving the evaluation of additive effects, two to four tissues were assessed in diffusion buffer only (controls) and four to eight tissues assessed in diffusion buffer containing additive(s), with any modified conditions/additives introduced following the buffer change at the end of the equilibration period.

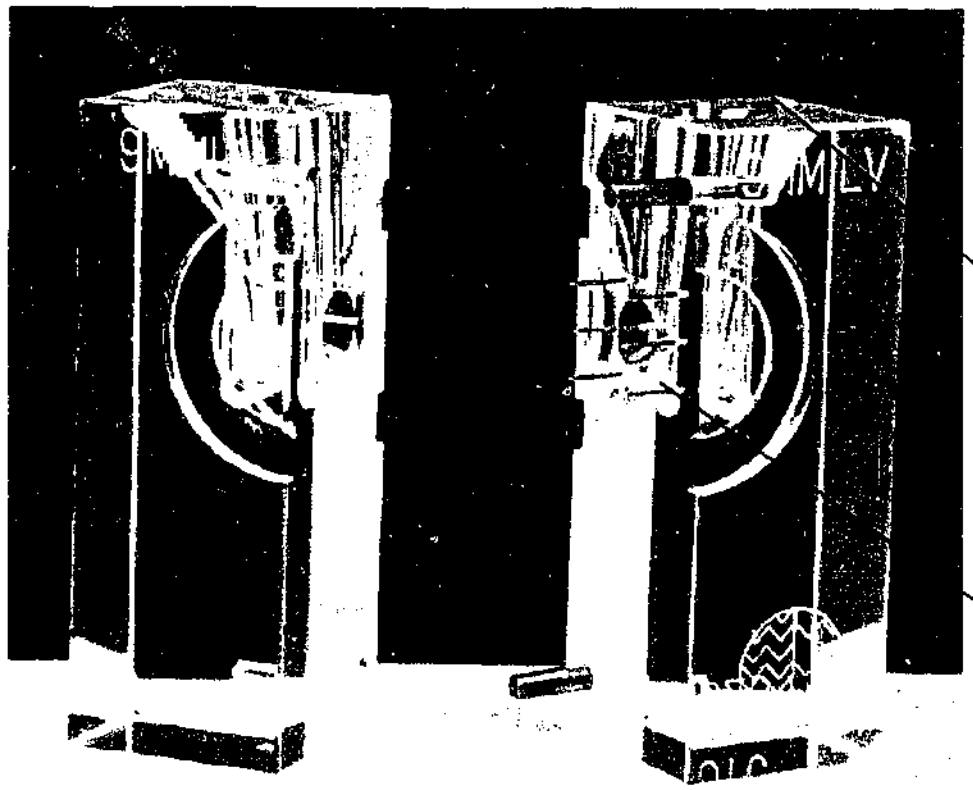


Figure 5.1 Photographs of the two halves of a single modified Ussing chamber (top plate) and a complete manifold incorporating six chambers set up for a typical diffusion experiment (bottom plate) as described in Section 5.2.3.2.

5.2.3.3 Sampling Procedure

Following the addition of hGH and/or radiolabelled markers, samples were withdrawn from the serosal (receiver) chamber every 20 min for up to 3 h, while samples were withdrawn from the donor chamber at the beginning and end of the experiment only.

Studies conducted with hGH: In order to determine hGH donor chamber concentration, a 50 µL sample from the mucosal chamber was taken at the beginning and the end of the experiment and immediately diluted with 50 µL of a PIC/buffer solution (final PIC concentration 2 µL/100 µL) to inhibit possible enzymatic degradation after sample collection. Samples were centrifuged at approximately 1200 *g* for 15 min and analysed on the day of the experiment. Analysis was conducted using the SEC assay, as described in Section 4.2.3.4.

A 20 µL aliquot of diffusion buffer was taken from the serosal (receiver) chamber at the beginning of the experiment (time zero) and then every 20 min from 40 min onwards. The sampled volume of hGH serosal samples was not replaced, as the volume removed was minimal. Following collection, samples were immediately diluted with horse serum (80 µL, to provide a 1 in 5 dilution) and stored at -20°C prior to analysis. The serosal chamber concentration of hGH was measured using the ELISA (described in Section 2.1.2.3.5).

Studies conducted with radiolabelled markers: The actual concentrations of radiolabelled compounds applied to the tissue (donor chamber concentration) are presented in Table 5.2. Additional unlabelled drug was added for [illegible]pam permeability experiments (0.1 mM) to prevent problems that may arise from non-specific binding of the compound. Samples (100 µL) were withdrawn from the donor chamber at the beginning and end of the experimental period only, while serosal samples (100 µL) were taken every 20 min throughout the duration of the experiment. The sampled volume of serosal samples was replaced with blank (drug free) buffer to maintain a constant volume, ionic composition and hydrostatic pressure. The effect of

Table 5.2 Specific activity and concentration of radiolabel applied to intestinal tissue for *in vitro* permeability determinations.

Drug	*Label*	*Specific activity (Ci/mmol)*	*Donor concentration (x 10^6 M)*
Mannitol	^{14}C	0.0515	6.5
D-glucose	^{14}C	0.0538	6.2
Diazepam	^{3}H	82.5	0.0081

this dilution was taken into account by the incorporation of appropriate correction factors into the permeability coefficient calculation.

5.2.3.4 Liquid Scintillation Counting

Samples containing radiolabelled compound were placed in 6 mL polyethylene scintillation vials, Starscint™ scintillation cocktail (3 mL) added and the vials then vortexed. Both ^{14}C and ^{3}H were analysed on a Packard Tri-Carb® liquid scintillation counter (Model 2000 CA, Packard Instrument Company, Downers Grove, IL). Counts per minute (cpm) were converted to disintegrations per minute (dpm) using the external standard channel's ratio. A blank (containing radiolabel free buffer) was included at the beginning of each set of samples to provide a background count, which was subsequently subtracted from all samples.

Linearity of the liquid scintillation counting was assessed by measuring the radioactivity of a set of ^{14}C-mannitol solutions prepared by serial dilution, with activities ranging from 110,000 dpm down to approximately 50 dpm. Precision was evaluated by counting each solution in triplicate and accuracy was determined by a back calculation of the result compared to the nominal activity of each solution.

5.2.3.5 Histology

Tissues collected for histological evaluation following experiments were quickly and carefully pinned onto a wax backing and fixed in a 10% buffered formalin solution. Following fixation, samples were sent to an external laboratory for processing and haemotoxylin and eosin (H & E) staining. Evaluation was conducted by an experienced veterinary pathologist in a blinded fashion using assessment criteria and a scoring system previously described by Swenson and Curatolo (Swenson et al., 1994). Histological features graded included the presence of mucus/debris, villous shortening, erosion, swollen epithelial cells, flat epithelial cells and

concentration of goblet cells. A scale of 0 to 3 was employed for each indication of histological abnormality, with 0 indicating no effect/no abnormality and 3 indicating severe damage.

5.2.3.6 Evaluation of hGH Stability in the Serosal Chamber

The stability of hGH in the Ussing chamber setup was evaluated at low concentration (representative of the receptor solution) by incubating the mounted intestinal tissues with approximately 200 ng/mL hGH in both the donor (mucosal) and receiver (serosal) chambers. These studies were conducted employing tissues from rabbit ileum, in addition to rabbit jejunum and colon (in preparation for later studies of regional permeability, described in Chapter Six). hGH concentration was monitored over a 3 h time period using the ELISA (as described in Section 2.1.2.3.5), with sampling carried out prior to addition of hGH, immediately after the addition of hGH (time zero), and at 90 and 180 min. Studies were performed on two tissues from each intestinal region.

5.2.4 Data Analysis

5.2.4.1 Permeability Coefficient Calculations

The apparent permeability coefficient (P_{app}) was calculated for each individual flux profile using the following equation:

$$P_{app} = \frac{(V_r \times dC_r)}{(A \times C_0 \times dt)} \qquad (5.1)$$

where V_r is the volume of the receiver chamber (1.5 mL), A is the exposed tissue surface area (0.64 cm^2), C_0 is the drug concentration in the donor chamber and dC_r/dt is the change in drug concentration in the receiver chamber with time (flux).

5.2.4.2 *Statistical Comparisons*

Statistical comparisons for the effect of excipients on marker and hGH permeabilities in rabbit ileum were conducted with the assistance of the Monash University Statistical Consulting Service. A repeated measures analysis of variance (ANOVA) was employed, utilising animal (rabbit) as the random factor and testing for significance at α=0.05 (MINITAB Statistical Software, version 6, Minitab Inc., PA). Each comparison was performed separately, since varying numbers of experiments for each test condition precluded fitting all conditions at once. Results are reported as mean ± standard error of the mean (SEM).

5.3 RESULTS AND DISCUSSION

A variety of tissue preparations from animal species including bullfrog (Armstrong et al., 1979), dog (Jezyk et al., 1992), turtle (Sarracino and Dawson, 1979), guinea pig (Cooke et al., 1983), chicken (Grubb et al., 1987), rat (Ungell et al., 1998) and rabbit (Yeh et al., 1994, Samanen et al., 1997) have been employed for intestinal permeability studies. Considerations for the choice of animal model included availability, tissue viability over the experimental period, relevance of data obtained to human absorption characteristics and cost. Although both rabbit and rat intestinal preparations have been well-characterised and are readily available at a reasonable cost, rabbit intestine was selected in the current study based on viability, ease of stripping and the reported good correlation between rabbit intestinal permeabilities and human absorption (Smith et al., 1988, Lee et al., 1993, Swaan et al., 1994). Rabbit intestine has been demonstrated to be viable in the modified Ussing chamber for up to four hours based on the measurement of electrical properties and both passive and active transport characteristics (Swaan et al., 1994, Yeh et al., 1994). The *in vitro* model also readily facilitates the examination of active transport characteristics through the use of bidirectional studies (i.e. mucosal to serosal and serosal to mucosal), with asymmetric transport generally indicative of either active absorption or efflux mechanisms.

5.3.1 Validation of Liquid Scintillation Counting

The range of counts chosen for the evaluation of liquid scintillation counting was that measured in the donor (high end of range) and receiver (down to low end of range) chambers for ^{14}C-mannitol in the *in vitro* permeability experiments. Linearity was confirmed over this range (110,000 to 50 dpm, r^2=1). Precision and accuracy of counting for all dilutions is described in Table 5.3. Precision was less than 8% and accuracy was within ±12% for each dilution investigated.

5.3.2 Use of Marker Compounds

Utilisation of a marker such as mannitol is essential, particularly in the absence of electrical measurements (e.g. transepithelial electrical resistance, TEER) in order to monitor tissue integrity. (Electrical measurements were not utilised in this study, as the facilities were unavailable in our laboratory) Mannitol is a small (MW 182), well-characterised, hydrophilic compound which is commonly used in permeability experiments to confirm tissue barrier function and provide a reference for paracellular transport (Marks et al., 1991, Swaan et al., 1994, Dowty and Dietsch, 1997, Jezyk et al., 1999). In the current study, mannitol was routinely included in permeability experiments, providing a reference for comparison to other drugs/markers included in the study and to mannitol permeability data published by other laboratories. Permeability measurements for mannitol also formed the basis of exclusion criteria, with animals exhibiting control tissue mannitol permeability coefficients (P_{app}) which deviated from the mean value by more than 30% not included in the final data analysis. Diazepam (MW 285) was used as a passive transcellular marker while D-glucose was used to confirm the presence of active transport processes (Grass and Sweetana, 1988, Jezyk et al., 1992, Ungell et al., 1998).

The mean flux data (± SEM) for mannitol and diazepam across stripped rabbit ileum are presented in Figure 5.2. The flux of diazepam is significantly greater than that of the poorly

Table 5.3 Validation data for liquid scintillation counting using serial dilutions of a solution of the radiolabelled marker, ^{14}C-mannitol. Counting was conducted as described in Section 5.2.3.4.

Nominal counts (dpm)	*Actual counts (dpm)*	*Accuracy (% deviation from nominal count)*	*Precision (% C.V. for mean count)*
110018.7	110149.3	0.1	0.2
110018.7	109770.3	-0.2	
110018.7	110150.0	0.1	
22003.7	22180.7	0.8	0.6
22003.7	22131.9	0.6	
22003.7	21934.3	-0.3	
11001.9	11046.9	0.4	0.1
11001.9	11042.6	0.4	
11001.9	11067.4	0.6	
1100.2	1026.4	-6.71	4.5
1100.2	1112.1	1.1	
1100.2	1110.4	0.9	
550.1	558.2	1.5	0.5
550.1	561.7	2.1	
550.1	556.1	1.1	
110.0	106.6	-3.1	7.3
110.0	97.5	-11.4	
110.0	113.0	2.7	
55.0	57.3	4.2	5.9
55.0	60.3	9.6	
55.0	53.6	-2.6	

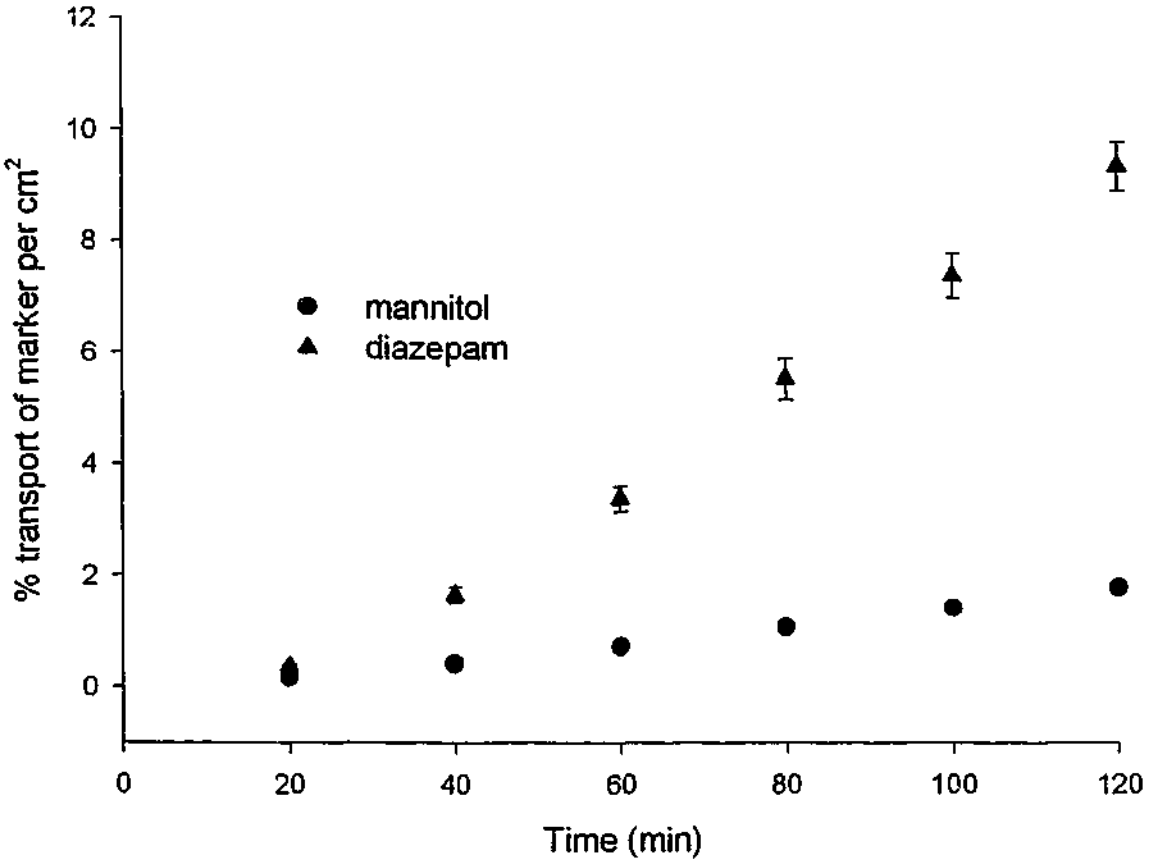


Figure 5.2 Flux of the marker compounds mannitol (circles) and diazepam (triangles) across stripped rabbit ileum in the modified Ussing chamber. Data were obtained using standard diffusion buffer in the absence of excipients according to the procedure described in Section 5.2.3.2.

permeable mannitol owing to the large contribution of the transcellular route to the surface area of the small intestine (99.99%) (Stenberg et al., 2000). Figure 5.3 presents the mean flux of D-glucose across rabbit ileum in both the mucosal to serosal and serosal to mucosal directions. The flux is significantly higher in the mucosal to serosal direction, reflecting the active nature of D-glucose transport. Apparent permeability coefficients are reported in Table 5.4 and compared to previously published values for the three markers, with P_{app}s for all compounds being consistent with literature data. This provided confirmation that the tissue collection and preparation technique employed allowed the maintenance of typical transport characteristics of isolated rabbit intestine throughout the experimental period.

The P_{app} values are consistent with those expected for such compounds. Diazepam is a moderately lipophilic (log P approximately 3.1) drug which is well absorbed following oral administration in humans, with the average fraction absorbed reported to be 97%. In comparison, the hydrophilic marker, mannitol, shows significant variability and often poor absorption following oral administration, with the fraction absorbed reported to be approximately 20% (Lee et al., 1997).

5.3.3 Histology

Although the primary method of monitoring tissue integrity in the *in vitro* model was the determination of mannitol permeability, a series of experiments was conducted in which intestinal tissues were collected and evaluated by light microscopy in order to provide additional confirmation of tissue integrity and viability. Intestinal segments undoubtedly suffer some degree of trauma in the process of excision, stripping, mounting and incubation in the modified Ussing chamber, while also being deprived of a normal blood supply and protective mucus layer. A histological assessment of tissues therefore offered a qualitative measure of the extent of any damage/abnormalities incurred and a physical check that the stripping process was removing the

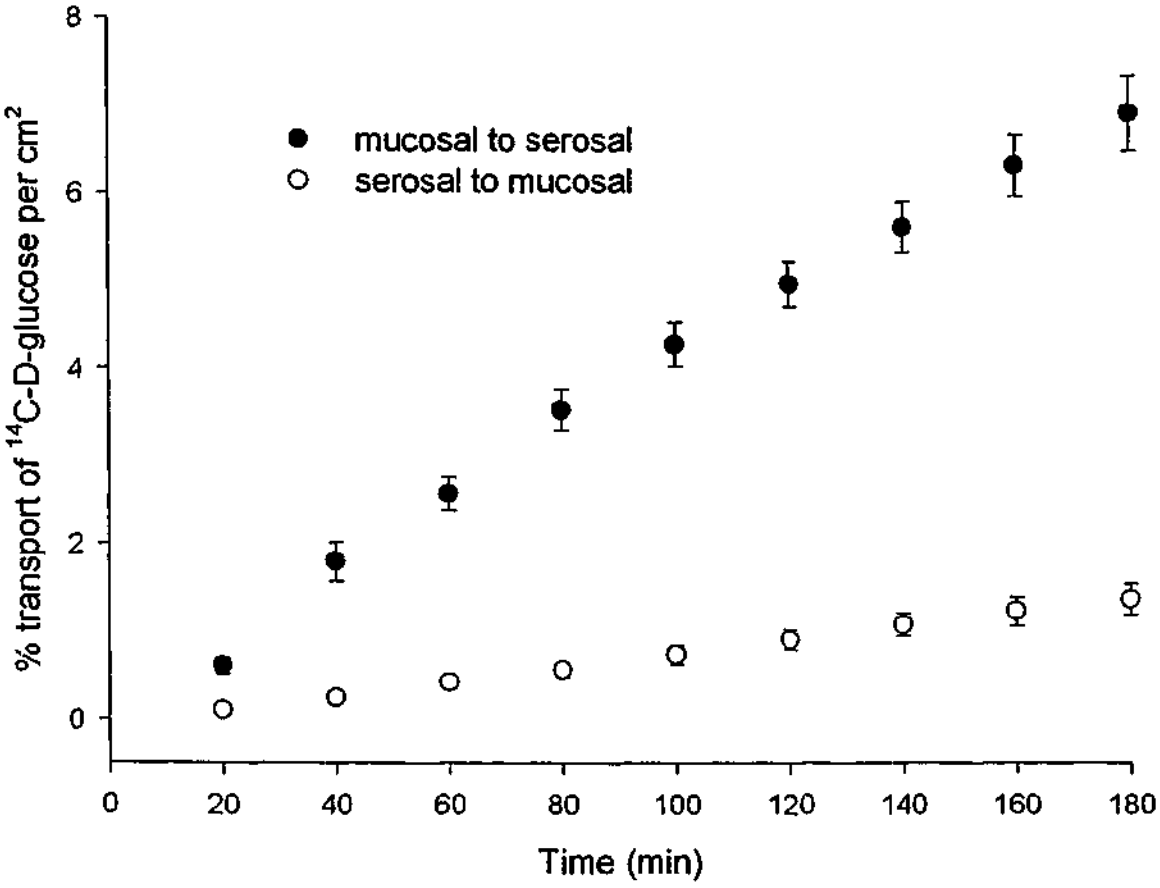


Figure 5.3 Flux of the marker compound D-glucose in the mucosal to serosal (closed circles) and serosal to mucosal (open circles) directions across stripped rabbit ileum in the modified Ussing chamber. Data were obtained using standard diffusion buffer in the absence of excipients according to the procedure described in Section 5.2.3.2.

Table 5.4 Permeability coefficients (P_{app}) for mannitol, D-glucose and diazepam obtained with stripped rabbit ileum.

	Drug	*MW*	*Permeability coefficient (cm/s x 10^{-6})* [a]	*N Tissues/Animals*
Experimental data	Mannitol	182	4.61 ± 0.20	23/5
	D-glucose	180	11.85 ± 0.40	7/2
	Diazepam	285	23.70 ± 1.67	23/6
Published data	Mannitol		4.02 ± 0.19[b]	7/3
			5.60 ± 1.2[c]	12-16/3-4
	D-glucose		14.50 ± 2.35[b]	15/4
	Diazepam		~ 28-30[d]	

[a] data reported as mean ± SEM
[b] from Jezyk et al 1992 (Jezyk et al., 1992)
[c] from Yu et al 1995 (Yu et al., 1995)
[d] estimated from graphical data presented by Smith 2000

serosal muscle layer as intended.

The evaluation and scoring system employed in the current work was adapted for use from a study published on the investigation of intestinal permeability enhancement in a rat intestinal perfusion model (Swenson et al., 1994). Swenson evaluated various surfactants for their enhancing effects on the absorption of a model drug. The appraisal of tissues involved assessment of both control tissues (i.e. tissues perfused with plain buffer) and tissues perfused with additives and/or enhancing agents.

Table 5.5 presents the results of a histological evaluation conducted by an experienced veterinary pathologist (Victorian Veterinary Pathology Service) on a set of rabbit ileal tissues from the current study. Tissues 1, 7 and 8 were sampled prior to mounting in the diffusion chamber, while all other tissues were mounted in the diffusion chamber and bathed in buffer for the times specified in Table 5.5. The pathologist's report described tissues numbered 1 to 4 as showing severe lesions of coccidiosis, evidenced by the presence of many coccidia parasites in the lamina propria and accompanied by the appearance of moderate to severe stunting of villi, attenuation of villous epithelium and, in tissue 2, widespread epithelial erosion. Moderate crypt epithelial hyperplasia and macrophage infiltration of the lamina propria were also observed in these tissues, all four of which were obtained from the one animal. Total scores for tissues infected with coccidia was poor (i.e. high), ranging from 5 for tissue number one, which had not been mounted in the diffusion chamber, to 10 for tissue number four, which had been incubated for 3 h in the diffusion chamber. In comparison, tissue number five had been incubated for 3 h in the diffusion chamber and scored a total of 2. Demonstrated to be free of coccidia, tissue five showed minor degenerative changes only, characterised by broadening and mild villous stunting in approximately half of the villi, accompanied by dilatation of lymph vessels in the lamina propria. These results indicate therefore that incubation of healthy tissues under control conditions in the modified Ussing chamber for up to 3 h allowed the maintenance

Table 5.5 Histological evaluation of rabbit ileal tissue. The evaluation was conducted by an experienced veterinary pathologist from the Victorian Veterinary Pathology Service.

Tissue no.	*Time in diffusion chamber (h)*	*Score for histological abnormality*[a]						
		Mucus/debris	*Villous shortening*	*Erosion*	*Swollen epithelial cells*	*Flat epithelial cells*	*[Goblet cells]*	*Total score*
1	0	0	2	1	0	2	0	5
2	1	1	3	2	0	3	0	9
3	2	2	3	2	0	2	0	9
4	3	2	3	2	0	3	0	10
5	3	1	1	0	0	0	0	2
6[b]	3	1	0	0	0	0	0	1
7[c]	0	0	0	0	0	0	0	0
8[c]	0	0	0	0	0	0	0	0

[a] *scores range from 0 (no abnormality) to 3 (serious abnormality/damage)*
[b] diffusion buffer contained 2 mM taurocholate
[c] tissues obtained from SPF (specific pathogen free) rabbits

of adequate tissue integrity and morphology. Tissues five and six are further described in Section 5.3.4.7.

Figure 5.4 presents two photographs of cross sections of rabbit intestinal mucosa following excision and stripping (approximately 200 x magnification). Normal healthy intestinal tissue is shown in the top plate (tissue 7), while the tissue depicted in the bottom plate (tissue 1) shows the infiltration of large numbers of coccidia and the associated deterioration of the mucosa as described above. The damage to tissues 2-4 (total scores 9-10) rated similarly to that reported for rat intestinal tissues exposed to the absorption enhancing surfactants in the study conducted by Swenson and Curatolo. The presence of damage and inflammation caused by the coccidiosis had the potential therefore to severely affect the resulting permeability measurements.

Coccidiosis is a common intestinal parasitic infection suffered by both domestic and wild rabbits, with symptoms of clinical disease including diarrhoea, weight loss and polydipsia. Many subspecies of the parasite *coccidia eimeria* exist, each with varying degrees of pathogenicity, although the subspecies infecting the New Zealand White rabbits in the current study was not identified. Rabbits were carefully monitored prior to use in experiments and none showed any signs of clinical disease, despite the damage detected in the intestinal tissue in several of the animals.

A brief search of the literature concerning coccidiosis in rabbits revealed that a "rabbit model of chronic ileal inflammation" has been induced by inoculation of healthy rabbits with the coccidian protozoan *Eimeria magna*, with the ensuing ileal inflammation associated with inhibition of Na^+-amino acid and Na^+-glucose cotransport (Sundaram et al., 1998a, Sundaram et al., 1998b). The chronic phase of the disease occurs 10-15 days post inoculation when the parasites have been eradicated from the gut, leaving an inflamed ileum exhibiting "massive lymphocytic and plasmocytic infiltration of the lamina propria and mucosa and mild villous blunting" (Hyun et al., 1995). Hyun and co-workers also utilised the rabbit model to

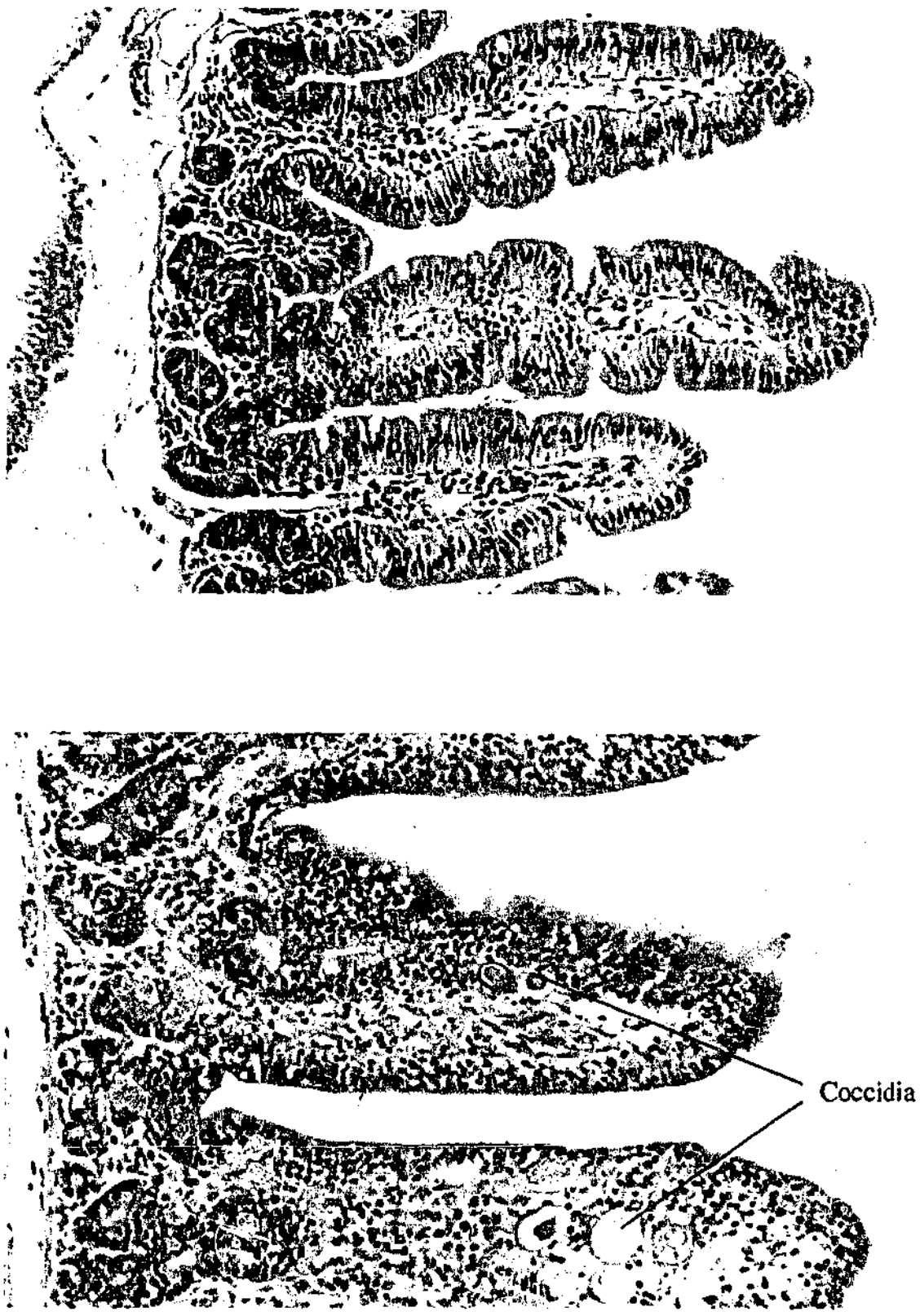


Figure 5.4 Photographs of rabbit ileum prior to use in the modified Ussing chamber, approximately x 200 magnification. The top plate shows normal rabbit ileum, while the bottom plate depicts ileum infested with the parasite, *coccidia eimera*.

examine the effect of ileal inflammation on paracellular permeability and found that ^{3}H-mannitol flux was markedly reduced in the chronically inflamed ileum, possibly resulting from alterations in mucosal and tight junctional structures. Altered intestinal permeability characteristics have also been observed in humans suffering from inflammatory conditions of the gut such as Crohn's disease, with increased permeability evident prior to inflammatory episodes (Fasano et al., 2000). Depending on the stage of the disease and the presence or absence of inflammation, measured P_{app} values may be either increased or decreased. In order to circumvent any further complications arising from the use of potentially infected animals, the remaining experiments were conducted using specific pathogen free (SPF) rabbits. The absence of coccidia in the SPF rabbits was verified by the pathologist, with tissues number 7 and 8 in Table 5.5 obtained from two separate SPF rabbits given a 'clean bill of health' and a total score of zero.

5.3.4 hGH in the Modified Ussing Chamber

5.3.4.1 hGH Stability Issues

Figure 5.5 presents size exclusion chromatograms of hGH in standard diffusion buffer sampled from the donor side of the modified Ussing chamber at time zero and 2 h of a typical diffusion experiment. It was evident that there was a significant loss of hGH from the solution over the 2 h time period. The reduction in hGH concentration was likely to be due to proteolytic degradation, aggregation or a combination of both of these processes. Degradation of hGH by intestinal proteases presented a major problem in the establishment of the rat intestinal perfusion model which was discussed in Chapter Four, although it was expected that this may be more readily controlled in the *in vitro* model. Carbogen bubbling in the diffusion chamber, necessary for mixing and tissue oxygenation, was the most likely source of aggregation-related loss of hGH. The significant tendency of hGH to aggregate in solution in the presence of a high air-water interface, such as that induced by agitation, has been widely reported

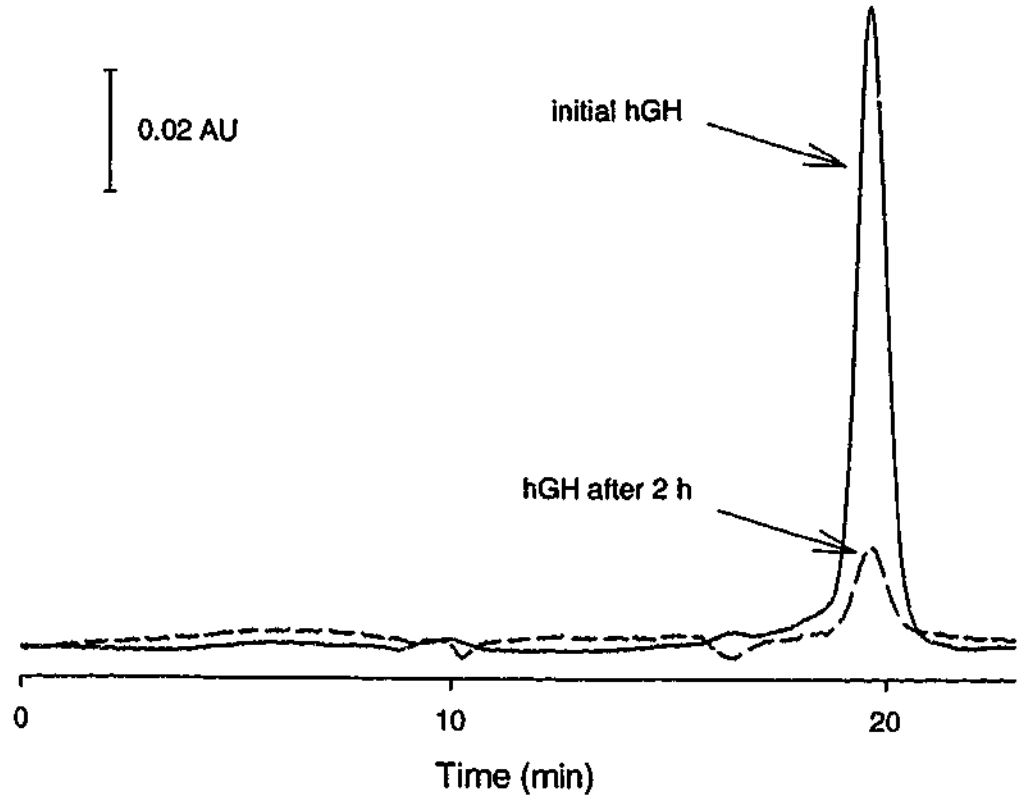


Figure 5.5 Representative chromatograms of hGH in the initial and final donor solutions in the absence of additives during a typical experiment in the modified Ussing chamber. Chromatograms were obtained using the SEC assay as described in Section 4.2.3.4.

(Hagenlocher and Pearlman, 1989, Maa and Hsu, 1997), with 67% of hGH lost in solution as insoluble aggregates following 1 min of vortexing (Katakam et al., 1995).

5.3.4.2 Use of Additives to Enhance hGH Stability

In order to measure hGH permeability in the diffusion chamber, it was necessary to maintain hGH stability within the *in vitro* model, with the only reduction in donor chamber hGH concentration being due to its passage across the intestinal barrier. The use of additives, including surfactants and a protease inhibitor, was explored to enhance hGH stability in the Ussing chamber.

The addition of aprotinin (250 μg/mL) to the donor chamber solution failed to prevent hGH loss, with still well under 50% hGH remaining after 2 h in the diffusion chamber. Aprotinin is an inhibitor of the luminally secreted pancreatic proteases trypsin and chymotrypsin (Trautschold et al., 1967) and has previously been shown to increase the *in vivo* absorption of insulin and ribonuclease from rat ileum thirty- and fifty-fold, respectively (Ziv et al., 1987). Based on the lack of an effect of aprotinin on percentage of hGH remaining, it was concluded that either this was not the most appropriate choice of protease inhibitor, or that loss of hGH was not the result of enzymatic degradation. A control experiment conducted in the absence of intestinal tissue in the diffusion chamber produced the same result, thereby confirming that enzymatic degradation was not responsible for the observed reduction in hGH concentration.

Table 5.6 describes the effects of the surfactants Pluronic® F127, Tween 20 and taurocholate on hGH stability in the modified Ussing chamber. Additives were included in both mucosal and serosal solutions at the concentrations specified, with all three effectively maintaining hGH stability over the 2 h time period, although the lower concentrations of Tween 20 failed to completely prevent hGH loss.

Figure 5.6 presents size exclusion chromatograms of hGH in diffusion buffer sampled from the donor side of the modified Ussing chamber at time zero and 2 h of a diffusion

Table 5.6 Effect of additives on hGH aggregation in the modified Ussing chamber. hGH aggregation/stability was determined using the SEC assay, as described in Section 4.2.3.4.

Additive	*Additive concentration in diffusion chamber*	*% hGH remaining after 2 h*
Nil		<50%
Pluronic® F127	0.1% w/v	100
	0.01% w/v	100
Tween 20	0.01% v/v	55
	0.03% v/v	70
	0.05% v/v	85
	0.1% v/v	99
	0.25% v/v	100
Taurocholate	2 mM	100
	5 mM	100

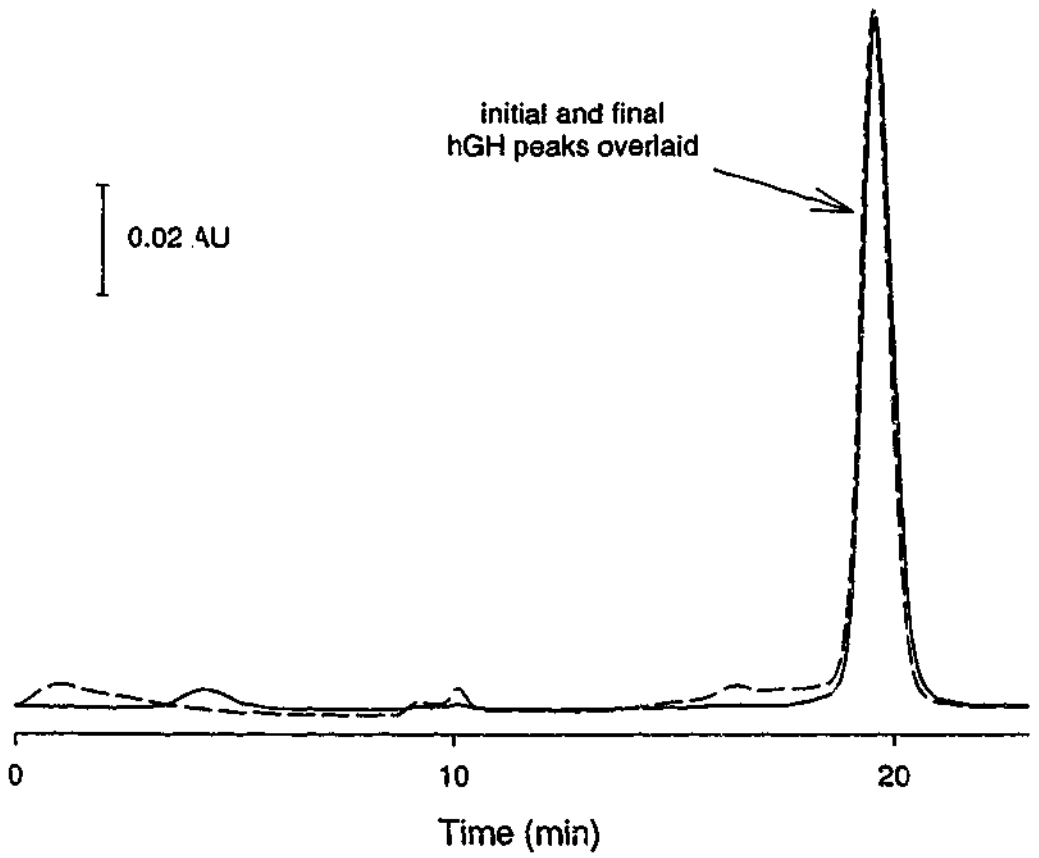


Figure 5.6 Chromatograms of hGH in the initial and final donor solutions in the presence of 2 mM taurocholate during a typical experiment in the modified Ussing chamber. Chromatograms were obtained using the SEC assay as described in Section 4.2.3.4.

experiment in which 2 mM taurocholate was included in the mucosal and serosal buffers. The two chromatograms are overlaid, indicating that no significant loss of hGH was detected following 2 h in the diffusion chamber. Similar results were obtained with Pluronic F127 (0.1% w/v, 0.01% w/v), Tween 20 (0.1% v/v, 0.25% v/v) and 5 mM taurocholate. hGH aggregation resulting from the carbogen bubbling in the chamber and the prevention of this aggregation by addition of a surface active agent was therefore the most likely explanation for the results obtained.

With several additives shown to effectively prevent loss of hGH from the donor chamber of the diffusion chamber, it was necessary to conduct further supportive studies to validate the use of an appropriate additive in the *in vitro* model. For example, detrimental effects of an additive on tissue integrity or interference with the hGH ELISA would preclude its further use in the study.

5.3.4.3 Effect of Additives on the ELISA

Low hGH concentrations (ng/mL) anticipated in the receiver side of the diffusion chamber necessitated the use of the ELISA. A study was necessary to verify that the presence of the additives investigated in the modified Ussing chamber model did not interfere with the assay method. The ELISA was used and the interference of additives investigated as described in Section 2.1.2.3.5.

Table 5.7 presents the effect of additives on the hGH ELISA at the additive concentrations used in the diffusion chamber. Pluronic® F127 was demonstrated to interfere with the assay at all concentrations utilised, even incorporating a higher dilution factor. This effect of surfactants on ELISAs is generally due to interference of the surfactant with the antibody antigen binding process, thus leading to artificially low protein concentration determinations. The effect of Pluronic® F127 on the ELISA therefore precluded its further use in the current study, as it was not possible to accurately measure hGH concentration in solutions

Table 5.7 Effect of additives on the hGH ELISA. Dilutions were performed in horse serum and analysis was conducted as described in Section 2.1.2.3.5.

Additive	*Additive concentration in diffusion chamber*	*Dilution*	*Interference*
Pluronic® F127	0.1% w/v	1:5	Yes
		1:10	Yes
	0.01% w/v	1:5	Yes
		1:10	Yes
Tween 20	0.1% v/v	1:5	No
	0.25% v/v	1:5	No
Taurocholate	2 mM	1:5	No
	5 mM	1:5	No

containing the poloxamer. In comparison, both Tween 20 and taurocholate were demonstrated not to interfere with the assay, with hGH concentrations measured in the presence of additives (after a 1:5 dilution with horse serum) determined to be within 20% of control (matrix containing no additive). High background absorbance values relative to that of blank matrix were also not detected in the presence of Tween 20 and taurocholate.

5.3.4.4 Effect of Additives on Marker Compound Permeability

In order to maintain the tissue integrity and viability established thus far in the development of the modified Ussing chamber model, it was necessary for any additives utilised to not affect the permeability of the transport markers, mannitol and diazepam.

Figures 5.7 and 5.8 present the effects of additives on the flux of mannitol and diazepam, respectively, in stripped ileum. Mannitol flux was greater in the presence of both 0.25% Tween 20 and 5 mM taurocholate compared to buffer, while diazepam flux was increased in the presence of 5 mM taurocholate. Table 5.8 describes the P_{app} values obtained for the markers in the presence and absence of additives. Consistent with the observations from the flux plots, mannitol P_{app} values were significantly higher upon inclusion of 0.25% Tween 20 and 5 mM taurocholate in the diffusion buffer ($p<0.005$), while diazepam P_{app} values increased upon the addition of 5 mM taurocholate to diffusion buffer ($p<0.005$). Diazepam permeability remained unchanged in the presence of 0.25% Tween 20 and the permeability of both markers was unaffected by the addition of either 0.1% Tween 20 or 2 mM taurocholate ($\alpha=0.05$).

Increased mannitol permeability indicated changes to the paracellular pathway most likely as a result of either altered tissue integrity or permeation enhancement and similarly, increased diazepam permeability reflected changes in transcellular transport characteristics. Bile salts and surfactants, including Tween 20 have been reported to act as absorption enhancers (Muranishi, 1990), therefore it was essential that these additives be used at concentrations which did not affect either the paracellular or the transcellular pathways. (Absorption enhancers and

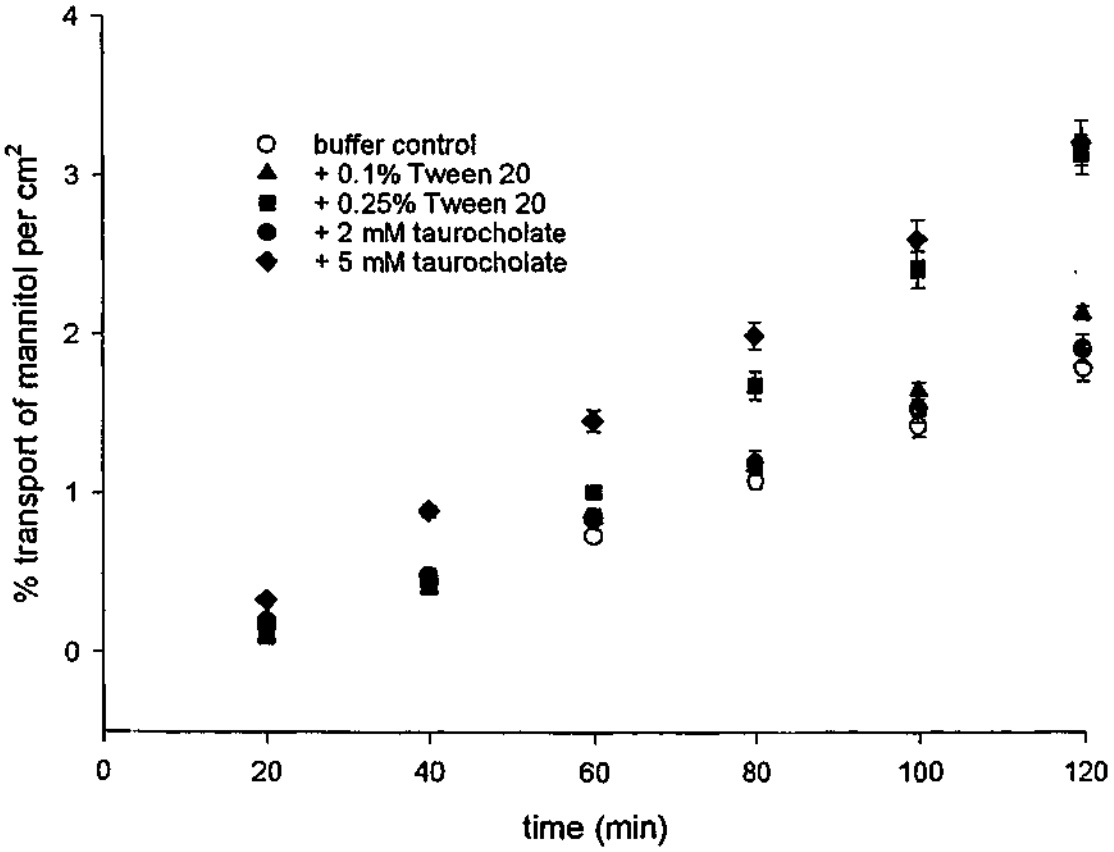


Figure 5.7 Flux of mannitol in the presence and absence of excipients across stripped rabbit ileum plotted as % transport/cm^2 versus time.

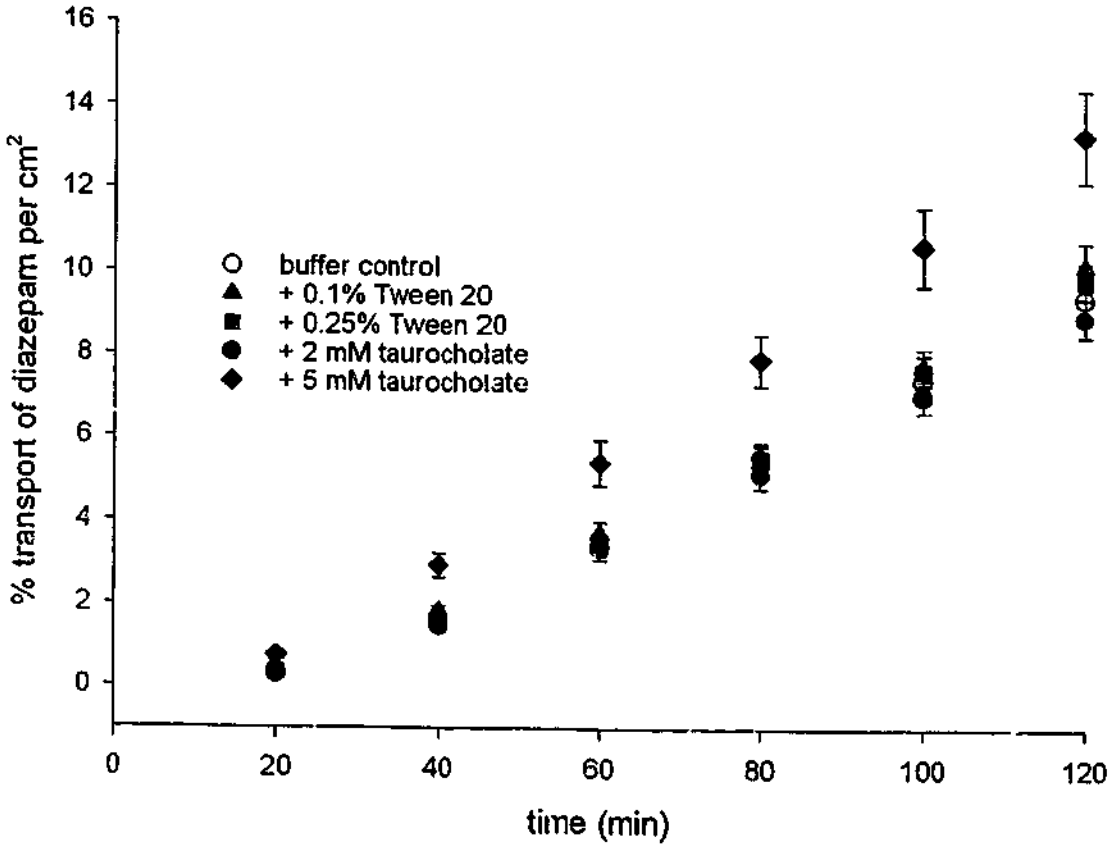


Figure 5.8 Flux of diazepam in the presence and absence of excipients across stripped rabbit ileum plotted as % transport/cm^2 versus time.

Table 5.8 Apparent permeability coefficients (P_{app}) for mannitol and diazepam in the presence and absence of excipients in stripped rabbit ileum.

Drug	*Additive*	*Additive concentration*	*P_{app} ($x\ 10^6$ cm/s)*[a]	*N*
Mannitol	**Nil**		**4.61 ± 0.20**	**23**
	Tween 20	0.1% v/v	4.98 ± 0.51	3
		0.25% v/v	7.87 ± 0.63[b]	3
	Taurocholate	2 mM	4.62 ± 0.31	24
		5 mM	7.03 ± 0.49[b]	6
Diazepam	**Nil**		**23.70 ± 1.67**	**23**
	Tween 20	0.1% v/v	26.99 ± 3.98	3
		0.25% v/v	26.60 ± 3.98	3
	Taurocholate	2 mM	23.75 ± 1.54	15
		5 mM	32.84 ± 3.53[c]	6

[a] data reported as mean ± SEM
[b] significantly different to mannitol control ($p<0.005$)
[c] significantly different to diazepam control ($p<0.005$)

mechanisms of enhancement will be discussed in detail in Chapter Six). Consequently, either 0.1% Tween 20 or 2 mM taurocholate would constitute appropriate choices for use with hGH in the *in vitro* model.

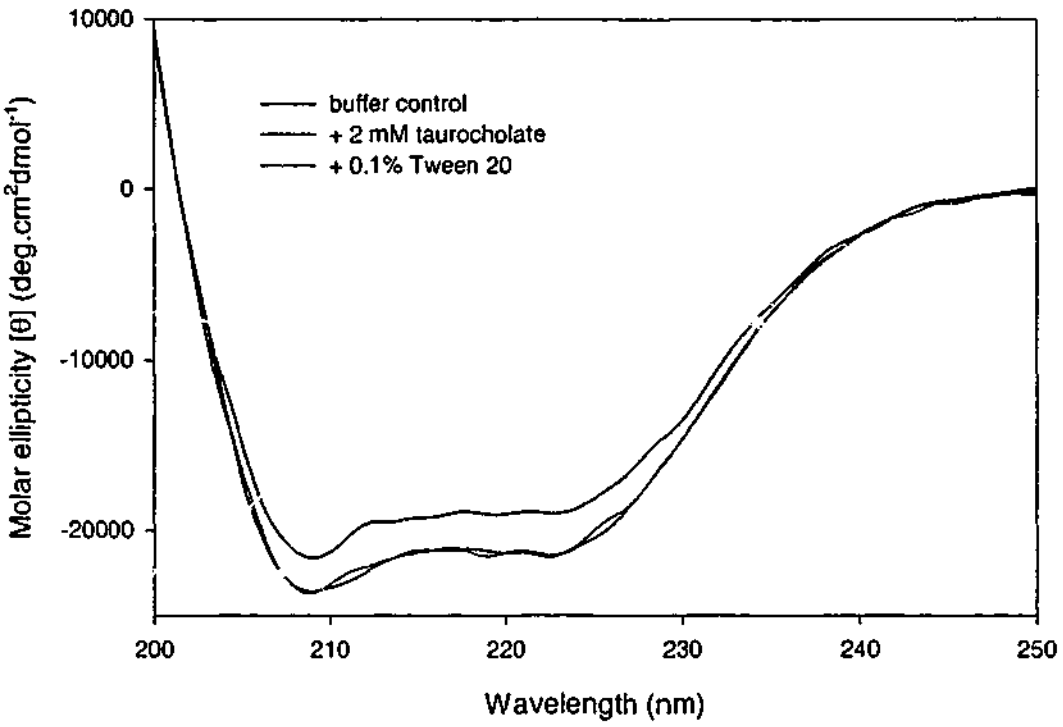
5.3.4.5 Effect of Additives on hGH Conformation

The possible impact of conformational changes on membrane translocation of proteins was discussed in Chapter Three where altered conformations of hGH were explored. It was essential that any additives employed in the *in vitro* model did not alter hGH conformation, as they could theoretically affect hGH permeability characteristics and interfere with the examination of hGH/carrier interactions, which will be further examined in the context of the *in vitro* model in Chapter Six.

The effect of additives on the conformation of hGH in diffusion buffer was evaluated using circular dichroism (CD). Wavelength scans were conducted as described in Section 3.1.2.2.3, with the exception of solution temperature, which was set to 37°C in order to mimic diffusion chamber conditions. Figure 5.9 presents the far (250 to 200 nm) and near (350 to 250 nm) UV CD spectra of hGH in the absence and presence of selected additives demonstrated not to alter marker compound permeabilities, i.e. 0.1% Tween 20 and 2 mM taurocholate. No changes were detected in the spectra of hGH in the presence of the additives, with the slight changes in molar ellipticity values apparent in the far UV scans most likely being the result of slight hGH concentration differences. The conformation of hGH therefore remained unchanged in the presence of either 0.1% Tween 20 or 2 mM taurocholate.

5.3.4.6 hGH Stability in the Serosal Chamber

Previous studies conducted using size exclusion analysis of the donor chamber solution have confirmed the absence of significant degradation over the course of the study with the inclusion of 2 mM taurocholate in the diffusion buffer (Section 5.3.4.2). The stability of hGH



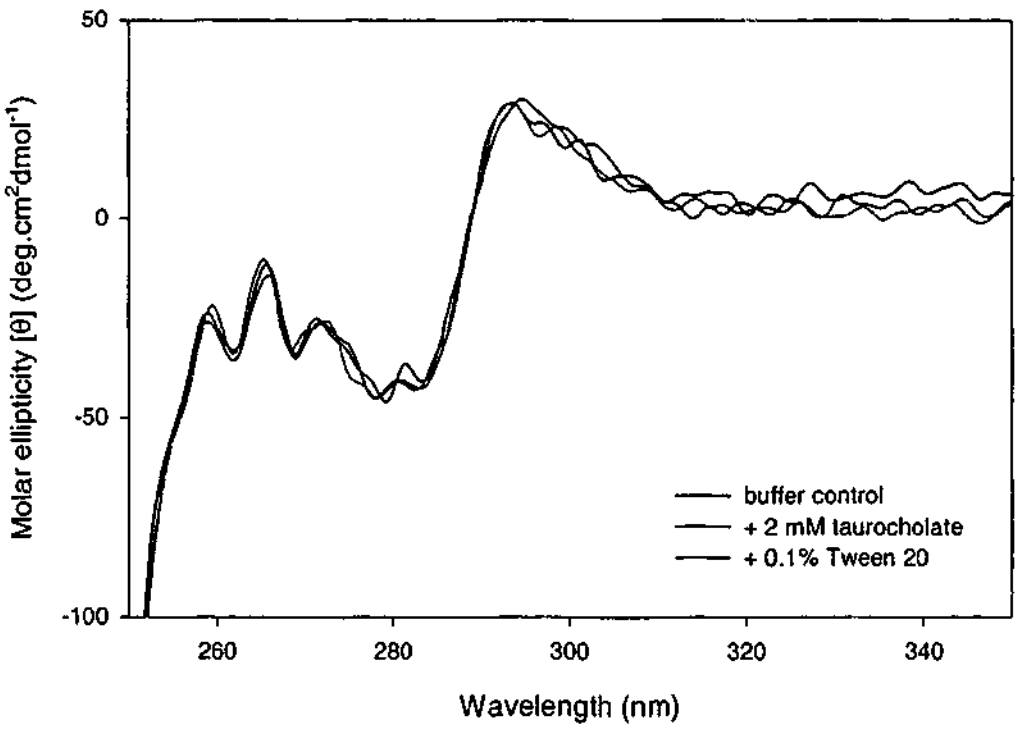


Figure 5.9 Far (top) and near (bottom) UV CD spectra for hGH (0.5 mg/mL) in diffusion buffer in the presence and absence of excipients at 37°C. Spectra acquisition was accomplished using the method outlined in Section 3.1.2.2.3.

in the Ussing chamber setup was evaluated at low concentration (representative of the receptor solution) by incubating mounted tissues (ileum, jejunum, colon) with hGH in both the donor (mucosal) and receiver (serosal) chambers. hGH was included in both half chambers in order to detect possible loss due to proteolysis on both sides and to minimise flux from one chamber to the other.

Table 5.9 presents the hGH concentrations measured over the time course of the experiment in each of the half chambers and for each of the different tissues. Although some variability was observed (<15%), there was no significant loss of hGH over the 3 h period from any of the chambers.

5.3.4.7 Measurement of hGH Permeability

Figure 5.10 presents the flux of hGH measured in the presence of 2 mM taurocholate (TC) in stripped rabbit ileum. A lag time of approximately 15 min was evident, possibly a result of a reduced bubbling rate, which was incorporated to further ensure minimal hGH aggregation and precipitation. The permeability coefficient obtained for hGH in the presence of 2 mM TC was $0.15 \pm 0.01 \times 10^{-6}$ cm/sec (n=16), a value still lower than the poorly permeable mannitol. Similar P_{app} values were determined for hGH in the presence of 5 mM TC and Tween 20 (0.1% and 0.25%, data not shown).

The 2 mM TC was selected for use in further studies utilising the *in vitro* model from the additives and concentrations assessed, primarily on the basis of its action in preventing hGH loss in the Ussing chamber, but also based on several other criteria. It did not interfere with the hGH ELISA, nor did it alter hGH conformation and importantly, for the purposes of maintaining typical tissue barrier properties, it did not affect marker compound permeabilities. Furthermore, the concentration of taurocholate employed is at the lower end of the range of physiological bile salt concentrations found in the fasted intestine and accordingly, the histological examination detected no apparent tissue degeneration from its inclusion in the bathing buffer.

Table 5.9 Stability of hGH at low concentration in the modified Ussing chamber. Concentrations were determined using the ELISA, as described in Section 2.1.2.3.5.

Tissue	*Mucosal/serosal chamber (M/S)*	*Conc. at 0 min (ng/mL)*[a]	*Conc. at 90 min (ng/mL)*[a]	*Conc. at 180 min (ng/mL)*[a]	*Mean conc.*	*C.V.(%)*
Jejunum	M	178.5	176.3	174.0	176.3	1.3
	S	204.0	195.8	217.5	205.8	5.3
Ileum	M	167.3	194.3	196.5	186.0	8.7
	S	188.3	173.0	217.5	192.9	11.7
Colon	M	206.3	198.8	208.5	204.5	2.5
	S	182.3	205.5	211.5	199.8	7.7

[a] data represent the average of two experiments for each intestinal region

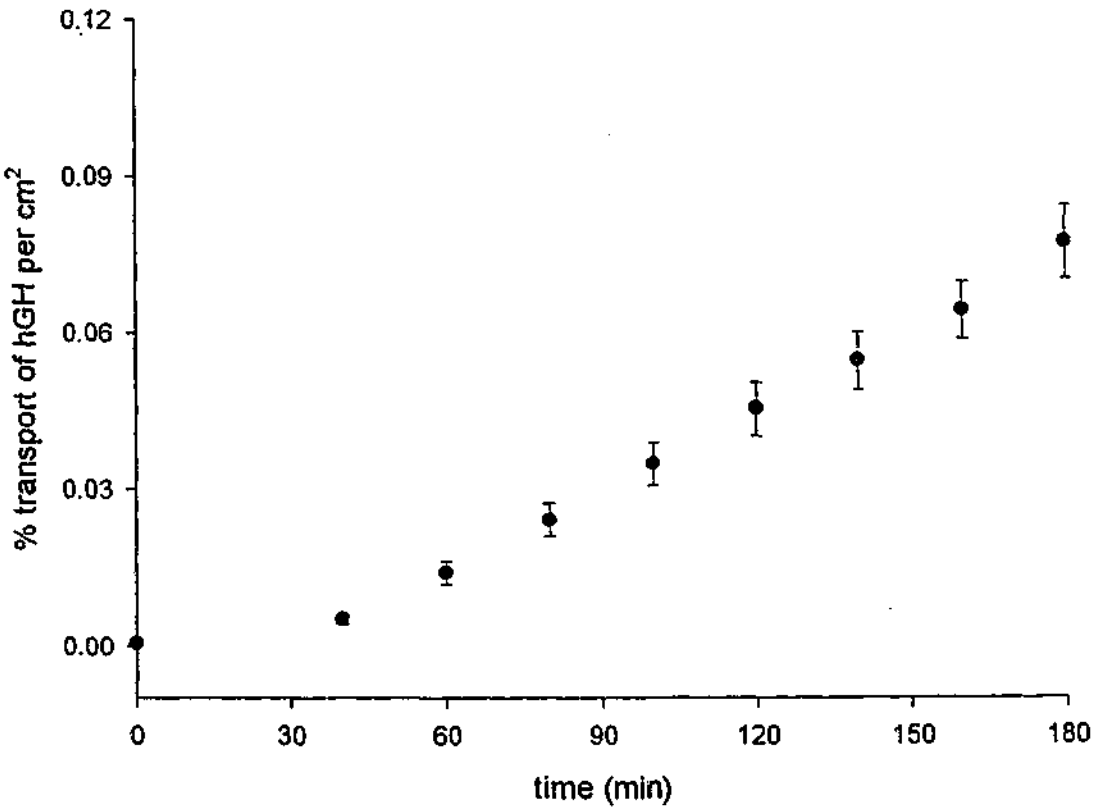


Figure 5.10 Flux of hGH in the presence of 2 mM taurocholate across stripped rabbit ileum. Data points represent the mean ± SEM.

Figure 5.11 presents two photographs of (healthy) rabbit ileal tissue, the top plate depicting tissue which has been bathed in unmodified buffer in the diffusion chamber for 3 h (number 5 from Table 5.5) and the bottom plate depicting tissue which has been bathed in buffer containing 2 mM taurocholate for 3 h (number 6 from Table 5.5). Both tissues showed evidence of mild degenerative changes following their incubation in the modified Ussing chamber. Tissue five exhibited broadening and mild stunting of approximately half the villi associated with lymphangiectasis of the lamina propria and tissue six displayed mild pyknosis of a few lymphoid cells in the lamina propria. Total scores for tissues 5 and 6 were low, indicating that tissue integrity and viability were adequately maintained in the modified Ussing chamber for up to 3 h both in standard buffer and in buffer containing 2 mM taurocholate.

The intestinal permeability of hGH has previously been examined using a modified Ussing chamber. Mlynek and co-workers determined the permeability of ^{125}I-hGH in rabbit du enum to be of the order of 1 x 10^{-6} cm/s, a value similar to that of mannitol (Mlynek et al., 2000). This permeability coefficient is surprisingly high, and may possibly have been due to the presence of free radiolabel since the method of analysis did not confirm the integrity of the iodinated hGH. In an earlier study, the permeability of hGH in rabbit colon was found to be 0.11 x 10^{-6} cm/s, with hGH concentration in this instance measured by an ELISA, thus verifying the presence of immunologically intact protein (Rubas et al., 1995). The low P_{app} values determined in the current work are more consistent with those reported by Rubas and more typical of a poorly permeable hydrophilic macromolecule such as hGH.

5.4 SUMMARY

A modified Ussing chamber model for determining the intestinal permeability of hGH was established and validated. The marker compounds mannitol, D-glucose and diazepam were employed to define tissue transport and barrier properties, with the similarity of marker permeability coefficients to those published by other laboratories confirming that tissue

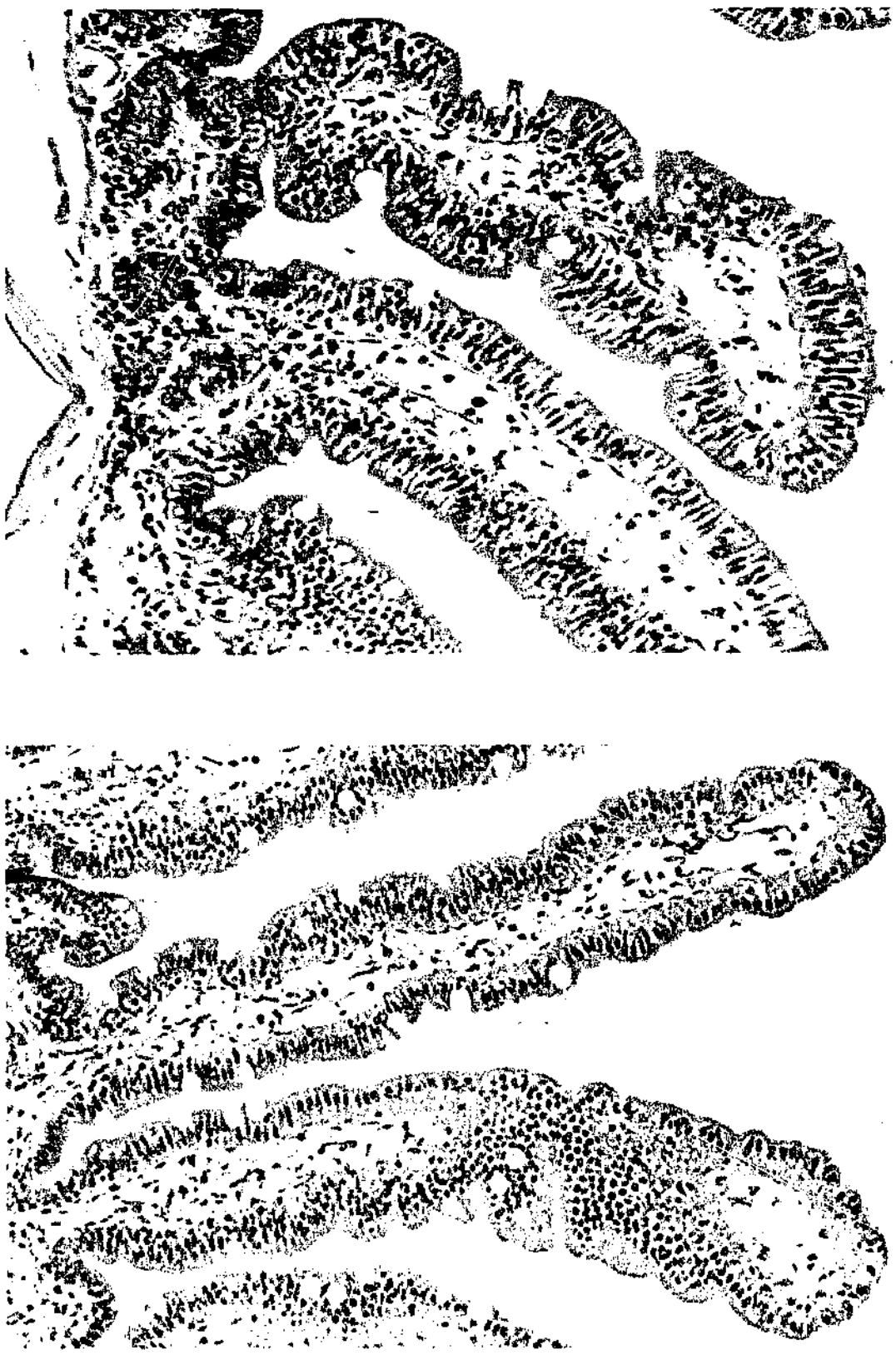


Figure 5.11 Photographs of rabbit ileum following a 3 h incubation in the modified Ussing chamber, approximately x 200 magnification. The top plate shows ileum which has been bathed in standard diffusion buffer, while the bottom plate shows ileum which has been bathed in diffusion buffer containing 2 mM taurocholate.

preparation and experimental procedures were appropriate in the *in vitro* model. Supportive histological observations indicated the absence of significant tissue damage to intestinal segments mounted and incubated in the diffusion chamber with the exception of tissues excised from rabbits found to be suffering intestinal coccidiosis. The possibility of damage and altered permeability measurements in coccidia affected tissues prompted the use of SPF rabbits in the remaining experiments.

The introduction of hGH into the diffusion chamber was initially complicated by a significant loss of hGH from the donor chamber, necessitating a search for an appropriate additive to enhance hGH stability. Inclusion of 2 mM taurocholate in the diffusion buffer effectively prevented the loss of hGH, most likely due to a reduction in interfacially-induced aggregation in response to the carbogen bubbling. Taurocholate did not interfere with the analysis of hGH by the ELISA and its presence did not affect the conformation of hGH. No difference was detected in marker compound permeability upon the addition of 2 mM taurocholate, indicating that the paracellular and transcellular pathways remained unaffected. The permeability coefficient of hGH measured in the presence of 2 mM taurocholate in rabbit ileum was comparable to previously reported data obtained in rabbit colon and consistent with that expected for a hydrophilic macromolecule.

The following chapter will report investigations incorporating the novel carrier compound into the *in vitro* model in order to quantify its effect on the intestinal permeability of hGH and examine absorption mechanisms.

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CHAPTER SIX

INVESTIGATION OF THE INTESTINAL PERMEABILITY OF hGH IN THE PRESENCE OF THE NOVEL CARRIER COMPOUND SNAC-2

6.1 GOALS

In vitro models are frequently employed to examine mechanisms of intestinal permeation in the absence and presence of absorption enhancing compounds. The modified Ussing chamber, incorporating either isolated intestinal tissues or Caco-2 cells, has been utilised to examine the mechanisms of absorption enhancement by agents such as sodium caprate (Anderberg et al., 1993, Tomita et al., 1996, Shimazaki et al., 1998, Soderholm et al., 1998), palmitoyl carnitine (Duizer et al., 1998, Shimazaki et al., 1998) and EDTA (Tomita et al., 1996). *In vitro* studies can provide valuable insight into the involvement of paracellular and/or transcellular routes, the presence of active and passive transport processes and the contribution to these by specific intra- and extra-cellular regulatory enzyme systems.

The development and validation of a modified Ussing chamber model for determining the intestinal permeability of hGH was presented in Chapter Five. The primary objective of the studies described in this chapter was to incorporate the model carrier, SNAC-2, into the *in vitro* model in order to probe the mechanisms of altered hGH permeability in the presence of the carrier.

One of the proposed hypotheses for the mechanism of absorption enhancement of hGH by SNAC-2 is that an altered conformation of hGH in the presence of the carrier may more readily translocate lipid membranes, thus giving rise to the observed oral absorption enhancement. Relatively high concentrations of SNAC-2 ($\geq$ 25 mg/mL) are likely to be required to effect hGH conformational changes (refer to Section 3.1.3.4) and hence carrier concentrations employed in the modified Ussing chamber were also relatively high (up to 50 mg/mL). Prior to the introduction of SNAC-2 into the diffusion chamber, careful consideration was given to the physicochemical characteristics of the carrier and the potential impact of these characteristics, including high carrier concentrations, on the solution conditions utilised in the chamber. As described in Section 1.4.1, SNAC-2 is an amphiphilic molecule and a weak acid, with a

calculated pKa of 4.77. Preliminary investigations involved an assessment of the effect of SNAC-2 on diffusion buffer osmolality and pH and the effects of SNAC-2 on the interfacial properties of the buffer in the presence of taurocholate (TC, added to prevent hGH aggregation in the modified Ussing chamber; see Chapter Five). The potential for a reduction in the free hGH concentration in the presence of SNAC-2 and TC in the diffusion buffer was explored in order to determine the effects on the apparent permeability coefficient of hGH.

Once the effects of SNAC-2 on the solution characteristics of the diffusion buffer and hGH were determined, the ileal permeability of hGH was examined in the presence of a range of SNAC-2 concentrations (10-50 mg/mL). Potential tissue damage was investigated through an assessment of the reversibility of the effect of SNAC-2 and a histological evaluation of tissues exposed to the carrier. Selected permeability measurements were also carried out in stripped rabbit jejunum and colon in order to explore regional differences in SNAC-2 (10 mg/mL) effects on hGH permeability.

The permeability of marker compounds (mannitol, PEG 4000 and diazepam) was determined in the absence and presence of SNAC-2 to study the effects of the carrier on paracellular and transcellular transport pathways. To assist in the interpretation of diazepam permeability data, the potential for solubilisation of the transcellular marker by the carrier was investigated.

Finally, the potential involvement of active transport processes in hGH flux in the absence and presence of SNAC-2 was probed by monitoring the effect of low temperature (10°C) on hGH permeation. These altered conditions also facilitated a study of the behaviour of marker compounds (mannitol, PEG 4000, diazepam, D-glucose) with and without SNAC-2 at low temperature.

6.2 EXPERIMENTAL

6.2.1 Materials

Diazepam (unlabelled) was obtained from Sigma Chemical Co. (St. Louis, MO) and glycerol was obtained from Ajax Chemicals (New South Wales, Australia). The marker compound, ^{3}H-PEG 4000, in addition to all other radiolabelled marker compounds, was obtained from NEN™ Life Science Products, Inc. (Boston, MA). All other reagents were of at least analytical grade. Water was obtained from a Milli-Q (Millipore, Bedford, MA) water purification system and was used throughout.

6.2.2 Methods

6.2.2.1 SNAC-2 Solution Preparation

SNAC-2 stock solutions (75 mg/mL) were prepared in a modified diffusion buffer using the sodium salt of SNAC-2. The modified diffusion buffer was prepared as described in Section 5.2.3.1, with the exception of Stock A, from which NaCl was omitted. Mucosal buffers for Ussing chamber experiments (with or without SNAC-2) were made fresh from stock solutions on the day of each experiment.

6.2.2.2 Osmolality Measurements

Osmolality measurements were performed on 10 µL aliquots of sample or standard solution using a Fiske One-Ten Osmometer (Fiske Associates, Needham Heights, MA). Prior to sample determinations, the osmolality of a 290 mOsm/kg (isotonic) standard solution was measured in duplicate and a calibration of the instrument was periodically conducted with standards encompassing a range of 50 to 850 mOsm/kg. Sample solution osmolality was

adjusted as required by the dropwise addition of a 2 M NaCl solution to give an isotonic solution where possible.

The effect of hypertonic donor solution conditions (in the absence of SNAC-2) on mannitol permeability (see Section 5.3.2) was assessed by adjusting the osmolality of donor solutions to either 350 or 500 mOsm/kg with 2 M NaCl and comparing the resulting permeability coefficients to those obtained using isotonic (290 mOsm/kg) solutions. Isotonic solution conditions were maintained in the receiver chamber for all experiments.

6.2.2.3 pH Effects

Diffusion buffer pH was monitored in the modified Ussing chamber under the solution conditions employed in permeability experiments. The pH of standard mucosal and serosal buffers in the absence and presence of 2 mM TC and SNAC-2 (10, 50 mg/mL) was investigated at ambient temperature (representative of buffers prior to use in experiments) and at 37°C in the diffusion chamber, while bubbling with carbogen (experimental conditions).

6.2.2.4 Surface Tension Measurements in the Presence of SNAC-2

Surface tension measurements were conducted using a Du Nouy tensiometer (Cambridge Instrument Company, Ltd., London) equipped with a glass vessel of 40 mm diameter and 20 mm depth. Measurements were performed in triplicate at ambient temperature with 10 mL of solution and water and glycerol were employed as reference standards. Prior to each measurement, the glass vessel was thoroughly rinsed and dried and the wire ring flamed and cooled.

The surface tension of SNAC-2 solutions in the concentration range of 1-40 mg/mL was determined, with solutions prepared by serial dilution. Experiments were conducted in the absence and presence of 2 mM TC. Surface tension (mN/m) was plotted against SNAC-2

concentration and the critical micellar concentration (CMC) estimated by determining the point of inflection of the resulting curve.

6.2.2.5 Interaction Between hGH and Solution Components

The potential for a decrease in the free hGH solution concentration due to an interaction with solution components (SNAC-2 and/or TC) was investigated by ultrafiltration utilising a 30 kDa molecular weight cutoff filter (Ultrafree-4, Millipore, Bedford, MA). Three different solution compositions were assessed and each was tested using 7-8 individual filters: (i) 0.5 mg/mL hGH alone, (ii) 0.5 mg/mL hGH and 2 mM TC and (iii) 0.5 mg/mL hGH, 2 mM TC and 50 mg/mL SNAC-2. hGH solutions were prepared in diffusion buffer as described in Section 5.2.3.1 and SNAC-2 solutions were prepared in the modified (Na^+ depleted) diffusion buffer as described in Section 6.2.2.1.

Filters were rinsed with Milli-Q water prior to use as recommended by the manufacturer. A 2 mL aliquot of hGH solution, with or without 50 mg/mL SNAC-2, was added to the donor side of each filter and centrifuged at approximately 500 *g* for 25 sec per spin for a total of three spins. Spin times were chosen to be long enough to allow sufficient volume through for sample collection, but short enough to prevent disturbance of solution equilibrium on the donor side of the filter. Immediately after each spin, samples of filtrate (50 μL) were withdrawn, diluted 1 in 2 with diffusion buffer, and analysed using the SEC assay (described in Section 4.2.3.4). Any filtrate remaining was discarded prior to the next spin. Following the final (third) filtrate collection, the donor side was also sampled and analysed for hGH content.

Control (unfiltered) samples were analysed to provide a comparison to the filtrate concentration and allow the determination of the free fraction of hGH. The free fraction of hGH was the concentration of hGH in the filtrate divided by the concentration of hGH present in the unfiltered control solution

6.2.2.6 Permeability Studies

Surgical procedures and permeability measurements in the absence of SNAC-2 were as described in Sections 5.2.3.2 and 5.2.3.3. For experiments performed in the presence of SNAC-2, the carrier was added to the donor (mucosal) chamber solution along with hGH or marker compounds following the equilibration period. Sampling was subsequently conducted as previously described (Section 5.2.3.3) for the remainder of the experiment. The ileal permeability of hGH and mannitol was determined in the presence of 10, 20, 30 and 50 mg/mL SNAC-2, with SNAC-2 solutions prepared in the modified diffusion buffer as described in Section 6.2.2.1. The permeability of the markers, PEG 4000 and diazepam, was also evaluated in the presence of the carrier.

The potential for active transport of hGH in the presence of SNAC-2 was investigated using low temperature conditions (10°C) in the modified Ussing chamber. The absence of active transport was confirmed by assessing the reduction in the permeability coefficient of the active transport marker, D-glucose. hGH ileal permeability was measured in the absence and presence of 50 mg/mL SNAC-2 at 10°C and the results obtained were compared to those obtained at 37°C. The effect of 50 mg/mL SNAC-2 on the intestinal permeability of the markers, mannitol, PEG 4000 and diazepam, was also examined under the low temperature conditions.

6.2.2.7 Reversibility of SNAC-2 Effects on hGH and Mannitol Permeability

The reversibility of the effect of SNAC-2 (10-50 mg/mL) on the permeability of hGH and the marker, mannitol, was studied by incubating the mounted rabbit ileal tissues with SNAC-2 in the diffusion chambers for 30 min prior to the commencement of the experiment. SNAC-2 solutions were then removed and discarded, fresh buffer without SNAC-2 was added and rapidly removed and discarded (to rinse out residual SNAC-2), and finally fresh standard diffusion buffer was added to the chambers before the addition of mannitol or hGH. Sampling procedures were as described in Section 5.2.3.3.

6.2.2.8 *Regional Studies of SNAC-2 Effects on hGH and Mannitol Permeability*

The effect of SNAC-2 (10 mg/mL) on hGH and mannitol permeability was evaluated in the jejunum and colon and data obtained were compared to those determined in the ileum. Surgery for these studies was as described in Section 5.2.3.2 except that jejunum was located and excised approximately 30 cm distal to the pylorus and colon tissue was removed from the distal region of the large intestine.

6.2.3 Data Analysis

The apparent permeability coefficient (P_{app}) was calculated using Equation 5.1 and statistical comparisons were conducted as described in Section 5.2.4.2. In order to compare the behaviour of hGH and marker compounds in the absence and presence of SNAC-2 under different temperature conditions, the difference of the log of the P_{app} values was used to calculate a P_{app} ratio (i.e. with:without carrier), with the errors for these comparisons determined using the 95% confidence interval for the ratio. Under selected conditions, hGH apparent permeability coefficients (P_{app}) were corrected for the free concentration by dividing the P_{app} by the free fraction of hGH, as described by Nerurkar et al. (Nerurkar et al., 1997).

6.3 RESULTS AND DISCUSSION

6.3.1 SNAC-2 in Diffusion Buffer – Effects on Osmolality

Physiological diffusion buffers employed in permeability studies are typically isotonic in order to assist in the preservation of tissue integrity and viability. It has been reported that solution hypertonicity can potentially affect both the permeability of marker compounds and the cellular morphology of tissues/cultured cells in the modified Ussing chamber (Lindmark et

al., 1995, Perez et al., 1996, Maitani et al., 1997). It was therefore necessary to investigate the effect of the inclusion of SNAC-2 on the osmolality of the diffusion buffer.

The addition of 50 mg/mL SNAC-2 to the standard diffusion buffer resulted in a very hypertonic solution, with a tonicity measuring approximately 650 mOsm/kg. Previous studies conducted by Lindmark et al. demonstrated that while hypertonic apical solution conditions (520 mOsm/kg) did not affect mannitol permeability or the cellular appearance of Caco-2 cells, the addition of the absorption enhancer, sodium caprate, produced markedly different effects depending on the tonicity of the buffer (Lindmark et al., 1995). In these studies, addition of caprate to standard apical diffusion buffer produced a solution tonicity of 520 mOsm/kg and a 70-fold increase in mannitol flux and severe cell damage. In contrast, the addition of caprate to a modified (Na^+ depleted) diffusion buffer resulted in a solution tonicity of 300 mOsm/kg (approximately isotonic) and gave rise to an 8-fold increase in mannitol flux with minimal cellular injury. The authors suggested that the net reduction in sodium ion concentration in the modified buffer was small, as it was replaced by the sodium salt of caprate. A similar substitution of the sodium salt of SNAC-2 (50 mg/mL) for NaCl in the diffusion buffer utilised in the present work resulted in a decrease in the tonicity of the solution from 650 to 350 mOsm/kg.

The effect of hypertonic solution conditions in the absence of carrier on the transport characteristics of rabbit ileum was assessed by measuring the permeability of the paracellular marker, mannitol, in the presence of donor solution tonicities of 290 (isotonic control), 350 and 500 mOsm/kg, each obtained by the addition of NaCl. Studies conducted by Lindmark and co-workers employed anisotonic conditions in the donor chamber, but maintained isotonic solution conditions in the receiver chamber throughout (Lindmark et al., 1995). In the current study, the receiver (serosal) chamber contained isotonic diffusion buffer in all experiments. Table 6.1 presents the P_{app} values determined for mannitol under these conditions, with the P_{app} values

Table 6.1 Apparent permeability coefficients (P_{app}) for ^{14}C-mannitol with stripped rabbit ileum employing three different donor solution osmolalities. Isotonic receiver solution conditions were maintained throughout. P_{app} values were calculated using Equation 5.1.

Donor solution osmolality (mOsm/kg)	*P_{app} (x 10^6 cm/sec)*[a]	*N (tissues/animals)*
290 (isotonic)	**4.61 ± 0.20**	**23/5**
350	4.49 ± 0.42	10/3
500	5.75 ± 0.45[b]	10/3

[a] data reported as mean ± SEM
[b] significantly different to isotonic control ($p<0.05$)

increasing slightly at 500 mOsm/kg ($p<0.05$) relative to the isotonic control, while remaining unchanged at 350 mOsm/kg.

Increased mannitol permeability under hypertonic solution conditions is thought to result from structural and functional alterations to tight junctions (Perez et al., 1996). Anderberg et al. utilised apical solution osmolalities of 350 mOsm/kg in Caco-2 cells describing this as a "slightly hyperosmotic solution" which did not affect monolayer integrity (Anderberg et al., 1993). Based on literature precedence and the results of the studies described above, it was concluded that the incorporation of 50 mg/mL SNAC-2 into a modified (Na^+ depleted) diffusion buffer provided the most favourable solution conditions regarding ion composition and osmolality (maximum of 350 mOsm/kg) and the lowest risk of tissue damage.

6.3.2 SNAC-2 in Diffusion Buffer – pH Effects

It has previously been demonstrated that mucosal buffer pH in the range of 7.4-5.5 has no effect on tissue viability as judged by mannitol permeability and electrical measurements in rabbit intestine, although altered pH may influence the active transport of selected compounds across the intestine (Hidalgo et al., 1993). Hidalgo and co-workers observed a three-fold increase in the flux of cephalexin in rabbit ileum at pH 5.5 compared to that observed at pH 7.4. The increase in cephalexin flux at the lower pH was suggested to result from pH dependent-carrier mediated transport. As the effect of pH on hGH flux was unknown, it was necessary to maintain the same pH for control buffers and buffers containing SNAC-2.

The pH of diffusion buffers used in these studies is presented in Table 6.2, with measurements recorded for buffers at ambient temperature and at 37°C bubbled with carbogen in the Ussing chamber. While the pH of solutions was reduced from approximately 8.2 to 7.7 in the presence of SNAC-2 at ambient temperature, the final pH of all buffers after 30 min in the

Table 6.2 pH measurements for buffers used in modified Ussing chamber experiments.

Solution composition	*pH at ambient temperature*	*pH at 37 °C[a] (15 min)*	*pH at 37 °C[a] (30 min)*
Standard mucosal buffer	8.21	7.73	7.66
Standard serosal buffer	8.10	7.66	7.65
Mucosal buffer + 2 mM TC	8.25	7.5	7.63
Serosal buffer + 2 mM TC	8.25	7.5	7.66
0.5 mg/mL hGH, 2 mM TC in standard mucosal buffer	8.19	7.8	7.70
0.5 mg/mL hGH, 10 mg/mL SNAC-2, 2 mM TC in modified diffusion buffer[b]	7.65	7.6	7.65
0.5 mg/mL hGH, 50 mg/mL SNAC-2, 2 mM TC in modified diffusion buffer[b]	7.81	7.71	7.71

[a] all measurements at 37°C were taken in the presence of carbogen bubbling
[b] modified diffusion buffer was prepared as described in Section 6.2.2.1

diffusion chamber at 37°C when bubbled with carbogen was comparable. Based upon these results, pH adjustments were not required when SNAC-2 was added to the diffusion buffer.

6.3.3 Surface Tension Measurements in the Presence of SNAC-2

The determination of the permeability coefficient (P_{app}) may be influenced by the micellar concentration of surfactants present in the diffusion buffer. Nerurkar and co-workers described the measurement of the permeability of a model peptide in the presence of Cremophor EL and Polysorbate 80 (Nerurkar et al., 1997). A decrease in peptide permeability observed in the presence of surfactant concentrations greater than the CMC was ascribed to a reduction in the thermodynamic activity of the peptide. Surface tension measurements were therefore performed on SNAC-2 containing diffusion buffer both in the absence and presence of TC in order to define the CMC of the carrier under the solution conditions employed in the modified Ussing chamber.

Surface tension measurements for the standards, glycerol and water, were found to be consistent throughout this series of experiments, with the surface tension varying by less than 2% for each (n=22 measurements for each over six days). Figure 6.1 presents the surface tension of a series of SNAC-2 solutions in diffusion buffer versus SNAC-2 concentration, in the absence and presence of 2 mM TC. In the absence of TC, the surface activity of the diffusion buffer decreased from an initial value of 77 to 60 mN/m over the concentration range of 0 to 40 mg/mL SNAC-2. The CMC of SNAC-2 in diffusion buffer was estimated from this profile to be 11.3 mg/mL. The surface tension of SNAC-2 solutions at all concentrations investigated remained at 58 to 60 mN/m in the presence of 2 mM TC, thus SNAC-2 had no effect on surface activity when combined with TC.

Previous studies have reported the CMC of taurocholate in 0.15 M NaCl (approximately isotonic) to be 3.2 mM at 20°C, increasing to 4.0 mM at 30-40°C

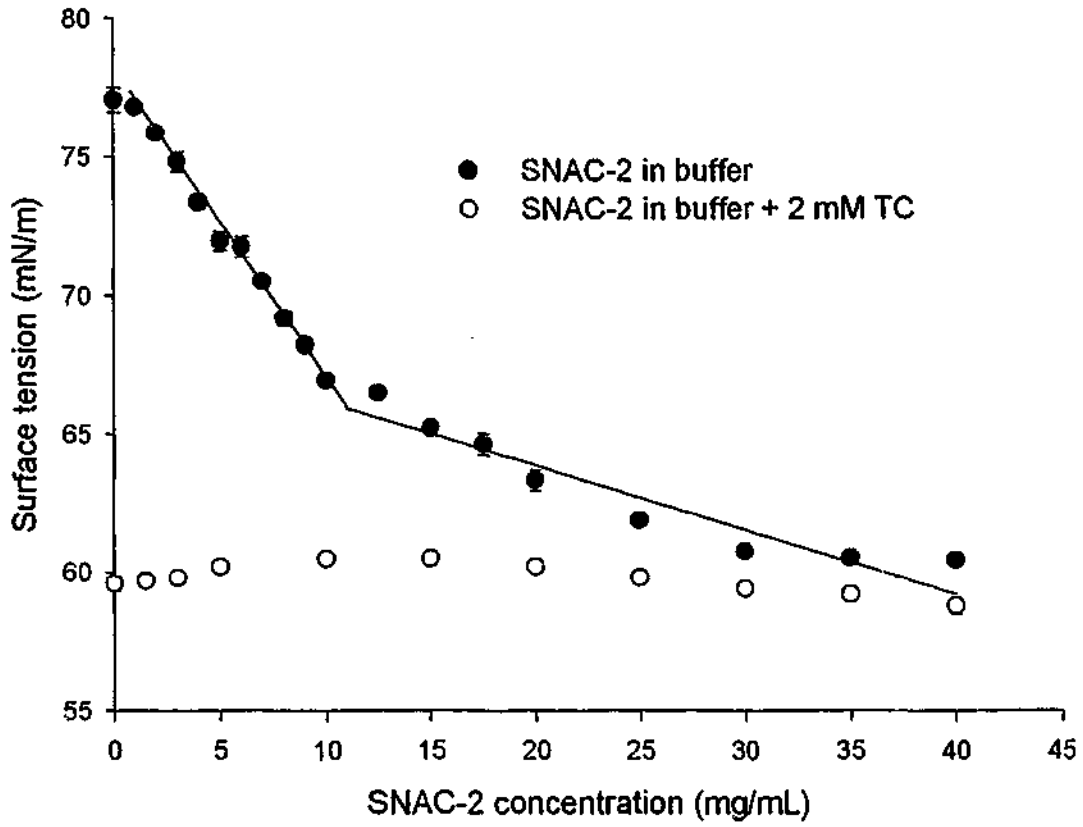


Figure 6.1 Surface tension (mN/m) versus SNAC-2 concentration in modified diffusion buffer. Symbols represent the mean ± SEM of three measurements.

(Carey and Small, 1972). These concentrations are higher than that employed in the diffusion buffer in the current study and as such, it is probable that the 2 mM concentration was below the CMC for TC in this system. Furthermore, it was unlikely that the inclusion of 2 mM TC in the diffusion buffer resulted in micellar solubilisation of the lipophilic marker, diazepam, with the P_{app} value of diazepam already demonstrated to be unaffected by the presence of 2 mM TC (Section 5.3.4.4). The effect of 2 mM TC on hGH permeability could not be defined owing to the [illegible] egation of hGH in the absence of TC, as described in Section 5.3.4. The [illegible] teractions in the presence of SNAC-2 and/or TC was further explored for [illegible] (Section 6.3.4) and diazepam (Section 6.3.7).

6.3.4 Ultrafiltration Studies to Determine Potential Interactions Between hGH and Solution Components

As discussed in Section 6.3.3, surface-active components in the diffusion buffer may interact with susceptible permeants (e.g. hGH, diazepam) through micellar solubilisation and affect the measurement of P_{app} values. The potential for an interaction between hGH and solution components (SNAC-2 and/or TC) in the modified diffusion buffer was assessed by ultrafiltration employing 30 kDa molecular weight cutoff filters. Three different solution compositions were evaluated: (i) 0.5 mg/mL hGH alone, (ii) 0.5 mg/mL hGH and 2 mM TC and (iii) 0.5 mg/mL hGH, 2 mM TC and 50 mg/mL SNAC-2. The first solution provided a control, used both to assist in the detection of an interaction between hGH and TC and to evaluate the possibility of hGH adsorbing to the filter, while the second and third solutions were selected based on conditions employed in the modified Ussing chamber.

Each solution condition was investigated in 7-8 filters in order to allow for any variation in the molecular weight cutoff range between individual filters. Filtrate samples were taken following each of the three spins and a donor sample was taken following the final spin.

The concentration of hGH determined in these samples was compared to the hGH concentration measured for the corresponding unfiltered solution. Donor samples for all solution conditions were within 15% of the unfiltered solution concentration, indicating that equilibrium conditions were maintained throughout the experiment.

The concentration of hGH in filtrate samples was compared to that in the unfiltered solution to allow the determination of the fraction remaining in solution following filtration. The free fraction of hGH in the control solution (i) was calculated to be 0.69 ± 0.07 (mean ± SEM, n=7 filters). It was noted that the control filtrate samples contained visible precipitate such that the measured free fraction could not be taken as an accurate reflection of the actual concentration of hGH passing through the filter. The presence of TC in solutions (ii) and (iii) would have most likely inhibited any aggregation and precipitation during filtration. The free fraction of hGH in the TC solution (ii) was determined to be 0.87 ± 0.02 (mean ± SEM, n=7 filters), while the free fraction of hGH in the 50 mg/mL SNAC-2 solutions (iii) was calculated to be 0.35 ± 0.04 (mean ± SEM, n=8 filters). No precipitate was visible in the filtrate of solutions (ii) and (iii). A small number of filtration experiments conducted in the presence of 10 mg/mL SNAC-2 and 2 mM TC gave values comparable to those obtained in the presence of 2 mM TC alone (data not shown).

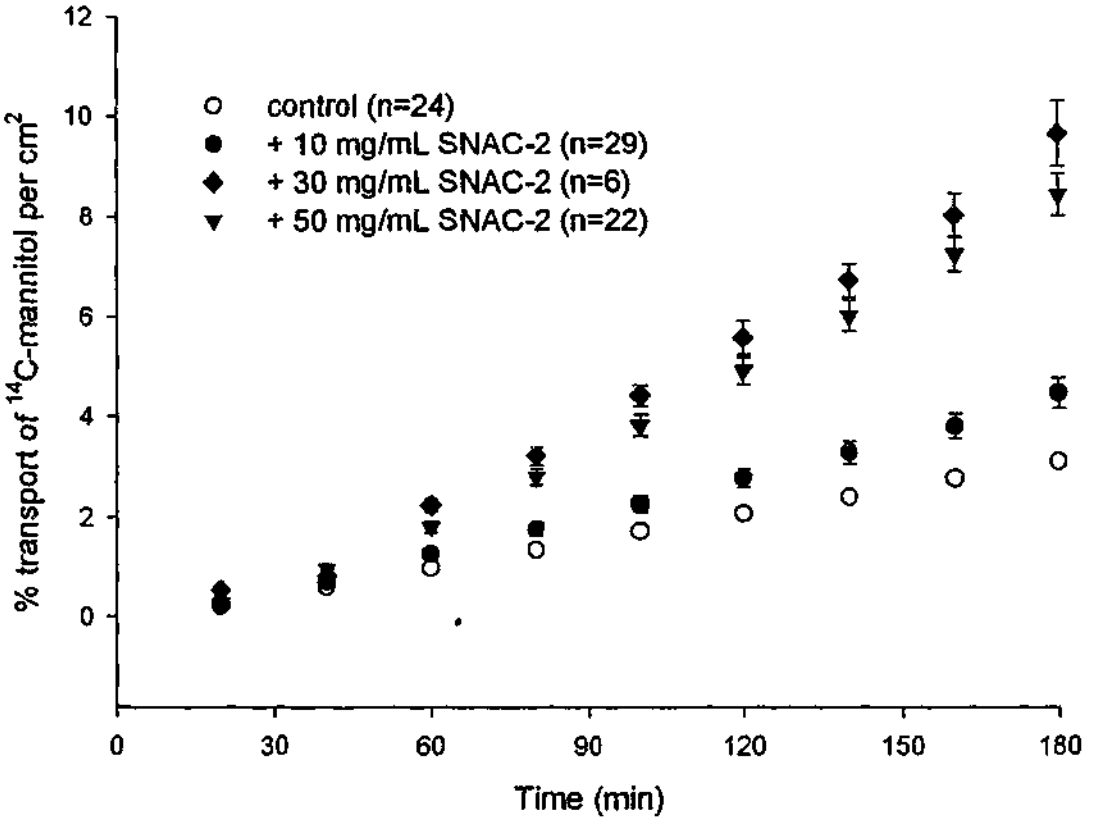
Owing to the presence of precipitate in control filtrate samples, the control conditions could not be used for comparative purposes. The free fraction of hGH in filtrate samples containing TC (ii) was within 15% of its corresponding unfiltered control, indicating that possible interactions between hGH and TC in solution were minimal. In comparison, the free fraction of hGH in filtrate samples containing SNAC-2 and TC was only 35% compared to the corresponding unfiltered control indicating the presence of a significant interaction between hGH and the solution components. The reduction in free hGH concentration in the solution containing 50 mg/mL SNAC-2 and TC therefore prompted a correction of hGH P_{app} values measured under these solution conditions.

Similar studies by Nerurkar and co-workers described the determination of the free fraction of a series of peptides in the presence of increasing concentrations of the surfactants, Polysorbate 80 and Cremophor EL (Nerurkar et al., 1997). The free fraction was used to correct the apparent permeability coefficients in Caco-2 cell monolayers for peptides in surfactant-containing diffusion buffers. Corrections were performed by dividing the P_{app} values by the free fraction of peptide. A likewise correction was made to P_{app} values measured for hGH in the current study, with those determined in the presence of 50 mg/mL SNAC-2 and 2 mM TC divided by the estimated free fraction of 0.35 (see Section 6.2.3).

6.3.5 Effect of SNAC-2 on the Permeability of the Paracellular Marker, Mannitol

6.3.5.1 SNAC-2 Concentration Effects on Mannitol Permeability

The permeability of mannitol in the presence of SNAC-2 was investigated in order to examine the effects of the model carrier on the paracellular pathway, one of the potential routes of hGH permeation. The flux of mannitol in the presence of 10, 20, 30 and 50 mg/mL SNAC-2 across stripped rabbit ileum is presented in Figure 6.2. All experiments presented in this figure and all subsequent experiments were conducted in the presence of 2 mM TC. Since the inclusion of 2 mM TC was required for hGH permeability measurements (2 mM TC demonstrated to prevent hGH aggregation in the diffusion chamber; see Section 5.3.4.2), this additive was also routinely included in all SNAC-2/marker compound permeability studies. Furthermore, similar P_{app} values were obtained for mannitol in the presence of 50 mg/mL SNAC-2 both with and without 2 mM TC (data not presented), indicating that any effects on mannitol permeability were predominantly due to the presence of SNAC-2. It is evident in Figure 6.2 that the inclusion of SNAC-2 in the donor chamber led to a significant increase in mannitol flux, as reflected in the P_{app} values reported in Table 6.3. Effects of the 20, 30 and 50 mg/mL concentrations were similar, while the 10 mg/mL SNAC-2 concentration caused only a small increase in the mannitol



Figu[illegible] Flux of ^{14}C-mannitol across stripped rabbit ileum plotted as % transport/cm^2 versus time. Data were obtained from experiments utilising donor (mucosal) solutions containing 0, 10, 30 and 50 mg/mL SNAC-2 in modified diffusion buffer. Symbols represent the mean ± SEM.

Table 6.3 Apparent permeability coefficients (P_{app}) for ^{14}C-mannitol in stripped rabbit ileum in the absence and presence of SNAC-2. P_{app} values were calculated using Equation 5.1. All diffusion buffers contained 2 mM taurocholate.

	SNAC-2 concentration (mg/mL)	*Permeability coefficient (x 10^6 cm/sec)*[a]	*N tissues/animals*
Control	**None**	**4.62 ± 0.31**	**24/10**
Effect of SNAC-2	10	6.47 ± 0.27[b]	29/5
	20	13.56 ± 0.44[b]	4/1
	30	15.57 ± 0.57[b]	6/1
	50	14.25 ± 0.46[b]	22/7
Effect of SNAC-2 pre-treatment[c]	10	3.90 ± 0.21	16/5
	20	8.46 ± 0.39[b]	4/1
	30	7.52 ± 0.38[b]	6/2
	50	9.21 ± 0.38[b]	14/3

[a] data reported as mean ± SEM
[b] significantly different to control ($p<0.001$)
[c] tissue exposed to SNAC-2 for 30 min prior to replacement of buffer (without SNAC-2) and addition of ^{14}C-mannitol

P_{app} value. Higher mannitol permeabilities indicate an effect of SNAC-2 on the paracellular route of absorption, either as a consequence of absorption enhancement, altered tissue integrity or a combination of both of these factors.

One possible mechanism of increased paracellular transport is that of calcium chelation/complexation, an ability common to many conventional absorption enhancers (Muranishi, 1990) and which may or may not play a role in the absorption enhancement afforded by compounds such as sodium caprate. Calcium chelating agents such as EDTA and EGTA are recognised to exert their absorption enhancing effects *in vitro* by sequestering calcium ions from tight junctions and subsequently increasing paracellular permeation. While these agents work *in vitro* as absorption enhancers, in order to be effective, their presence is generally required in both mucosal and serosal buffers. *In vivo*, oral drug formulations only have access to the mucosal surface, and this, in addition to potentially large doses of chelating agents needed to complex with variable luminal calcium concentrations, makes it highly unlikely that calcium chelation would be responsible for any significant *in vivo* absorption enhancement (Hochman and Artursson, 1994). Based upon these observations, while it was unlikely that calcium chelation played a major role in the *in vivo* absorption enhancement of hGH by SNAC-2, it was possible that calcium chelation by SNAC-2 affected paracellular transport in the *in vitro* model.

A brief investigation of the complexation of calcium by SNAC-2 was conducted using an ion specific electrode for measuring free calcium ion concentrations in order to assess this phenomenon as a potential mechanism of enhancement of hGH permeation in the *in vitro* model. The study demonstrated a reduction in the concentration of free calcium ions in solution in the presence of the carrier, although a binding constant could not be calculated owing to the non-ideal solution conditions and the likely presence of micelles and/or mixed micelles (data not shown). It was therefore possible that complexation of calcium ions by SNAC-2 contributed to the observed increase in the permeability of the paracellular marker, mannitol, in the *in vitro* model. Furthermore, if hGH intestinal permeation were to occur to any significant extent via the

paracellular route, it is possible that this may be enhanced *in vitro* at least in part as a result of calcium complexation by SNAC-2. However, this phenomenon is unlikely to be primarily responsible for the *in vivo* absorption enhancement of hGH.

Previous investigations by Milstein et al. into the intestinal permeability of hGH and marker compounds in the presence of another carrier, 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid (referred to as E198 by Milstein), revealed that 150 mg/mL 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid had no significant effect on the permeability of mannitol (Milstein et al., 1998). These results were in contrast to earlier studies, which explored the effect of several different Emisphere carrier compounds (but not SNAC-2 or 4-(4-(N- phenylsulfonyl)aminophenyl)butyric acid) on the intestinal permeability of sodium cromolyn and the paracellular marker, lucifer yellow (Leone-Bay et al., 1996b). It was determined that the permeabilities of both cromolyn and lucifer yellow were significantly greater (up to 5-7-fold higher) in the presence of selected carrier compounds, indicating that the paracellular pathway was affected by the presence of these carriers.

Having demonstrated an effect of SNAC-2 on the paracellular pathway, the possibility existed for the mechanism of absorption enhancement of hGH to result from increased paracellular permeability. The contribution of the paracellular route to the surface area of the small intestine is however minimal (0.01%) and is not likely to play a major role in the absorption of most drugs. (Stenberg et al., 2000). Despite the limited surface area for absorption, increased paracellular permeability to macromolecules has been reported. Fasano and Uzzau described the enhanced absorption of insulin and immunoglobulins via the paracellular route in a rabbit model, with enhancement by Zonula occludens toxin (Zot) mediated via opening of tight junctions (Fasano and Uzzau, 1997). Additionally, sodium caprate was shown to reversibly increase the ileal tight junction permeability of macromolecules in rats (Soderholm et al., 1998). The disadvantage of the non-specific absorption enhancement via the paracellular pathway is the

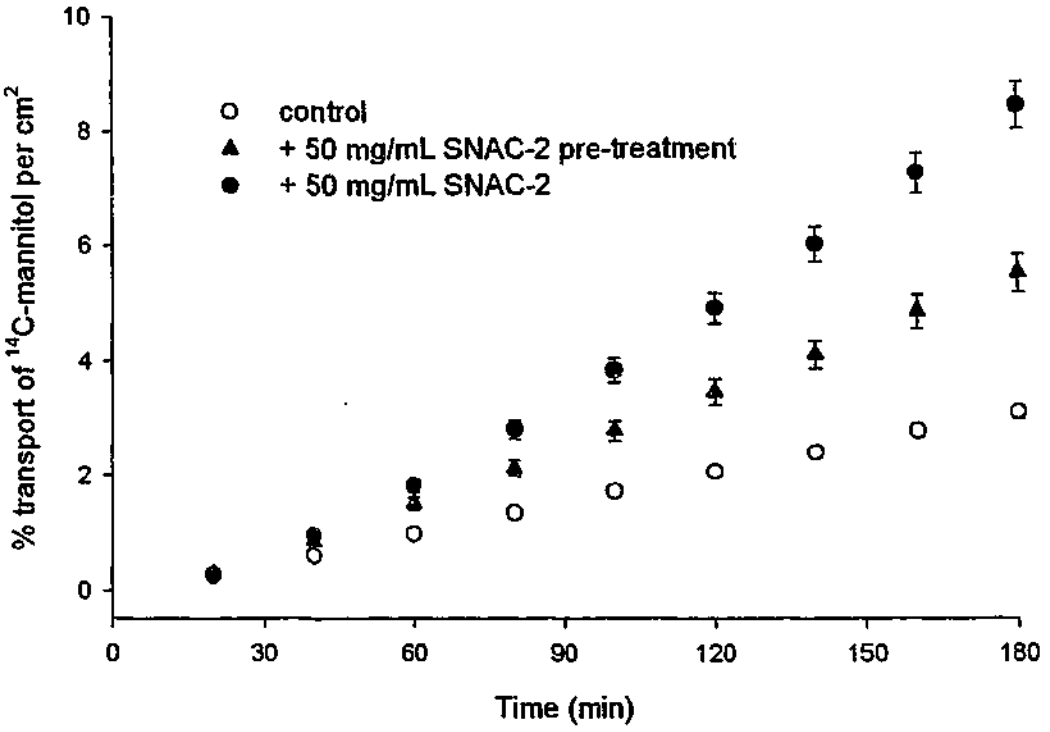
potential for concomitant unwanted absorption of antigenic macromolecules which may be instrumental in the development of autoimmune disease (Fasano et al., 2000).

6.3.5.2 Reversibility of SNAC-2 Effects on Mannitol Permeability

The reversibility of the effect of absorption enhancers is frequently studied for the purpose of evaluating the potential for irreversible alterations to tissue (Anderberg et al., 1992, Yeh et al., 1994, Oberle et al., 1995, Quan et al., 1998, Soderholm et al., 1998). The absorption enhancer is added to the diffusion chamber for a short period of time (e.g. 10-30 min) prior to the experiment, removed, drug added and permeability measurements conducted in the usual manner for the remainder of the experimental period. Incomplete reversibility of effect (failure of the apparent permeability coefficient to return to the control value) may indicate tissue damage, a time dependent effect or a combination of the two.

Mannitol and other hydrophilic markers including PEGs and dextrans may be used to assist in the assessment of the reversibility of absorption enhancers (Anderberg and Artursson, 1993, Anderberg and Artursson, 1994, Quan et al., 1998). Anderberg and Artursson developed a routine screen for the evaluation of potential absorption enhancing compounds utilising mannitol as a marker to demonstrate both altered permeability in the presence of enhancers and the reversibility of effect in cultured cells (Anderberg and Artursson, 1994). In these studies, transepithelial electrical resistance (TEER) measurements were omitted from the screen in favour of mannitol owing to the greater sensitivity of the latter assay. In the current series of experiments, mannitol was employed to observe the reversibility of the effect of SNAC-2 at concentrations of 10-50 mg/mL in order to gain insight into the time dependency of SNAC-2 effects and the potential for toxicity.

Figure 6.3 presents the reversibility of the effect of 10 and 50 mg/mL SNAC-2 on mannitol flux. While the flux of mannitol following pre-treatment with 10 mg/mL SNAC-2 was comparable to that of control, the flux of mannitol after tissue exposure to 50 mg/mL SNAC-2



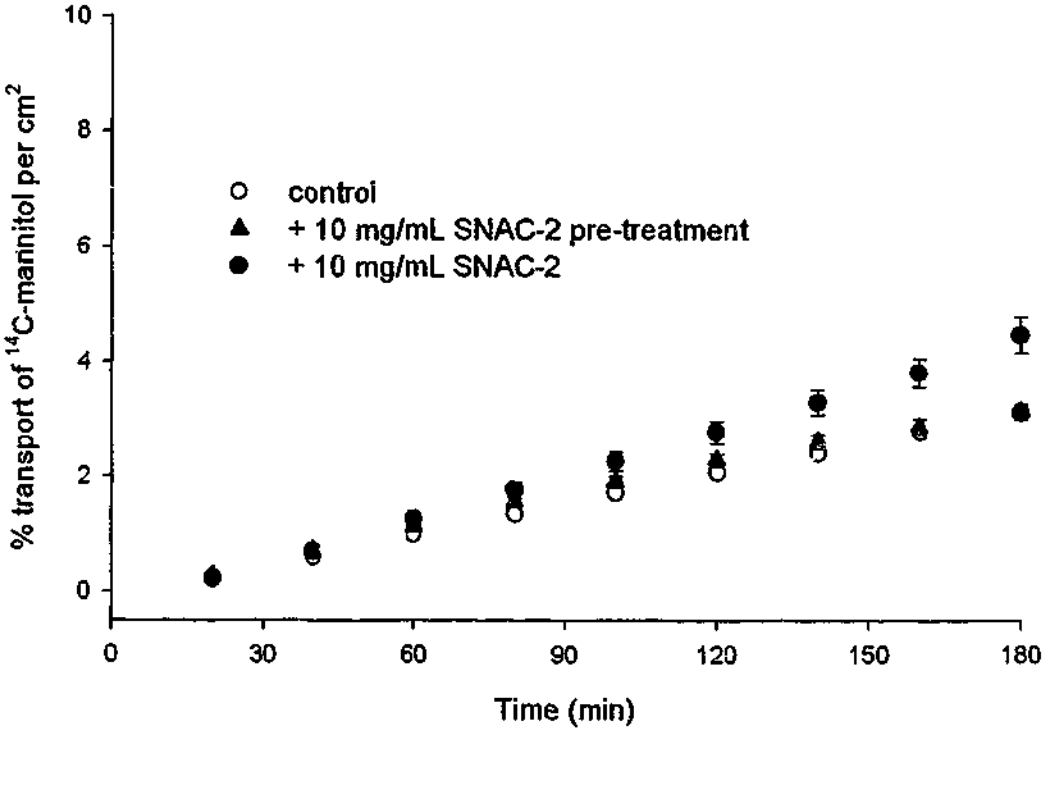


Figure 6.3 Flux of ^{14}C-mannitol across stripped rabbit ileum plotted as % transport/cm^2 versus time. Tissues were treated with 50 mg/mL SNAC-2 in modified diffusion buffer for 30 min or 3 h (top) and 10 mg/mL SNAC-2 in modified diffusion buffer for 30 min or 3 h (bottom). Symbols represent the mean ± SEM.

remained markedly higher than that of the control value. Table 6.3 lists the P_{app} values for mannitol measured in tissues pre-treated with 10, 20, 30 and 50 mg/mL SNAC-2. Complete reversibility of effect was demonstrated by 10 mg/mL SNAC-2 only, with P_{app} values actually slightly lower than those of control ($p=0.039$), while a highly significant increase relative to control values was apparent for all other concentrations of SNAC-2 ($p<0.001$). The reason for the slight reduction in P_{app} values observed in tissues pre-treated with 10 mg/mL SNAC-2 was not further investigated.

Irreversibility of effect may be a consequence of insufficient tissue recovery time and/or tissue damage. Observation of at least partial reversibility of effect on the mannitol permeability coefficient at all SNAC-2 concentrations suggests that some recovery of tissue barrier properties was achieved within the experimental period. From a viability standpoint, the use of isolated rabbit intestinal tissues in the modified Ussing chamber is restricted to a maximum of four hours total experimental time (Swaan et al., 1994), limiting the time available for tissue recovery. Caco-2 cells have some advantage in reversibility studies, as cell monolayers may be allowed many hours recovery time prior to permeability measurements. *In situ* models such as the rat intestinal perfusion model also allow greater flexibility in this regard, with viability preserved through the aid of a reasonably intact intestine and blood supply.

Despite the possibility of tissue damage indicated by a lack of complete reversibility of mannitol permeability in the *in vitro* model, it is important to note that extensive *in vivo* toxicity studies with the Emisphere carriers have consistently demonstrated the absence of tissue damage (Leone-Bay et al., 1995, Leone-Bay et al., 1996a, Leone-Bay et al., 1998, Milstein et al., 1998). The issue of potential tissue damage was further addressed by conducting a histological examination of tissues exposed to SNAC-2 in the diffusion chamber as described in Section 6.3.8.3.

6.3.6 Effect of SNAC-2 on the Permeability of PEG 4000

PEG 4000 is commonly utilised in permeability studies as a large hydrophilic "non-absorbable" marker (Cogburn et al., 1991, Rubas et al., 1993, Yu et al., 1995, Jezyk et al., 1999). PEG 4000 exhibits neglible oral bioavailability in humans (Rubas et al., 1993) which reflects its poor intestinal permeability characteristics. It was included in the current study to provide a representative hydrophilic, high molecular weight marker for determining the effects of SNAC-2 on paracellular transport.

The ileal permeability of PEG 4000 in standard diffusion buffer containing 2 mM TC was determined to be $0.86 \pm 0.07 \times 10^{-6}$ cm/sec (mean ± SEM). This permeability coefficient compared favourably to literature data, with Yu and co-workers reporting a P_{app} for PEG 4000 in rabbit ileum of $1.30 \pm 0.19 \times 10^{-6}$ cm/sec (Yu et al., 1995). The addition of 50 mg/mL SNAC-2 to the donor chamber resulted in an increase in the P_{app} value to $2.20 \pm 0.16 \times 10^{-6}$ cm/sec, which is approximately 2.5-fold higher than the control value ($p<0.001$), a magnitude of effect similar to that observed for the small, hydrophilic mannitol (see Section 6.3.5.1). The increased permeability of PEG 4000 in the presence of SNAC-2 also indicates an effect on the paracellular pathway, most likely resulting from non-specific absorption enhancement, altered tissue integrity in the *in vitro* model or a combination of the two.

The P_{app} value of PEG 4000 under control conditions was approximately five-fold higher than that measured for hGH. Even though PEG 4000 is a large hydrophilic marker, it is still approximately five times smaller than hGH (MW 22 kDa) based on MW. It is an oversimplification, however, to compare the intestinal permeability of these two macromolecules based on MW alone. Other factors affecting intestinal permeation include shape and physicochemical characteristics, such as the proposed altered conformation of hGH in the presence of SNAC-2. He and co-workers described PEGs in solution as "flexible" and "more elongated than spherical" which may make them more able to diffuse through tight junctions

than more spherical proteins of a comparable or larger size (He et al., 1998). In conjunction with the difference in molecular weight, this may partially explain the intestinal permeability of PEG 4000 still being five times higher than that of hGH under control conditions. Despite these differences, PEG 4000 was used as a well-characterised, macromolecular marker of the effects of SNAC-2 on the paracellular pathway, providing a more relevant comparator for hGH than mannitol (MW 182).

6.3.7 Effect of SNAC-2 on the Permeability of the Transcellular Marker, Diazepam

The effect of SNAC-2 on diazepam permeability was assessed in order to examine carrier effects on the transcellular transport pathway. Figure 6.4 presents the flux of the transcellular marker, diazepam, in stripped rabbit ileum in the absence and presence of 10 and 50 mg/mL SNAC-2. Diazepam flux was markedly reduced in the presence of both concentrations of SNAC-2, with a 20-30 min lag time also evident in the presence of the carrier. Table 6.4 lists the P_{app} values obtained for diazepam in stripped rabbit ileum, with a significant reduction in the P_{app} value for both 10 and 50 mg/mL SNAC-2 ($p<0.005$). The most likely explanation for the reduction in P_{app} values observed was the solubilisation of diazepam by SNAC-2. The calculation of P_{app} (see Equation 5.1) incorporates the initial donor concentration of drug, which theoretically equates to the free concentration of drug. The free concentration may be reduced as a consequence of micellar solubilisation and thus the "actual" P_{app} may be underestimated (also observed for hGH, as discussed in Section 6.3.4). To enable the calculation of the free concentration of diazepam in the donor chamber, the solubility of diazepam in SNAC-2 solutions (in modified diffusion buffer) was studied as a function of SNAC-2 concentration. As the concentration of SNAC-2 increased (0-50 mg/mL), there was a significant increase in the solubility of diazepam, occurring in a non-linear fashion, with a ten-fold solubility enhancement observed in the presence of 50 mg/mL SNAC-2 (data not presented).

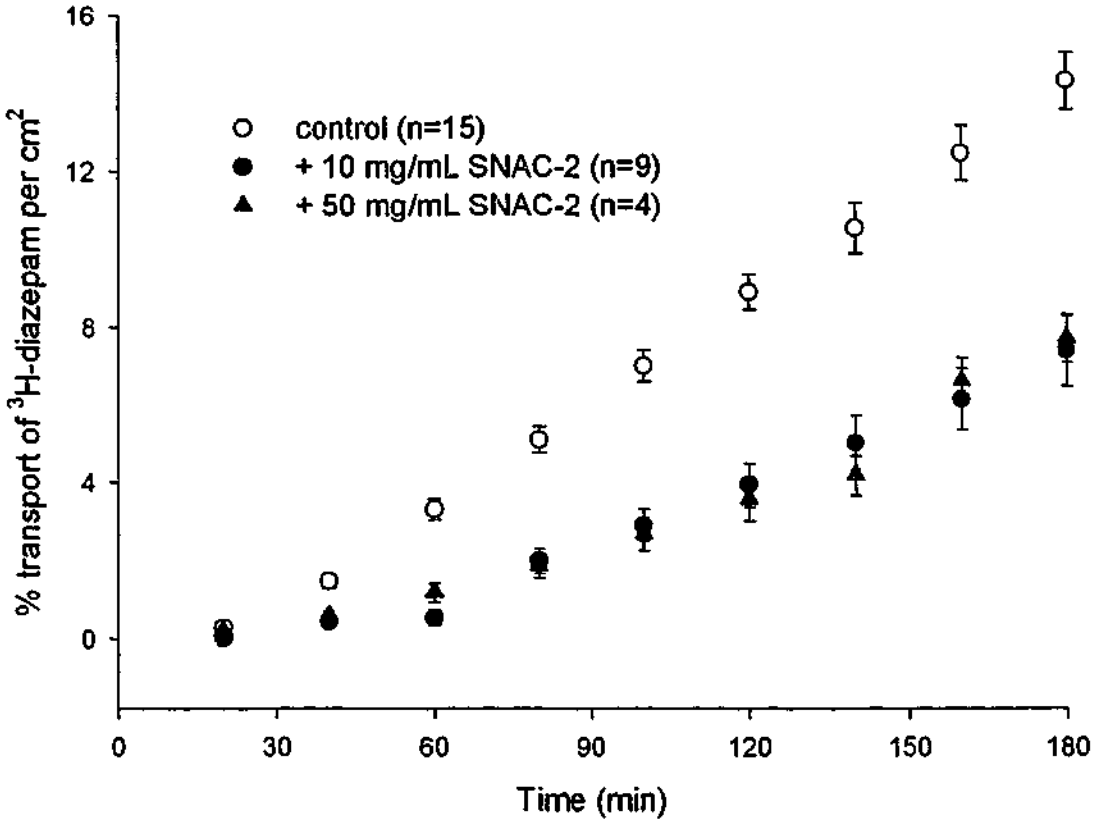


Figure 6.4 Flux of ^{3}H-diazepam across stripped rabbit ileum plotted as % transport/cm^2 versus time. Data were obtained from experiments utilising donor (mucosal) solutions containing 0, 10, and 50 mg/mL SNAC-2 in modified diffusion buffer. Symbols represent the mean ± SEM.

Table 6.4 Apparent permeability coefficients (P_{app}) for ^{3}H-diazepam in stripped rabbit ileum in the absence and presence of SNAC-2. P_{app} values were calculated using Equation 5.1. All diffusion buffers contained 2 mM taurocholate.

	SNAC-2 concentration (mg/mL)	*Permeability coefficient (x 10^{6} cm/sec)*[a]	*N tissues/animals*
Control	**None**	**23.75 ± 1.54**	**15/4**
Effect of SNAC-2	50	10.38 ± 1.06[b]	4/1
	10	13.25 ± 1.15[b]	9/3

[a] data reported as mean ± SEM
[b] significantly different to control ($p<0.005$)

Calculation of the free fraction of drug in surfactant solutions utilising solubility profiles has been described by Amidon et al. for the purpose of determining the free fraction of a model lipophilic drug in the presence of varying concentrations of Tween 80 (Amidon et al., 1982). A requirement of this method of calculation is linearity of the solubility profile, a trend not seen in the diazepam/SNAC-2 profiles obtained in the current study. The absence of linearity precluded the calculation of the free diazepam concentration by this method. Similar (non-linear) solubility profiles obtained by O'Reilly and co-workers for the lipophilic drug, clofazimine, in the presence of bile salts were ascribed to atypical micelle formation (O' Reilly et al., 1994). As neither SNAC-2 nor TC could be described as conventional surfactants, it is likely that the atypical nature of micelles and/or mixed micelles formed was also responsible for the observed non-linearity in the current study.

It is probable that the reduction in P_{app} values determined for diazepam in the presence of SNAC-2 resulted from micellar solubilisation of the lipophilic marker, although as the free concentration could not be determined, the P_{app} value could not be corrected to allow for this effect. Consequently, the effect of SNAC-2 on the transcellular pathway could not be defined.

6.3.8 Effect of SNAC-2 on the Permeability of hGH

6.3.8.1 SNAC-2 Concentration Effects on hGH Permeability

Figure 6.5 presents the flux of hGH across stripped rabbit ileum in the absence and presence of 10, 20, 30 and 50 mg/mL SNAC-2. hGH flux was significantly greater in the presence of SNAC-2, with a lag time of approximately 60-70 min noted in the presence of carrier concentrations of 30 and 50 mg/mL. The basis for the extended lag time was not readily apparent, but may result from a time-dependent absorption enhancing effect. Importantly, there was no evidence of hGH instability in the presence of SNAC-2 in the diffusion chamber, as

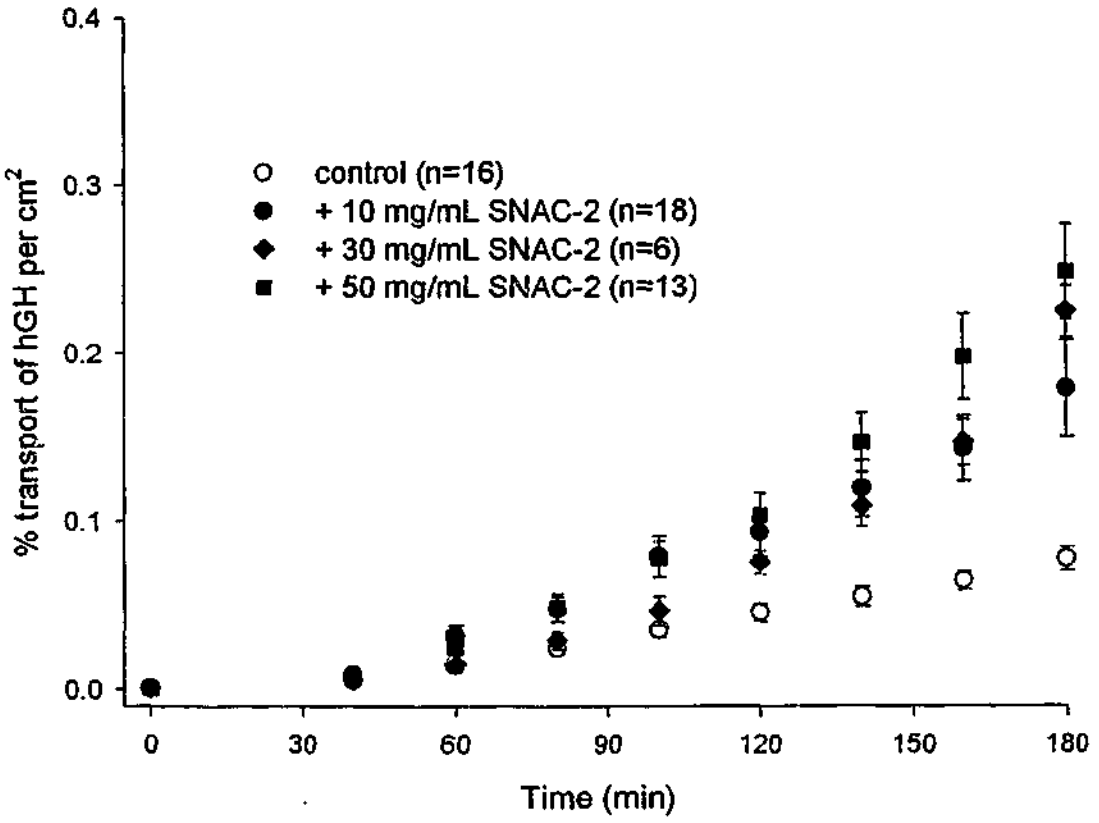


Figure 6.5 Flux of hGH across stripped rabbit ileum plotted as % transport/cm^2 versus time. Data were obtained from experiments utilising donor (mucosal) solutions containing 0, 10, 30 and 50 mg/mL SNAC-2 in modified diffusion buffer. Symbols represent the mean ± SEM.

demonstrated by the consistent initial and final donor chamber hGH concentrations determined for each experiment using the SEC assay.

Table 6.5 lists the P_{app} values for hGH in stripped rabbit ileum, with values determined in the presence of the carrier significantly greater than control values ($p<0.005$). Addition of 20, 30 and 50 mg/mL SNAC-2 resulted in similar increases in the P_{app} of hGH (approximately three-fold) compared to the addition of 10 mg/mL SNAC-2, which led to a two-fold increase relative to that measured in buffer alone. Correction of the P_{app} value for the free fraction of hGH in solutions containing 50 mg/mL SNAC-2 (described in Section 6.3.4) increased the P_{app} such that the enhancement afforded by SNAC-2 was approximately nine-fold, which was almost an order of magnitude higher than the control. Correction of the P_{app} values for hGH in the presence of the intermediate concentrations (20 and 30 mg/mL) of SNAC-2 was not investigated, as the majority of the permeability experiments, including mechanistic studies, were conducted using the 50 mg/mL carrier concentration.

The permeability of the large hydrophilic marker, PEG 4000, was increased approximately 2.5-fold in the presence of 50 mg/mL SNAC-2 (described in Section 6.3.6), a much smaller change than the nine-fold increase in the hGH P_{app} value under the same solution conditions. If the transport route of hGH in the presence of SNAC-2 was wholly paracellular, it is likely that the increase in the P_{app} value for the 22 kDa hGH would be less than that observed for PEG 4000. Consequently, assuming that there is minimal interaction between PEG 4000 and SNAC-2 in solution, it is probable that a significant component of the transmucosal passage of hGH in the presence of SNAC-2 is via the transcellular route.

The permeation of hGH under control conditions (i.e. in the absence of carrier) may occur via either the transcellular or the paracellular route or a combination of both. Gan and co-workers described the transport of thyrotropin-releasing hormone across Caco-2 cell monolayers as "predominantly paracellular" (Gan et al., 1993), while Marcon-Genty et al. showed

Table 6.5 Apparent permeability coefficients (P_{app}) for hGH in stripped rabbit ileum in the absence and presence of SNAC-2. P_{app} values were calculated using Equation 5.1.

	SNAC-2 concentration (mg/mL)	*Permeability coefficient (x 10^6 cm/sec)*[a]	*N Tissues/animals*
Control	**None**	**0.15 ± 0.01**	**16/7**
Effect of SNAC-2	10	0.30 ± 0.02[b]	18/5
	20	0.36 ± 0.05[b]	4/1
	30	0.40 ± 0.03[b]	6/1
	50	0.48 ± 0.03[b]	13/5
	50	1.37 ± 0.08[b,c]	-
Effect of SNAC-2 pre-treatment[d]	10	0.11 ± 0.02	8/3
	50	0.38 ± 0.06[b]	7/2

[a] data reported as mean ± SEM
[b] significantly different to control ($p<0.005$)
[c] values corrected for the free fraction of hGH as described in Section 6.3.4.
[d] tissue exposed to SNAC-2 for 30 min prior to replacement of buffer (without SNAC-2) and addition of hGH

transcellular transport of β-lactoglobulin across rabbit ileum (Marcon Genty et al., 1989). The affinity of macromolecules for a permeation pathway in the presence of enhancers in the colon has been described, with macromolecules the size of hGH (approximately 20 kDa) thought to pass partly via the transcellular route and partly via the paracellular route (approximately 50% through each) (Muranishi, 1990). Knipp and co-workers restricted the conformation of a series of hydrophilic peptides by various cyclisation methods, subsequently observing greatly enhanced absorption of the peptides in Caco-2 cells (Knipp et al., 1997). The enhanced permeation was ascribed to a shift from paracellular to transcellular absorption, a result of heightened lipophilic characteristics of the cyclic peptides. The increase in hGH permeability upon the addition of 50 mg/mL SNAC-2 may possibly result from an enhanced lipophilic nature of hGH in the presence of SNAC-2 due to conformational changes accompanied by a shift to a predominantly transcellular route of absorption.

Studies by Mlynek and co-workers investigating hGH permeability in rabbit intestine reported an increase in ileal permeability from $3.09 \pm 0.25 \times 10^{-6}$ cm/sec to $5.81 \pm 0.49 \times 10^{-6}$ cm/sec upon the addition of the carrier, 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid (referred to as E198 by Mlynek) (Mlynek et al., 2000). The actual values of control permeability coefficients obtained in the current study are approximately 20-fold lower than the control values determined by Mlynek, with the most likely reason for this discrepancy being the detection of free radiolabel with the use of ^{125}I-hGH as discussed in Section 5.3.4.7. The observation of a greater effect of SNAC-2 on hGH permeability in this study compared to the effect of 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid described by Mlynek (nine-fold compared to two-fold) may be the result of one or more contributing factors. It has previously been demonstrated that the Emisphere carriers vary in their ability to deliver hGH across the intestinal mucosa (Milstein et al., 1998) and hence SNAC-2 may be more effective in promoting the transmucosal delivery of hGH than 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid. In addition, the potential for an interaction between hGH and 4-(4-(N-

phenylsulfonyl)aminophenyl)butyric acid was not evaluated nor corrected for by Mlynek, with measured P_{app} values possibly artificially low due to a reduction in the free concentration of hGH. Mlynek attributed the mechanism of 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid - mediated hGH absorption enhancement in rabbit duodenum to a passive transcellular process based on the lack of effect of 4-(4-(N- phenylsulfonyl)aminophenyl)butyric acid on paracellular marker permeability and the observations of no asymmetry of transport and no change in hGH permeation in the presence of active transport inhibitors.

An *in vitro* assessment of hGH permeability in the presence of carrier compounds was also conducted by Wu and Robinson, who showed the enhanced permeation of hGH across Caco-2 cells by the Emisphere carriers, SNAC and SABA (referred to as E414 and E352, respectively, by Wu) (Wu and Robinson, 1999a, Wu and Robinson, 1999b). They observed twelve- and nine-fold increases in hGH permeability with SNAC and SABA, respectively. Although it is difficult to compare permeability coefficients and permeation enhancement obtained in Caco-2 cells to those obtained in isolated tissues, the results of the current study are broadly consistent with those reported by Wu with regard to the increased permeability of hGH in the presence of carrier compounds. Interestingly, Wu also observed an asymmetry of hGH permeation in the presence of SNAC and SABA, suggesting the involvement of a "P-glycoprotein-like efflux system". This is in direct contrast to the results obtained by Mlynek for hGH permeation in the presence of 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid in rabbit duodenum (discussed above), although the discrepancy may possibly be due to the overexpression of P-glycoprotein in Caco-2 monolayers relative to intestinal tissues, a characteristic noted by Wu. The potential for the involvement of active transport processes in the absorption enhancement of hGH by carriers is further discussed in Section 6.3.10, in which results of studies to investigate the possibility of active transport of hGH in the presence of the model carrier, SNAC-2, are also described.

6.3.8.2 *Reversibility of SNAC-2 Effects on hGH Permeability*

The reversibility of the effect of SNAC-2 on hGH intestinal permeability was assessed employing carrier concentrations of 10 and 50 mg/mL. Table 6.5 presents the effect of a 30 min exposure of rabbit ileum to 10 and 50 mg/mL SNAC-2 on hGH P_{app} values. Tissues pre-treated with 10 mg/mL SNAC-2 prior to experiments demonstrated complete reversibility of effect, with P_{app} values actually slightly lower than those of control ($p=0.031$), while tissues pre-treated with 50 mg/mL showed incomplete reversibility, returning to a P_{app} value of slightly over double that of the control. Similar results were seen for mannitol, as described in Section 6.3.5.2.

Studies conducted by Milstein and co-workers on the permeability of hGH in the presence of the Emisphere carrier, 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid, showed complete reversibility of effect on hGH after a 30 min exposure of rabbit duodenum to 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid (Milstein et al., 1998). Several possibilities may account for the discrepancies between Milstein's observations and those of the current study, with different carriers and carrier concentrations likely to exhibit different tissue and different reversibility effects. The two studies were also performed on tissues excised from different intestinal regions; Milstein's data was obtained in duodenum, rather than ileum, thus making a direct comparison difficult. Furthermore, there remains the possibility of residual damage to the excised intestinal segments which are inherently deprived of a protective mucus layer and blood supply and exposed to high and extended local concentrations of the carrier. Potential tissue damage in the presence of SNAC-2 will be further discussed in the context of histological changes as described below.

6.3.8.3 *Histology*

Evaluation of tissues by light microscopy to confirm viability and an explanation of the scoring system employed in these studies was discussed in detail in Section 5.3.3. Scoring of tissues was generally low indicating that minimal tissue damage was sustained following

incubation in modified diffusion buffer containing 10 or 50 mg/mL SNAC-2 for both 30 min (pre-treatment only) and 3 h. Moderate disruption of the mucosa was observed in all tissues, with mild villous stunting and an increase in the proportion of goblet cells noted in the segment pre-treated with 10 mg/mL SNAC-2.

Despite the overall assessment indicating the absence of severe tissue damage in the presence of SNAC-2, results were interpreted with caution owing to poor staining and visualisation of tissues exposed to the weakly acidic carrier. The principle behind the haemotoxylin and eosin staining procedure is the selective colouring and definition of cell structures based on their acid and alkali characteristics. The presence of relatively large concentrations of a weak acid such as SNAC-2 may have interfered with the staining process, making interpretation of results difficult. Consequently, the tabulated results of the pathologist's evaluation are not presented.

Anionic surfactants are well known to adversely affect tissue morphology (Gullikson et al., 1977, Swenson et al., 1994). Sodium dodecyl sulphate (SDS) is an example of this group of compounds and is known to interfere with lipid membranes and denature proteins, accounting for its efficiency as an absorption enhancer. Adverse effects reported in rat intestinal mucosa perfused with buffered 1% SDS include villous erosion and degradation of epithelial cells (Nadai et al., 1972). Such histological abnormalities have also been demonstrated to be reversible in the rat intestine (Nakanishi et al., 1983, Swenson et al., 1994). While the morphological effects of SNAC-2 were unlikely to be as severe as those caused by a strong surfactant such as SDS, the possibility of a degree of local toxicity induced by the carrier in isolated tissues would not be unexpected.

6.3.9 Regional Comparisons of SNAC-2 Effects on hGH and Mannitol Permeability

Since the oral absorption of drugs typically occurs in the proximal small intestine, the focus of *in vitro* permeability studies is generally in this region. Regional studies (jejunum, ileum, colon) may nevertheless provide a useful comparison and assist in the identification of regions of optimal absorption/absorption enhancement of drugs. Environmental variations along the length of the gastrointestinal tract that can influence drug absorption include changing membrane characteristics (e.g. surface area for absorption), pH, the presence of proteolytic enzymes and the expression of active transport systems. An investigation of the intestinal permeability of hGH and the marker, mannitol, in the presence of SNAC-2 was conducted in stripped rabbit jejunum and colon to assess the absorption enhancement afforded by SNAC-2 in these regions and compare to data obtained in rabbit ileum.

6.3.9.1 SNAC-2 Effects on Mannitol Permeability in Rabbit Jejunum and Colon

The flux of mannitol across stripped rabbit jejunum and colon in the absence and presence of 10 mg/mL SNAC-2 is presented in Figure 6.6. Addition of SNAC-2 led to an increase in mannitol flux in both regions, although the increase was significantly greater in the colon. Table 6.6 lists the P_{app} values determined for mannitol, with approximately two- and eight-fold increases shown in the presence of SNAC-2 in jejunum and colon, respectively. Owing to the magnitude of the change in mannitol flux and the potential for tissue damage, a small number of experiments were conducted to explore the reversibility of the effect of SNAC-2 in colon tissue. A degree of reversibility was observed over the 3 h experimental period, indicating at least partial recovery of tissue barrier properties in this time, however, recovery was not complete.

Based on changes in the permeability of mannitol observed in all intestinal regions investigated (jejunum, ileum, colon), with the greatest increase shown in the colon, it was evident that the paracellular route of transport was affected by the presence of SNAC-2 in all

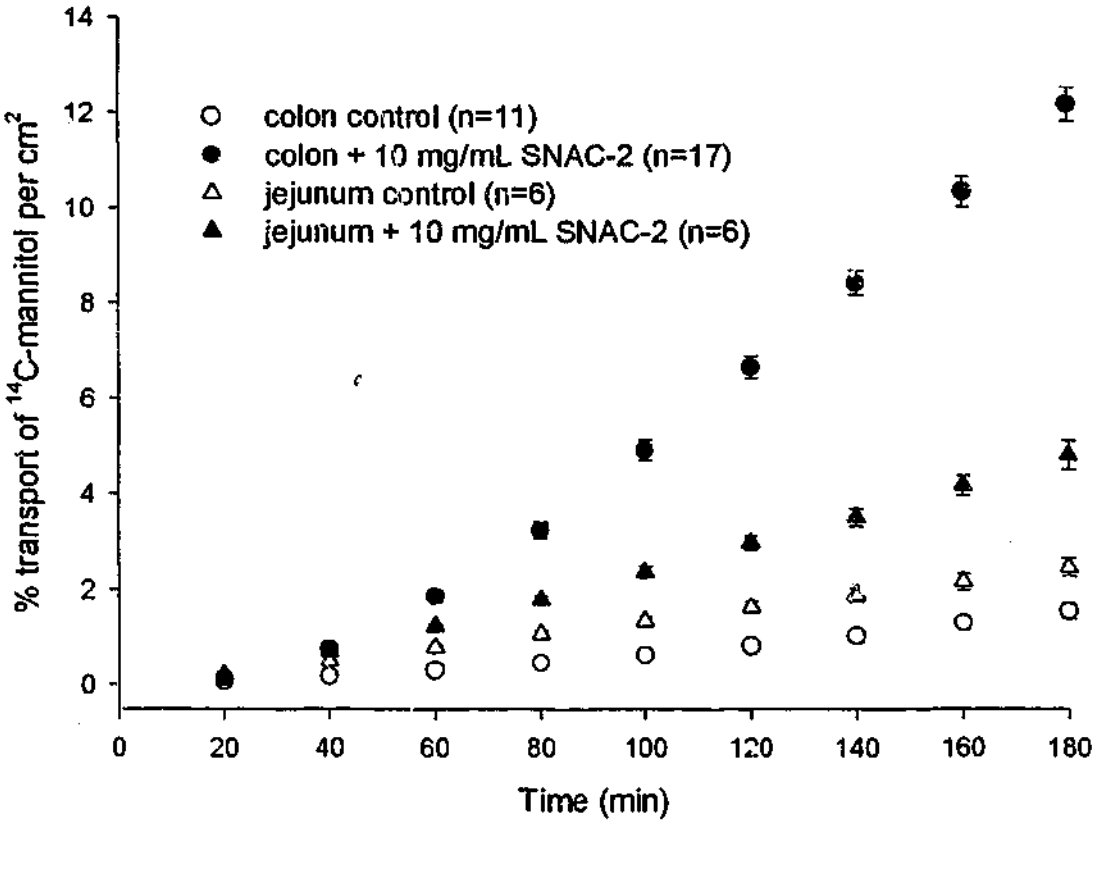


Figure 6.6 Flux of ^{14}C-mannitol across stripped rabbit jejunum and colon plotted as % transport/cm^2 versus time. Data were obtained from experiments conducted in the absence (open symbols) and presence (closed symbols) of 10 mg/mL SNAC-2. Symbols represent the mean ± SEM.

Table 6.6 Apparent permeability coefficients (P_{app}) for ^{14}C-mannitol in stripped rabbit jejunum and colon. P_{app} values were calculated using Equation 5.1. All diffusion buffers contained 2 mM taurocholate.

	Region	*SNAC-2 concentration (mg/mL)*	*Permeability coefficient ($\times 10^6$ cm/sec)*[a]	*N Tissues/animals*
Control	**jejunum**	**0**	**3.40 ± 0.35**	**6/2**
Effect of SNAC-2		10	7.60 ± 0.35[b]	6/2
Control	**colon**	**0**	**3.01 ± 0.38**	**11/5**
Effect of SNAC-2		10	22.56 ± 0.63[b]	17/6
Effect of SNAC-2 pre-treatment[c]		10	9.18 ± 1.14[b]	3/1

[a] data reported as mean ± SEM
[b] significantly different to control ($p<0.001$)
[c] tissue exposed to SNAC-2 for 30 min prior to replacement of buffer (without SNAC-2) and addition of ^{14}C-mannitol

regions. The possible basis for the observed increases in mannitol permeability was discussed in Section 6.3.5.

6.3.9.2 SNAC-2 Effects on hGH Permeability in Rabbit Jejunum and Colon

Figure 6.7 presents the flux of hGH across stripped rabbit jejunum and colon in the absence and presence of 10 mg/mL SNAC-2. hGH flux in both intestinal regions was increased in the presence of SNAC-2, although to a much larger extent in the colon. The P_{app} values reported in Table 6.7 show approximately five- and twenty-fold increases in hGH permeability in jejunum and colon, respectively. Pre-treatment of colonic tissue with 10 mg/mL SNAC-2 demonstrated incomplete reversibility, a result similar to that obtained for mannitol (Section 6.3.9.1).

Mlynek and co-workers investigated the permeability of hGH in the presence of the carrier, 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid (referred to as E198 by Mlynek), in rabbit jejunum and colon (Mlynek et al., 2000). The authors reported a 1.5- fold increase in hGH permeability in jejunum in the presence of 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid, a significant increase (4-fold) in hGH permeability in "upper colon", but surprisingly, little difference in the P_{app} of hGH in "lower colon" (presumably distal colon, as used in the current study). The greater sensitivity of rabbit colon to the effects of SNAC-2 seen in the current study is broadly consistent with literature reports of heightened sensitivity of the colorectal region to the action of conventional absorption enhancers (Muranishi and Yamamoto, 1994).

A possible explanation for the large difference between SNAC-2 effects in proximal small intestine (jejunum, ileum) and distal colon is the existence of a different mechanism of absorption enhancement in the colon. Previous studies examining absorption mechanisms of a poorly absorbed drug in the presence of sodium caprate across rat jejunum and colon found that the predominant effect of the enhancer in the colon was to increase paracellular permeability,

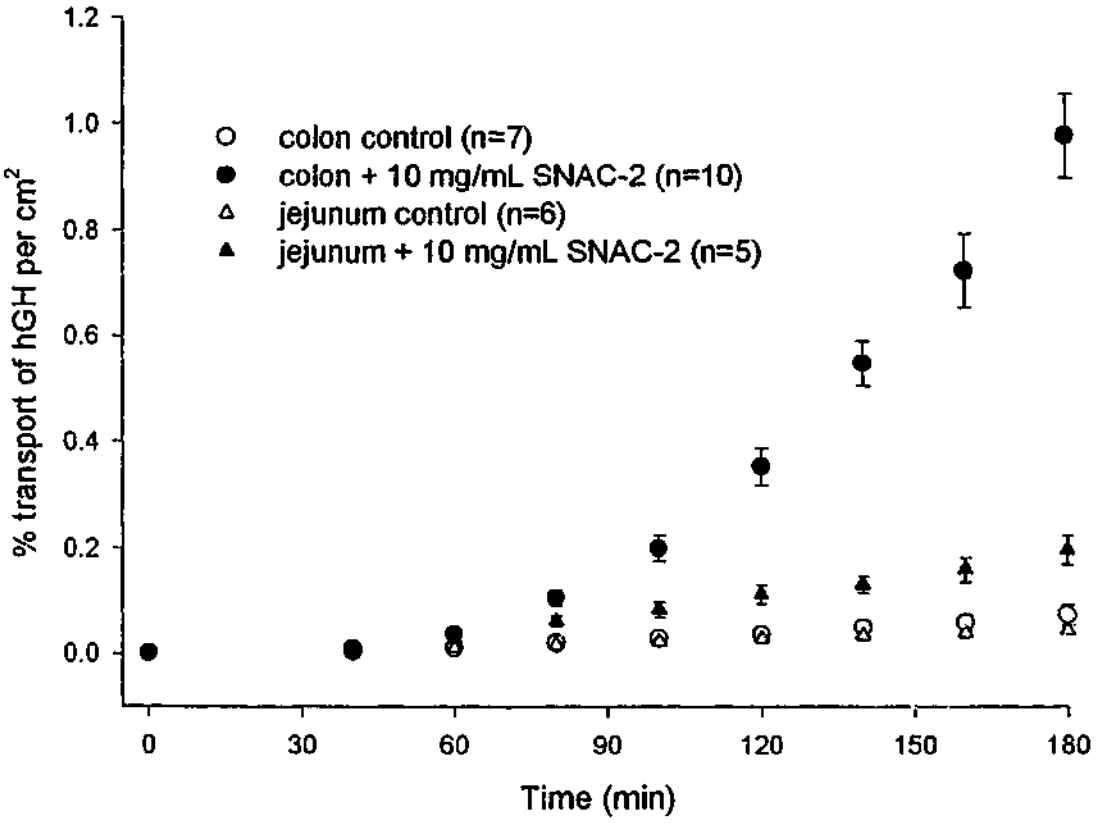


Figure 6.7 Flux of hGH across stripped rabbit jejunum and colon plotted as % transport/cm^2 versus time. Data were obtained from experiments conducted in the absence (open symbols) and presence (closed symbols) of 10 mg/mL SNAC-2. Symbols represent the mean ± SEM.

Table 6.7 Apparent permeability coefficients (P_{app}) for hGH in stripped rabbit jejunum and colon. P_{app} values were calculated using Equation 5.1. All diffusion buffers contained 2 mM taurocholate.

	Region	*SNAC-2 concentration (mg/mL)*	*Permeability coefficient (x 10^6 cm/sec)*[a]	*N Tissues/animals*
Control	**jejunum**	**0**	**0.07 ± 0.03**	**6/2**
Effect of SNAC-2		10	0.33 ± 0.04[b]	5/2
Control	**colon**	**0**	**0.10 ± 0.03**	**7/3**
Effect of SNAC-2		10	2.33 ± 0.18[b]	10/4
Effect of SNAC-2 pre-treatment[c]		10	1.17 ± 0.30[b]	3/1

[a] data reported as mean ± SEM
[b] significantly different to control ($p<0.001$)
[c] tissue exposed to SNAC-2 for 30 min prior to replacement of buffer (without SNAC-2) and addition of hGH

while the effect in the jejunum was found not to be due to increased paracellular permeability (Tomita et al., 1992).

Based on the limited regional permeability experiments conducted as part of the current work, it was not possible to postulate a mechanism of enhancement of hGH absorption in the presence of SNAC-2 in colon tissue. Furthermore, increased colonic absorption was unlikely to be responsible for the increase in hGH bioavailability following oral administration, although this study indicated that colonic administration may provide an effective means of hGH delivery. This is consistent with studies reported by Leone-Bay and co-workers describing the significant serum levels of hGH obtained following colonic administration of hGH combined with various Emisphere carriers (but not SNAC-2) to rats (Leone-Bay et al., 1996a).

6.3.9.3 Histology Assessment in Regional Studies

The maximum concentration of SNAC-2 employed in regional permeability measurements was 10 mg/mL and this was the concentration used in the regional histological evaluation. Segments of isolated jejunum and colon that had not been mounted in the diffusion chamber exhibited no abnormalities, as would be expected for freshly excised healthy tissues. Jejunum tissue samples incubated in the chamber in the presence of 10 mg/mL SNAC-2 exhibited only mild degenerative changes, with moderately disrupted villi noted and some pyknotic cells evident in the lamina propria. Colon tissue samples incubated in the chamber with SNAC-2 demonstrated minimal adverse morphological alterations.

Overall, tissues evaluated in regional histological studies demonstrated only minor changes in the presence of SNAC-2, notwithstanding the concerns raised in the discussion in Section 6.3.8.3 regarding the consequence of poorly stained tissues in the presence of the carrier (pathologist's grading not shown; see Section 6.3.8.3 for possible tissue effects). These results suggest that increased permeability of hGH in the modified Ussing chamber was not simply a consequence of tissue damage.

6.3.10 Investigation of Active Transport of hGH in the Presence of SNAC-2

In order to further probe the mechanism of absorption enhancement of hGH by SNAC-2, the possible involvement of active transport processes was investigated. The presence of active transport may be assessed by a number of different means including the evaluation of concentration dependence, asymmetry of transport (bidirectional studies), effect of inhibitors (e.g. ouabain) and temperature dependence.

Different donor concentrations of hGH were not assessed in the current study for several reasons. Firstly, increasing the hGH concentration would most likely have resulted in further aggregation of hGH (aggregation of hGH described in Section 5.3.4.1). Secondly, reduction of the donor concentration of hGH to any significant extent may not have allowed the accurate determination of receiver chamber concentrations by the ELISA due to the concentrations falling below the LOQ of the assay.

Bidirectional transport was briefly evaluated, although this was complicated by increased hGH aggregation in the serosal chamber (data not shown). The reason for the observed increase was not readily apparent, but may possibly have been due to the absence of mucosal surface-derived amphiphilic compounds contributing to the prevention of hGH aggregation. Such difficulties were not reported by Wu (Wu and Robinson, 1999a, Wu and Robinson, 1999b) and Mlynek (Mlynek et al., 2000), who described bidirectional studies of hGH in the presence of carriers, although both of these studies employed smaller concentrations of (radiolabelled) hGH.

An attempt was made to study the effect of selected flux the presence of SNAC-2. The use of the Na^+/K^+ ATPase inhibi ole h preliminary studies first conducted to assess the effect of the ad ai the serosal buffer on mannitol permeability. This concentration of the ected based on previous studies demonstrating the abolishment of short-circuit current across stripped rabbit ileum in the presence of 0.1 mM serosal ouabain (Swaan et al., 1994). Addition of ouabain to the serosal buffer in a control experiment (standard mucosal and serosal buffers with no additives)

resulted in a marginal increase in mannitol permeability, while the inclusion of serosal ouabain in a permeability experiment conducted in the presence of 50 mg/mL SNAC-2 led to a consistently larger mannitol P_{app} value than that observed in the presence of SNAC-2 alone (data not shown). The observed increases in mannitol permeability are consistent with reports of ATP depletion resulting in the disruption of tight junctions and altering paracellular permeability (Canfield et al., 1991). The use of inhibitors specific for alternate active transport pathways was not further explored owing to the possibility of tissue damage in the presence of inhibitors combined with SNAC-2 in the diffusion chamber.

Evaluation of the temperature dependence of transport has frequently been reported as a means of assessing the potential for active transport of compounds in both cultured cells and isolated tissues (Yamazaki et al., 1994, Rubas et al., 1995, Matsukawa et al., 1996, Makhey et al., 1998, Mlynek et al., 2000). Permeability measurements were not conducted at temperatures higher than 37°C, owing to the possibility of tissue damage occurring at higher temperatures over the experimental period (3 h, allowing for the 60-70 min lag time observed for hGH in the presence of SNAC-2). The permeation of hGH in the absence and presence of SNAC-2 was investigated in a series of experiments conducted at low temperature (10°C).

6.3.10.1 Permeability Studies of hGH and Marker Compounds at Low Temperature in the Absence of SNAC-2

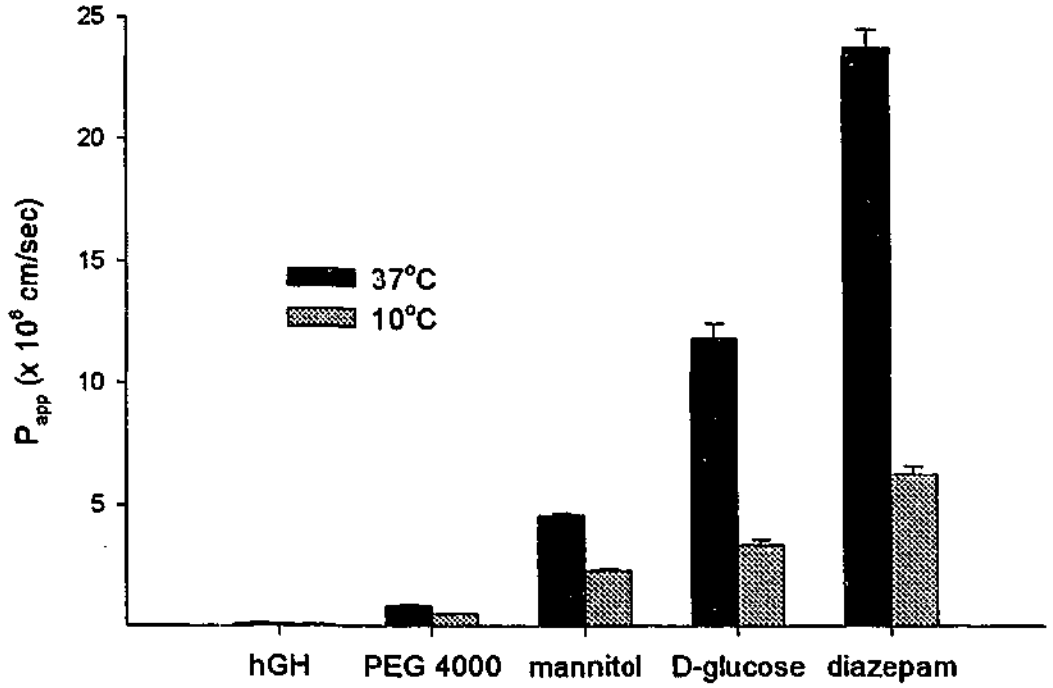
The permeability of hGH and the marker compounds, D-glucose, mannitol, PEG 4000 and diazepam was measured in the absence of the carrier at 37°C and 10°C. Inhibition of active transport at 10°C was verified by the reduction in the permeability of the actively transported marker, D-glucose, relative to the permeability of the passively transported marker, mannitol, i.e. a reduction in the glucose:mannitol P_{app} ratio. The glucose:mannitol P_{app} ratio decreased from 2.56 measured at 37°C to 1.47 at 10°C. Despite possible minor differences in

regional permeabilities, these ratios are consistent with results reported by Jezyk et al. for describing the inhibition of D-glucose transport in rabbit jejunum using the specific Na^+/glucose co-transporter inhibitor, phlorizin (Jezyk et al., 1992). Jezyk observed a reduction in the glucose:mannitol P_{app} ratio from 2.87 to 1.51 in the presence of 1.0 mM phlorizin.

Figure 6.8 presents bar graphs which illustrate the P_{app} values for compounds at 37°C and 10°C. The top plate incorporates a linear scale, while the bottom plate shows a log scale for the magnitude of the P_{app} values (y axis). The log scale was utilised in order to demonstrate the changes to hGH and PEG 4000 permeability relative to the highly permeable transcellular marker, diazepam. The P_{app} values for the hydrophilic markers, mannitol and PEG 4000, decreased by approximately 50%, most likely reflecting a contraction of tight junctions in response to the low temperature, a phenomenon previously described by Gonzalez-Mariscal et al. (Gonzalez-Mariscal et al., 1984). The change in diazepam permeability with the reduction in temperature was not significantly different to that observed for D-glucose, and is consistent with the decrease in diazepam P_{app} values determined at low temperature (4°C) in Caco-2 cells by Cogburn et al. (Cogburn et al., 1991). The reduction in hGH permeability was similar in magnitude to that observed for mannitol and PEG 4000 and may be a consequence of hGH permeation occurring at least in part via the paracellular pathway in the *in vitro* model. Table 6.8 lists the P_{app} values measured for all compounds at 10°C and 37°C.

6.3.10.2 Permeability Studies of hGH and Marker Compounds at Low Temperature in the Presence of SNAC-2

The permeability of hGH and the markers, mannitol, PEG 4000 and diazepam, was measured in the presence of 50 mg/mL SNAC-2 at 10°C and compared to data obtained at 37°C in order to examine the potential for the involvement of active transport in hGH intestinal permeation. Comparisons were performed using P_{app} ratios in a similar manner to that described



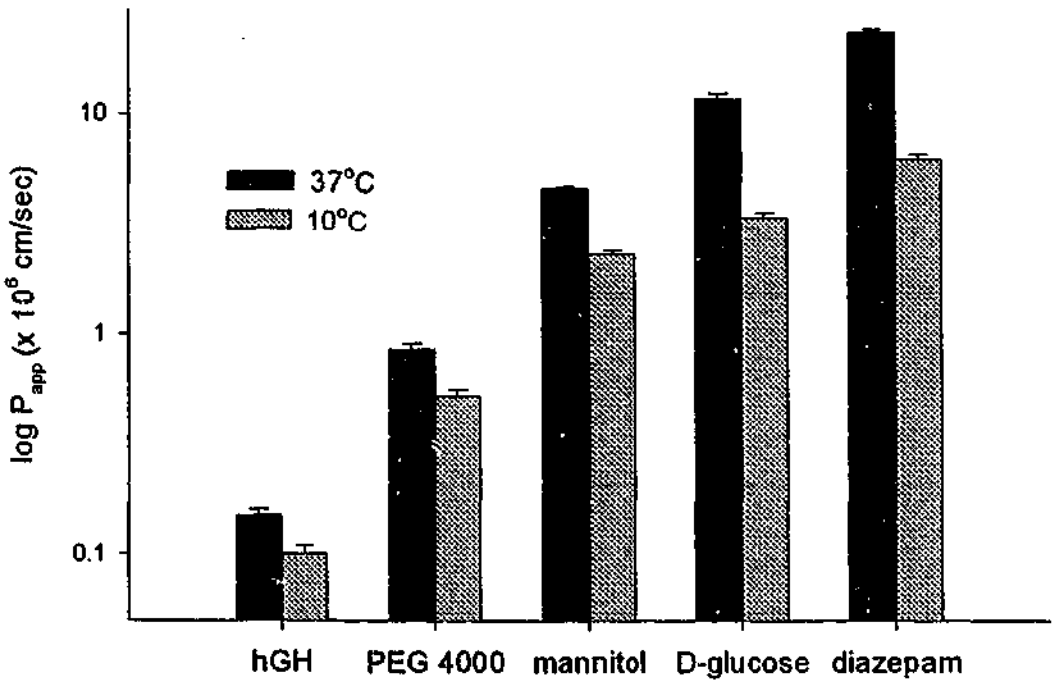


Figure 6.8 Apparent permeability coefficients measured in the absence of SNAC-2 at 37°C and at 10°C for hGH and the marker compounds, ^{3}H-PEG 4000, ^{14}C-mannitol, ^{14}C-D-glucose and ^{3}H-diazepam represented on both a linear scale (top plate) and a log scale (bottom plate). Error bars represent the SEM.

Table 6.8 Apparent permeability coefficients (P_{app}) for hGH, ^{14}C-mannitol, ^{3}H-PEG 4000, ^{14}C-D-glucose and ^{3}H-diazepam in stripped rabbit ileum at 37°C and 10°C. P_{app} values were calculated using Equation 5.1. All diffusion buffers contained 2 mM taurocholate.

	P_{app} at 37°C[a] (x 10^6 cm/sec)		*P_{app} at 10°C[a] (x 10^6 cm/sec)*	
	Control	*+ 50 mg/mL SNAC-2*	*Control*	*+ 50 mg/mL SNAC-2*
hGH	0.15 ± 0.01 (16)	1.37 ± 0.08 (13)[b]	0.10 ± 0.01 (9)	0.87 ± 0.03 (12)[b]
^{14}C-mannitol	4.62 ± 0.31 (24)	14.25 ± 0.46 (22)	2.30 ± 0.20 (15)	5.93 ± 0.22 (9)
^{3}H-PEG 4000	0.86 ± 0.07 (14)	2.20 ± 0.16 (6)	0.52 ± 0.02 (10)	0.65 ± 0.04 (6)
^{14}C-D-glucose	11.85 ± 0.40 (7)	-	3.38 ± 0.43 (6)	-
^{3}H-diazepam	23.75 ± 1.54 (15)	10.38 ± 1.06 (4)	6.26 ± 0.29 (6)	2.38 ± 0.17 (3)

[a] data are reported as mean ± SEM, with figures in parentheses representing the number of experiments

[b] values corrected for the free fraction of hGH as described in Section 6.3.4.

in Section 6.3.10.1 for the D-glucose:mannitol P_{app} ratios. "SNAC-2 P_{app} ratios" were calculated for hGH, mannitol, PEG 4000 and diazepam by determining the difference in the log of the P_{app} values obtained in the presence of 50 mg/mL SNAC-2 for each compound and the log of its P_{app} value determined under control conditions (i.e. in the absence of the carrier) at the corresponding temperature (37°C or 10°C). The P_{app} values for all compounds in the absence and presence of 50 mg/mL SNAC-2 at 37°C and 10°C are listed in Table 6.8. The P_{app} measured for hGH in the presence of 50 mg/mL SNAC-2 at 10°C was corrected for the free fraction of hGH as described in Section 6.3.4, assuming that the interaction between hGH and solution components is comparable at both temperatures.

Figure 6.9 presents a bar graph that illustrates the SNAC-2 P_{app} ratios for hGH, mannitol, PEG 4000 and diazepam at 10°C and 37°C. Ratios for hGH were similar at both temperatures and reflected an approximate nine-fold increase in hGH permeability in the presence of 50 mg/mL SNAC-2. The SNAC-2 P_{app} ratio for diazepam remained unaltered at 10°C, a likely indication that the interaction between the passively transported diazepam and the solution components (SNAC-2 and/or TC, described in Section 6.3.7) was also similar at both temperatures. Interestingly, the SNAC-2 P_{app} ratio for PEG 4000 was significantly reduced by approximately 50% at 10°C, and while the effect on mannitol was reduced by around 20%, this was found not to be statistically significant. This was possibly due to a combined effect of the contraction of tight junctions, coupled with a better "preserved" tissue under perturbed solution conditions (i.e. in the presence of SNAC-2).

The temperature independence of the effect of SNAC-2 on hGH permeability suggests that active transport is not likely to be involved in the absorption enhancement of hGH by SNAC-2. Studies by Mlynek on hGH permeability in the presence of the Emisphere carrier, 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid, in rabbit duodenum conducted at 2°C showed a reduction in the effect of the carrier at low temperature, although this was ascribed to the

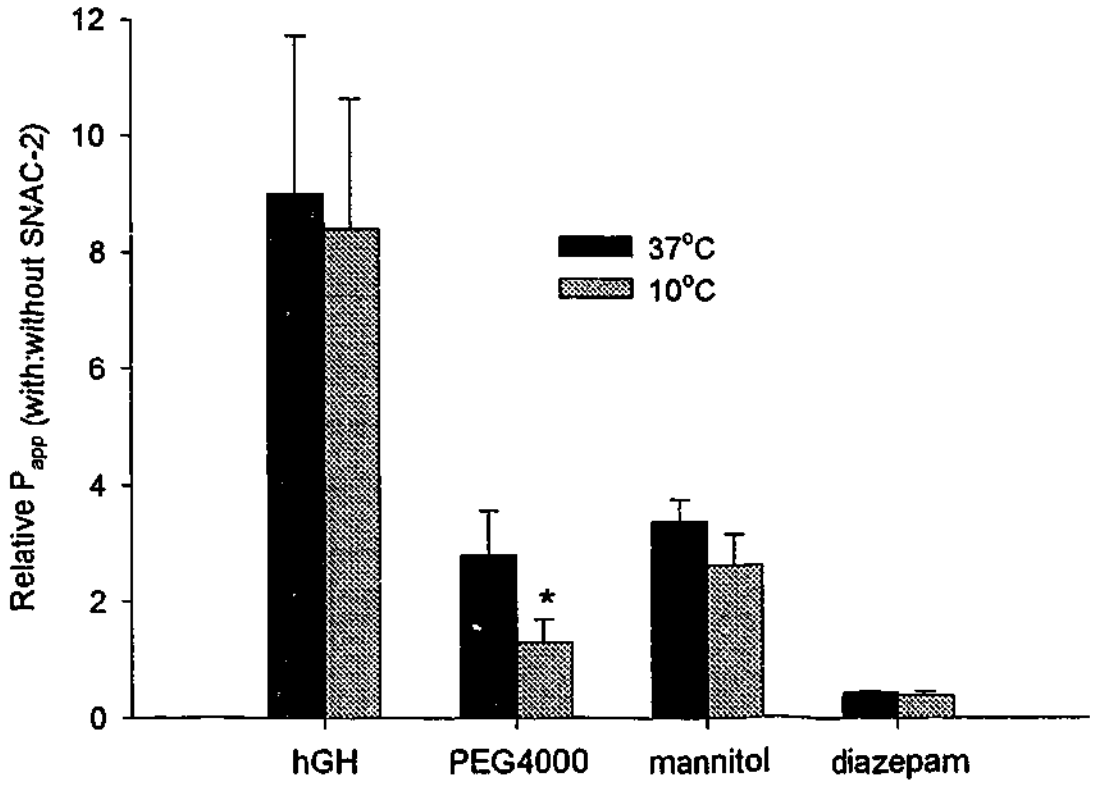


Figure 6.9 Relative apparent permeability coefficients (P_{app}, with:without 50 mg/mL SNAC-2) for hGH and the marker compounds, ^{3}H-PEG 4000, ^{14}C-mannitol and ^{3}H-diazepam, at 37°C and 10°C. Error bars represent the 95% confidence interval for the ratios and asterisks denote a significant difference between the relative permeability coefficients at 37°C and 10°C.

precipitation of 4-(4-(N-phenylsulfonyl)aminophenyl)butyric acid at 2°C (Mlynek et al., 2000). The precipitation of SNAC-2 (50 mg/mL) was not observed at 10°C in the current study. As discussed in Section 6.3.8.1, Wu and Robinson described hGH permeability in the presence of the Emisphere carriers, SABA and SNAC (referred to as E352 and E414, respectively, by Wu), in Caco-2 cells, proposing the involvement of P-glycoprotein mediated efflux in hGH co-transport with the carriers (Wu and Robinson, 1999a, Wu and Robinson, 1999b). If this were the case in the current study, one would expect the P_{app} measured for hGH in the presence of the carrier at 10°C to be significantly higher than that measured at 37°C. Such a change was not observed and therefore it is likely that hGH permeation in the presence of SNAC-2 was not subject to efflux in the rabbit ileum.

The behaviour of the paracellular markers, mannitol and PEG 4000, in the presence of SNAC-2 at low temperature indicated that the increased permeation observed for these markers was most likely occurring via a different transport pathway to that followed by hGH. If the absorption enhancement of hGH occurred as a result of enhanced paracellular permeability, then it is probable that the ratio of effect of SNAC-2 on hGH at low temperature would be reduced in a similar manner to that observed for PEG 4000 and mannitol.

Results of the temperature dependence studies indicate that the absorption enhancement of hGH in the presence of SNAC-2 may occur via a passive transcellular process. With increased paracellular marker permeability measured in the presence of SNAC-2 possibly an artefact of the *in vitro* model, the significantly greater magnitude of effect of the carrier on hGH permeation, demonstrated at both 10°C and 37°C, also suggested a specificity of SNAC-2 for hGH.

6.4 SUMMARY

The *in vitro* modified Ussing chamber model developed for measuring the intestinal permeability of hGH (described in Chapter Five) was utilised to evaluate the effect of the carrier, SNAC-2, on hGH permeability and examine absorption mechanisms.

Preliminary investigations were conducted to assess the effect of SNAC-2 in the diffusion buffer on solution osmolality, pH and surface activity. Donor solutions containing 50 mg/mL SNAC-2 were determined to be considerably hypertonic (650 mOsm/kg), necessitating the use of a modified (Na^+ depleted) buffer in order to prevent potential tissue damage caused by hypertonic solution conditions. The pH of buffers in the modified Ussing chamber at 37°C, bubbled with carbogen, was unaffected by the addition of SNAC-2. The CMC of SNAC-2 (11.3 mg/mL in diffusion buffer) could not be determined by surface tension measurements in the presence of 2 mM TC.

An investigation of the interaction between hGH and solution components including SNAC-2 and TC was conducted using ultrafiltration. The free fraction of hGH in solutions containing 50 mg/mL SNAC-2 was determined to be 0.35. This allowed the correction of hGH P_{app} values measured under these solution conditions.

Using rabbit ileum, the addition of 10-50 mg/mL SNAC-2 to the donor solution was shown to increase the P_{app} values for mannitol, with the 50 mg/mL concentration increasing mannitol permeability three-fold. The addition of 50 mg/mL SNAC-2 was also demonstrated to increase the P_{app} of a representative large hydrophilic marker, PEG 4000, by approximately 2.5-fold. Alterations in the permeability of mannitol and PEG 4000 in the presence of SNAC-2 indicated an effect of the carrier on the paracellular pathway. Reversibility of effect was demonstrated for mannitol at the 10 mg/mL concentration of SNAC-2 only, with all other concentrations showing incomplete reversibility.

The potential for calcium chelation as a possible mechanism of increased paracellular transport by SNAC-2 in the *in vitro* model was briefly investigated. Using an ion specific

electrode, a reduction in the concentration of free calcium ions in SNAC-2 solutions was noted, and while it was unlikely that calcium chelation played a major role in the *in vivo* absorption enhancement of hGH by SNAC-2, it was possible that this phenomenon may have contributed to the observed effects of SNAC-2 on paracellular transport in the *in vitro* model.

The permeability of diazepam decreased in the presence of SNAC-2, most likely as a result of micellar solubilisation. Diazepam solubility increased in a non-linear fashion in the presence of increasing concentrations of SNAC-2, precluding calculation of the free fraction of diazepam in solution by this method.

The addition of 10-50 mg/mL SNAC-2 to the donor solution was shown to increase the P_{app} values for hGH in rabbit ileum, with the 50 mg/mL concentration shown to increase hGH permeability nine-fold. Reversibility of the effect was demonstrated for hGH at the 10 mg/mL concentration of SNAC-2, while 50 mg/mL showed incomplete reversibility. Histological studies on selected tissues exposed to the carrier verified the absence of significant damage, although interference by SNAC-2 with the staining process complicated this assessment.

Regional studies were conducted in which the permeabilities of hGH and mannitol in the absence and presence of SNAC-2 in rabbit jejunum and colon were assessed. Increased P_{app} values for hGH and mannitol were observed in both regions in the presence of SNAC-2, with a large increase noted in the colon, possibly reflecting the greater sensitivity of this region to absorption enhancing compounds.

Finally, the possible involvement of active transport in the enhancement of hGH permeability by SNAC-2 was evaluated at low temperature (10°C) in the modified Ussing chamber. The relative change in hGH permeability in the presence of SNAC-2 remained the same at 10°C and 37°C, while a reduced effect of SNAC-2 on the permeability of the hydrophilic markers, mannitol and PEG 4000, was observed. The temperature independence of the carrier effect on hGH suggested that active transport was not responsible for the increased

permeability of hGH in the presence of the carrier. The reduced effect of SNAC-2 on the paracellular markers, mannitol and PEG 4000, indicated that it was unlikely that hGH permeation in the presence of SNAC-2 occurred via the paracellular route alone. The enhanced permeation of hGH effected by the model carrier, SNAC-2, is most likely due to a passive transcellular process, with the greater effect of the carrier on hGH permeability compared to that observed with the marker compounds also indicating a degree of specificity of SNAC-2 for hGH.

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CHAPTER SEVEN

SUMMARY AND PERSPECTIVES

The aim of this research was to investigate the mechanism of oral absorption enhancement of hGH by a representative Emisphere carrier compound, SNAC-2. These studies focussed primarily on the hypothesis that altered intestinal permeability is responsible for the observed increase in oral absorption. Both *in situ* and *in vitro* permeability models were evaluated in order to facilitate the measurement of hGH intestinal permeability in the presence of SNAC-2. The putative role of an intermediate hGH conformation stabilised by SNAC-2 was considered, with a series of CD spectroscopic experiments performed to characterise the interaction between hGH and SNAC-2. Selected *in vivo* studies were also performed to firstly, confirm the utility of the carrier for improving hGH oral absorption and secondly, to assess the possible contribution of intestinal lymphatic transport to the SNAC-2 enhanced intestinal absorption of hGH.

In vivo studies in rats demonstrated that the oral administration of a simple solution of hGH combined with SNAC-2 in buffer lead to maximum serum concentrations approximately fourteen times higher than those measured in control animals administered an oral dose of hGH in buffer alone (Chapter Two). The bioavailability of hGH from the oral hGH/SNAC-2 solutions was approximately 2% relative to subcutaneously administered hGH, and five-fold higher than that measured for control animals dosed orally with hGH alone.

The contribution of intestinal lymphatic transport to hGH absorption in the presence of SNAC-2 was evaluated in a pilot study employing an unconscious lymph-cannulated rat model (Chapter Two). No hGH was detected in lymph following the intraduodenal administration of an hGH/SNAC-2 solution to rats. Since the appearance of as little as 0.1% of the administered dose could have been detected in the lymph, it was unlikely that the observed oral absorption enhancement of hGH by SNAC-2 was the result of significantly increased lymphatic transport.

In order to explore the possibility of an altered hGH conformation in the presence of the carrier, circular dichroism spectroscopy was utilised to probe hGH conformation and thermal

stability in the presence of SNAC-2. Significant background absorbance in the presence of the model carrier imposed severe limitations on the SNAC-2 concentrations that could be evaluated in these studies (Chapter Three). No changes were detected in hGH spectra at ambient temperature in the presence of SNAC-2 at concentrations below 2 mg/mL and minimal changes were observed in the thermal transition temperature of hGH, indicating that hGH conformation remained unaltered under these conditions. The use of an alternative non-spectroscopic analytical technique, such as microcalorimetry, would be required to facilitate further studies of hGH thermal stability in the presence of higher carrier concentrations (e.g. 25-50 mg/mL).

The role of altered intestinal permeability in the absorption enhancement of hGH by SNAC-2 was investigated firstly by means of an *in situ* rat intestinal perfusion model (Chapter Four). Significant blood levels of hGH were observed in rats during the perfusion of an hGH/SNAC-2 solution through an isolated segment of jejunum, with higher carrier concentrations in perfusate giving rise to higher hGH blood levels. A substantial and variable loss of hGH from the perfusion solution due to enzymatic degradation was observed in all intestinal perfusion experiments precluding the calculation of hGH intestinal permeability. Interestingly, enzymatic degradation of hGH was unaffected by the presence of SNAC-2 suggesting that proteolytic enzyme inhibition was not primarily responsible for the carrier-induced improvement in hGH oral absorption. While the use of a proprietary protease inhibitor cocktail was shown to effectively prevent hGH degradation in collected perfusate, strategies to inhibit the significant *in situ* degradation were unsuccessful and consequently, hGH permeation could not be reliably quantitated using this model.

A modified Ussing chamber model incorporating isolated rabbit intestinal tissue was subsequently established and validated for examining hGH intestinal permeability (Chapter Five). The marked tendency of hGH to aggregate in the presence of carbogen bubbling/mixing necessitated the inclusion of 2 mM sodium taurocholate (TC) in the tissue bathing buffer. TC was demonstrated not to alter hGH conformation (CD spectra were unchanged) and no

difference was detected in mannitol and diazepam permeability upon the addition of 2 mM TC indicating that the paracellular and transcellular pathways, respectively, remained unaffected. Furthermore, a histological assessment did not detect a difference between rabbit ileal tissues maintained in the diffusion chamber in standard physiological buffer and a similar buffer containing 2 mM TC.

The addition of SNAC-2 to the donor side of the modified Ussing chamber was shown to increase the permeability coefficients (P_{app}) of the paracellular markers, mannitol and PEG 4000, by up to approximately three-fold in rabbit ileum indicating an effect of the carrier on the paracellular pathway (Chapter Six). The effect of the carrier on paracellular permeability was shown to be partially reversible and may reflect altered tissue integrity due to high and extended local carrier concentrations, which may not be relevant in an *in vivo* situation. Such effects are difficult to assess in view of the absence of the normal physiological protective effects of dilution, an intact blood supply and a mucous la[illegible] In comparison, the P_{app} value of hGH in ileum was up to nine-fold higher in the presence of SNAC-2. Given that the MW of hGH (22 kDa) is five times larger than that of PEG 4000, it is likely that either the carrier is more specific for hGH or that the permeation of hGH in the presence of the carrier occurs at least in part via the transcellular route.

The possible involvement of active trans[illegible]ort in the enhancement of hGH permeability by SNAC-2 was evaluated at low temperature (10°C) in the modified Ussing chamber (Chapter Six). The magnitude of the increase in hGH permeability in the presence of the carrier remained the same at 10°C and 37°C, while a reduced effect of SNAC-2 on the permeability of the paracellular markers, mannitol and PEG 4000, was observed. The temperature independence of the carrier effect on hGH suggested that active transport was not responsible for the increased permeability of hGH in the presence of SNAC-2. The reduced effect of SNAC-2 on the paracellular markers was attributed to a contraction of tight junctions at low temperature and

indicated that it was unlikely that the carrier-induced increase in hGH permeation occurred via the paracellular route alone.

The use of the *in vitro* model provided insight into possible transport mechanisms of hGH in the presence of the model carrier. Results indicated that both active transport processes and paracellular processes were not likely to be primarily responsible for hGH transport. The findings of this study are consistent with the possible involvement of a passive transcellular process, although further studies are necessary to fully characterise the transport mechanisms of hGH in the presence of SNAC-2.