ELSEVIER

International Journal of Pharmaceutics 197 (2000) 13–21

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Carrier-enhanced human growth hormone absorption across isolated rabbit intestinal tissue

Gwen M. Mlynek *, Laura J. Calvo, Joseph R. Robinson

School of Pharmacy, University of Wisconsin, Madison, WI 53706, USA

Received 11 June 1999; received in revised form 27 August 1999; accepted 9 September 1999

Abstract

Small molecular weight alpha acid derivatives are able to enhance the intestinal absorption of human growth hormone through isolated rabbit intestinal tissue. The enhancement is not through the usual tissue modification associated with traditional penetration enhancers nor is it through an active transport process. Rather these small molecules associate with human growth hormone in solution to make it more transportable through intestinal tissue. It is shown that the enhancer has specificity for a particular protein and the enhancer and human growth hormone must be in solution together to be effective, i.e. pretreating the tissue with enhancer and then adding the protein does not increase tissue permeability. Moreover, the enhancer does not increase the permeability of mannitol or progesterone, thus providing additional evidence of specificity and establishing that these agents are not classical penetration enhancers.

Keywords: Intestinal tissue; Enhancers; Permeability enhancer; Human growth hormone; Oral delivery

1. Introduction

Absorption of peptides, proteins and other large water soluble compounds across mucosal tissues is significantly impeded because of molecular size and resistance created by hydrogen bonding of the solute as it moves across the tissue. Indeed, there are only a few examples of therapeutic levels being achieved via the oral route for peptides and proteins. A number of approaches have been reported in the literature to enhance oral absorption of these agents, including various penetration enhancers, structural modification of the peptides and, in some cases, taking advantage of the tissues various transporter systems. The present work expands on the newer concept (Leone-Bay et al., 1995; Milstein et al., 1998) that certain small molecules, primarily alpha acid derivatives, can bind to peptides and proteins in solution and make them more transportable across intestinal tissue. To date the detailed mechanism(s) of enhancement have not been com-

Abbreviations: hGH, Human Growth Hormone; KRB, Krebs-ringer bicarbonate buffer; NaDC, sodium deoxycholate; TDC, taurodeoxycholate; TEER, transepithelial electrical resistance; Ω cm^2, Resistance.

* Corresponding author.

0378-5173/00/$ - see front matter
PII: S0378-5173(99)00322-1

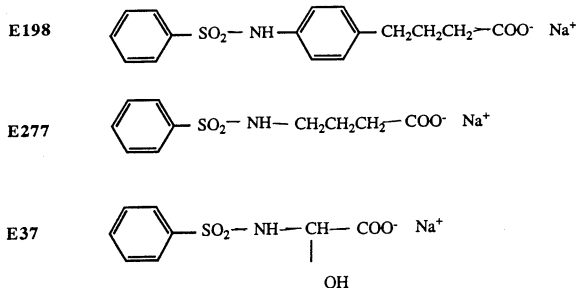

Fig. 1. Structures of enhancers used in permeability studies.

pletely understood and the present work focuses on delineating primary absorption characteristics of this new approach to oral absorption of peptides/proteins and other relatively large molecular weight water soluble compounds.

Earlier work (Leone-Bay et al., 1995; Milstein et al., 1998) has established the synthesis and ability of certain small molecules to enhance oral delivery of rhGH and salmon calitonin in rats: High sensitivity differential scanning calorimetry (DSC) was used to verify that a non-covalent interaction between these proteins and small molecules occurred in solution as well as to explore the effects of these molecules on the structural stability of the proteins under study. What is not yet available is a detailed understanding of how these small molecules enhance the absorption of peptides/proteins and the present work is an attempt to provide some elements of understanding.

Table 1
Radiolabel, specific activity and applied concentration of compounds utilized for in vitro permeability studies

Compound	Specific activity (mCi mmol^{-1})	Solution concentration
^{125}I-hGH	2.15×10^{6}	0.05 nM
^{3}H-Mannitol	2.25×10^{4}	35.5 nM
^{14}C-Progesterone	48.9	1.32 μM
^{14}C-Glycerol	43.0	2.3 μM

2. Materials and methods

2.1. Materials

Radiolabeled drug molecules, ^{125}I-human growth hormone (hGH), ^{14}C-progesterone, ^{3}H-mannitol, and ^{14}C-glycerol, were all purchased from NEN Radiation Products (Dupont Chemical, Wilmington, DE). Purity of all probes was at least 99%, as verified by the supplier. Enhancers were manufactured and supplied by Emisphere Technologies (Hawthorne, NY). See Fig. 1 for structures, chemical names and abbreviations are as follows; 4-(4-(N-phenylsulfonyl) aminophenyl) butyric acid, sodium salt (E198); 4-(N-benezene sulfonyl) amino butyric acid, sodium salt (E277); N-phenylsulfonyl-L-serine sodium salt (E37). Purity of all enhancers, determined by reverse phase chromatography was $>$ 98%. All other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Preparation of drug solutions

The bathing solution used in all diffusion studies was Kreb's ringer bicarbonate (KRB) buffer. The pH was adjusted to 6.5–6.6 with concentrated HCl, and maintenance of this pH was verified at the end of a diffusion experiment. Osmolarity of solutions was measured on a Wescor 5500 Vapor Pressure Osmometer (Logan, UT)

Solutions of the radiolabeled substances were prepared in KRB as indicated in Table 1, non-aqueous solvents were removed by evaporation prior to addition of KRB. In experiments involving enhancers, the enhancer was added at 10, 50, or 150 mg ml^{-1} in KRB. The higher concentrations of some enhancer formulations required sonication to achieve dissolution (Branson 5200, Branson Ultrasonics Corporation, Danbury, CT). Radiolabeled compounds were added to the enhancer solution in microliter quantities after a clear enhancer solution was obtained. All solutions were made prior to animal sacrifice and tissue harvesting, approximately 25–35 minutes before start of the permeability experiment.

2.3. In vitro perfusion studies

Male New Zealand rabbits (Bakkom Rabbitry, Viroqua, WI) weighing approximately 2.3 kg were used for all in vitro permeability studies. Rabbits were sacrificed by an overdose of sodium pentobarbital, 200–220 mg kg^{-1} via injection into a marginal ear vein. Prior to euthanasia, rabbits were given an intramuscular injection of Ketamine 20 mg kg^{-1} and Xylazine 4 mg kg^{-1}. After sacrificing, the abdominal cavity was opened via a midline incision from the xyphoid process to the pubic symphysis. The section of intestine of interest (upper duodenum, lower duodenum, jejunum, ileum, Peyer's patches, upper colon, and lower colon), was cut from the surrounding tissue and mesentery and placed immediately in ice cold normal saline. A lengthwise cut along the line of mesenteric attachment opened the lumen, and the tissue was gently rinsed in cold saline to remove food/fecal material. The rinsed tissue was pinned mucosal side down onto a dissecting pad, and for all segments except the lower colon, the muscle layer was gently peeled away using fine forceps.

The diffusion cell used in all permeability experiments was a modification of a cell described by Schoenwald and Haung (1983). The temperature for all experiments was maintained at 37°C, unless otherwise stated, and the bathing solutions were continually gassed with 95% O_2, 5% CO_2 to both mix the solution and aerate the tissue. A 0.67 cm^2 area of tissue was exposed to the donor and receiver compartment, each having a volume of 7 ml. At specified times, 1 ml of the receiver solution was sampled and replaced with KRB buffer which was both radiolabel and enhancer free. Samples were placed in liquid scintillation vials for ^{3}H and ^{14}C isotopes, and 4ml of scintillation cocktail added (BioSafe II, Research Products International, Mount Prospect, IL). These samples were counted for total disintegrations per minute (dpm) on a Beckmann LSC 6000IC (Beckmann Instruments, Arlington Heights, IL). For ^{125}I labeled growth hormone, the 1 ml samples were placed in gamma tubes with plug stoppers and counted for total counts per minute (cpm) on a Cobra 5002 Gamma Counter (Packard Instrument, Meridan, CT). In order to determine the initial donor concentration, a 1 ml sample was taken from solutions before the study began, and counted for total dpm or cpm in the same manner as timed samples. At the termination of the experiment, a 1 ml sample was removed from the donor chamber. This was done to verify mass balance of the radiolabel, i.e. to ensure that significant amounts had not adsorbed to the diffusion cell or tissue. Diffusion cells and mounting rings were cleaned with a strong surfactant, Radiacwash (VWR Scientific, Chicago, IL), copious rinsing with warm water followed to ensure no subsequent radioactive contamination.

The transepithelial electrical resistance of intestinal tissues was measured using a four-electrode system prepared according to previous methods, (Rojanasakul and Robinson, 1989; Jantzen et al., 1992; Liaw et al., 1992). Measurements were conducted in diffusion cells using solutions of KRB and enhancer concentrations of 150 mg ml^{-1}.

2.4. Calculations and statistical analysis

The in vitro apparent permeability coefficients (P_{coeff}) were calculated from the following equation:

$$P_{coeff} = \left(\frac{V}{A}\right)\left(\frac{\mathrm{d}\,(\%\ \text{transported})}{\mathrm{dt}}\right) \quad (1)$$

were V is the volume of the diffusion half cell, A is the area of tissue exposed, and the quantity [d (% transported)/dt] is the slope of the percent radiolabeled drug transported into the receiver compartment versus time plot.

All statistical analysis was done using the Student's t-test, unpaired and two-tailed, through the computer program STATVIEW. Only p-values less than 0.005 are considered statistically significant, the actual confidence interval is given if the level of significance is not obvious from graphical illustration. Averages reported are determined from $n = 4–6$, and all uncertainties are expressed as standard errors of the mean.

Table 2
Summary of ^{125}I human growth hormone apparent permeability coefficients in the rabbit duodenum

Experimental design	P_{coeff} (x10^{-6} cm s^{-1})
Temperature 2°C	1.75 ± 0.39
Temperature 18°C	1.74 ± 0.12
Temperature 37°C (control)	1.62 ± 0.32
Temperature 45°C	1.74 ± 0.12
^{125}I-hGH 0.03 nM	1.23 ± 0.40
^{125}I-hGH 0.06 nM	1.52 ± 0.36
^{125}I-hGH 0.12 nM	1.40 ± 0.40
^{125}I-hGH 0.05 nM + hGH 15 nM	1.47 ± 0.39
^{125}I-hGH 0.05 nM ± hGH 60 nM	1.25 ± 0.26
Serosal to mucosal transport	1.41 ± 0.38
Mannitol 0.55 M	1.66 ± 0.34
Amiloride 3 mM	1.35 ± 0.17
Ouabain 10 mM + amiloride 3 mM	1.39 ± 0.23
Monensin 25 μM	1.00 ± 0.27
Chloroquine 1 mM	1.22 ± 0.20
Urea 0.1 M	0.79 ± 0.10*
Urea 5.0 M	0.41 ± 0.19*
SDS 1 mM	0.66 ± 0.22*
Guanidine 4.5 mM	1.01 ± 0.17*

* Denotes statistical significance from control experiment. Mucosal concentration of ^{125}I-hGH, unless otherwise indicated, is 0.05 nM.

3. Results and discussion

3.1. Intestinal transport of ^{125}I-hGH

The findings for labeled human growth hormone permeability in the duodenum are summarized in Table 2. The permeability of growth hormone was shown to be independent of temperature over the range of 2–45° C. Transport was not affected by concentration of growth hormone so that increasing the amount of labeled protein by four-fold had no effect on permeability. Saturating with unlabeled protein (up to 60 nM) likewise had no effect on the transport of the labeled protein. Permeability was independent of the direction of transport, i.e. there is no change if the ^{125}I-hGH is placed on the mucosal or the serosal side of the tissue. Increasing the osmotic load in the donor solution from $\approx$295 mOsm to over 3-fold using mannitol, did not significantly alter the permeation. Traditional inhibitors of active transport processes, namely ouabain and amiloride were also investigated. Amiloride, which selectively inhibits the Na^+ cotransporter, did not affect the permeability of ^{125}I-hGH. Ouabain, also selective in its action, inhibits the sodium/potassium ATPase system by competing for the K^+ binding site, and even a substantial dose, 10 mM, in addition to amiloride, did not significantly affect the permeability coefficient. Agents that block transcytosis, monensin and chloroquine, did not significantly inhibit the transport of ^{125}I-hGH. The effect of protein denaturants on permeability was also investigated, using sodium dodecyl sulfate (SDS), guanidine hydrochloride and urea. Growth hormone transport was significantly reduced by all three agents.

There have been many studies to suggest that protein molecules can be transported across the intestinal epithelium. Both the mechanism and extent of transport vary widely, depending on the protein and the system used, be it in vitro, in vivo, or cultured epithelial cells. Some proteins that have been examined include; insulin, bovine serum albumin, horseradish peroxidase, α-lactalbumin, β-lactoglobulin (Schichiri et al., 1973; Gonella and Walker, 1987; Gardner, 1988; Marcon-Genty et al., 1989; Lee et al., 1991). Other non-protein macromolecules, such as dextrans, have also been shown to permeate intestinal tissue (Masuda et al., 1986; Rubas et al., 1995b).

The latter authors concluded that 70 kD dextran was transported passively and transcellularly, since the diameter of the extracellular space is approximately 5–8 Å, and would limit passage of a molecule of that size. Using electron microscopy, they visualized dextrans in the cytosol of cultured Caco-2 cells, while the paracellular space appeared to be devoid of the macromolecule. Other mechanisms of protein transport have been demonstrated involving endocytosis, both non-selective pinocytosis and receptor-mediated (Pusztai, 1989).

We have found that the transport of ^{125}I-hGH in the intestinal tissue is primarily passive in nature. It is independent of temperature, concentration, osmotic pressure, directionality, and is not affected by known inhibitors of active transport processes. A literature value for the permeability coefficient of recombinant human growth hor-

Table 3
Effect of various enhancers on ^{125}I human growth hormone permeability in the rabbit duodenum

Experimental design	P_{coeff} (x10^{-6} cm s^{-1})
Control (no enhancer)	1.62 ± 0.32
E277 150 mg ml^{-1}	1.71 ± 0.47
E37 150 mg ml^{-1}	1.73 ± 0.28
E198	
10 mg ml^{-1}	1.34 ± 0.27
50 mg ml^{-1}	1.95 ± 0.21
150 mg ml^{-1}	3.45 ± 0.34*

* Denotes statistical significance from control experiment.

mone in the rabbit colon of $0.12 \pm 0.01 \times 10^{-6}$ cm s^{-1} (Rubas et al., 1995a), is significantly lower than that obtained in this study, $4.59 \pm 0.84 \times 10^{-6}$ cm s^{-1} for the upper colon, and $4.13 \pm 0.19 \times 10^{-6}$ cm s^{-1} in the lower colon, as shown in Table 5. However, the cited study used rabbits that were significantly larger, ≈ 4.5 kg, which translates into an age of 4.5 months, compared to less than three months for this work. It is known that the intestinal permeability to proteins decreases dramatically with age, a phenomenon called ''closure''. The authors also did not define colonic tissue used as to upper and lower segments. In addition, iodinated hGH is more hydrophobic than hGH. These differences, along with tissue preparation and other differences in technique involved, could possibly account for the variation in permeability coefficients.

Table 4
Apparent permeability coefficients of ^{125}I human growth hormone in the presence of E198 150 mg ml^{-1}, in the rabbit duodenum

Experimental design	P_{coeff} (x10^{-6} cm s^{-1})
E198 150 mg ml^{-1} [a]	3.45 ± 0.34[b]
Temperature 2°C	1.15 ± 0.41*
Temperature 18°C	2.33 ± 0.59
Temperature 45°C	8.15 ± 0.76*
Ouabain 2 mM	3.44 ± 0.26
Amiloride 3 mM	3.25 ± 0.33
Serosal to mucosal transport	3.46 ± 0.38
Urea 0.1 M	1.93 ± 0.18*
E198 150 mg ml^{-1} (pre-treatment)	1.43 ± 015*

* Denotes statistical significance from control experiment.
[a] Designated control experiment for Table 4, conducted at 37°C.

In summary, the transport of radiolabeled growth hormone, in the absence of enhancer, is passive, with the bulk of the transport being transcellular diffusion. Also, it appears that aggregation of the protein may limit the amount of protein available for transport.

3.2. Effect of enhancer on intestinal transport of ^{125}I-hGH

Several enhancers, E277, E37, and E198 were examined as to their effect on the permeability of growth hormone through duodenal tissue, of these, only E198 significantly effected the apparent permeability coefficient. The results are shown in Table 3. The enhancement was only seen at high concentrations of enhancer. This enhancement was also stimulated by an increase in temperature and was independent of the directionality of the tissue, provided the enhancer and growth hormone were on the same side, these results are listed in Table 4. Reduced temperature alone could eliminate enhancement, and was just as effective when combined with amiloride and ouabain. Additional studies on temperature effects established that at low temperatures the enhancer partially precipitated from the solution. Neither amiloride or ouabain alone significantly altered the effect of E198 on ^{125}I-hGH transport (results also shown in Table 4). However, the addition of urea 0.1 M could completely counteract the enhancement effect of E198. Pre-treatment of the tissue with enhancer, followed by addition of the protein, did not result in any increase in permeability. This indicates that the protein and enhancer must be delivered to the site of absorption together, or that the enhancer needs to have contact with the protein, to prepare it to permeate through the tissue.

The permeability of ^{125}I-hGH varied along the intestinal tract, as did the effect of enhancer E198. Actual values for the apparent permeability coefficients are given in Table 5. The upper colon had by far the best response, eliciting an increase of over three-fold in the permeability coefficient.

Table 5
Regional variation in apparent permeability coefficients of ^{125}I hGH and the effect of enhancer E198

Tissue[a]	P_{coeff} (x 10^{-6} cm s^{-1}) ^{125}I-hGH	^{125}I-hGH +E198	R.I.[b]
Duodenum, upper	1.64 ± 0.32	3.45 ± 0.34	2.10
Duodenum, lower	1.02 ± 0.18	2.85 ± 0.34	2.79
Duodenum, upper (rat)	3.25 ± 0.92	5.62 ± 1.08	1.73
Jejunum	2.86 ± 0.28	4.31 ± 0.51	1.51
Ileum	3.09 ± 0.25	5.81 ± 0.49	1.88
Peyer's patches	1.01 ± 0.24	2.55 ± 0.34	2.52
Colon, upper	4.59 ± 0.84	17.35 ± 0.68	3.78
Colon, lower	4.13 ± 0.19	3.71 ± 0.29	0.90

[a] Tissue of rabbit origin, unless indicated otherwise.
[b] R.I. is the relative increase in P_{coeff} as a result of E198.

Ileal tissue was next in magnitude of response, followed by the duodenum. Interestingly, the lower colon showed no statistical change. The enhancement in the upper duodenal tissue was verified in another species, the rat, and although the variability was greater, there was still a positive response seen with enhancer E198.

3.3. What is the pathway of enhancer effects?

It is clear that hGH absorption, with or without enhancers, is not through an active transport process and thus the enhancing effect cannot be ascribed to this pathway. A more likely explanation is that the enhancers are functioning in a classical sense by modifying the tissue. Several lines of evidence show that this is not the case.

3.3.1. Inability to enhance absorption of other probes

The effect of enhancer on the duodenal tissue permeability of other probes was investigated using ^{14}C-progesterone, ^{3}H-mannitol, and in the upper colon, ^{14}C-glycerol. Table 6 shows that neither E198 or E277, both at a concentration of 150 mg ml^{-1} had any significant effect on the permeabilities in any section of tissue.

Progesterone was chosen as a model transcellular path indicator. Being of the steroid family, it is exceedingly lipophilic, having a log octanol/water partition coefficient of 3.89 (Grass, 1985). Two compounds were used as paracellular markers, glycerol and mannitol. Both are hydrophilic, with partition coefficients of -1.79 and -3.10, respectively (Madara and Pappenheimer, 1987). It was hoped that by using these model compounds and examining any changes in their permeability profiles in the presence of enhancer, information could be obtained on the mechanism of enhanced growth hormone permeability, and whether the enhancer causes damage to the tissue, resulting in an increase in permeability.

3.3.2. Changes in tissue electrical resistance are not related to permeability

The transepithelial electrical resistance of intestinal tissue, and the effects of enhancer on the resistance were examined. Table 7 lists the values of these parameters for all tissues under all conditions. E198 effects the resistance most significantly in the colon, reducing the average value by over 100 $\Omega \cdot cm^2$, while E37 only reduces the resistance by 30 $\Omega \cdot cm^2$. However, this is not the case in the jejunum and ileum, where the enhancers both lower the epithelial resistance by the same amount, roughly 60–80 $\Omega \cdot cm^2$.

Table 6
Effect of enhancers on permeability of various probe molecules[a]

Drug	Tissue	P_{coeff} (x10^{-6} cm s^{-1})		
		Control	E198	E277
^{14}C-Progesterone	Duodenum	0.27 ± 0.05	0.36 ± 0.06	0.27 ± 0.03
^{3}H-Mannitol	Duodenum	1.26 ± 0.24	0.98 ± 0.30	0.98 ± 0.28
^{14}C-Glycerol	Colon (upper)	1.18 ± 0.08	1.27 ± 0.09	nd

[a] Concentration of Enhancers E198 and E277 is 150 mg/ml; nd not determined.

Table 7
Transepithelial electrical resistance ($\Omega \cdot cm^2$) values for various regions of intestinal tissue[a]

Tissue	Control	E198	E37
Duodenum	332.5 (16.1)	141.9 (10.2)	150.6 (20.8)
Jejunum	171.6 (24.0)	114.8 (13.4)	104.8 (13.4)
Ileum	131.0 (17.5)	45.8 (11.2)	68.5 (17.5)
Colon (upper)	181.9 (21.9)	66.4 (11.6)	151.5 (23.4)

[a] Concentration of Enhancers E198 and E277 is 150 mg ml^{-1}.

An attempt to correlate the changes in transepithelial electrical resistance with the change in permeability coefficient of ^{125}I-hGH is depicted in Fig. 2. The y axis and bars represent the resistance values and the double y axis and lines show the permeability coefficients. The biggest change in the resistance values, seen in the duodenum, does not result in the greatest increase in permeability, likewise the tissue with the greatest change in permeability, the colon, has only a moderate drop in electrical resistance.

3.4. Tentative mechanism of enhancer effect

Several analytical techniques, including DSC, show that hGH and the enhancer interact non-covalently in solution. It is argued that the resulting complex is sufficiently hydrophobic to permit enhanced absorption across biological membranes.

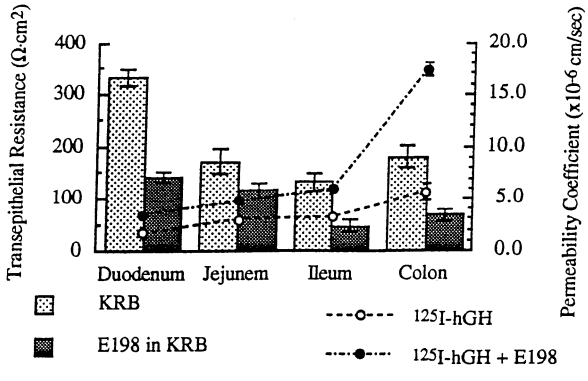

Fig. 2. Variation in transepithelial electrical resistance and the permeability coefficient of ^{125}I-hGH with tissue origin. The y axis and bars represent the resistance values and the double y axis and lines are the permeability coefficients.

Growth hormone and enhancer needed to be presented together to the tissue in order to achieve permeability enhancement, i.e. pre-treatment of the tissue with enhancer did not have the desired effect. It would therefore be concluded that the primary mechanism by which E198 enhances permeability is not through tissue effects, but rather through a change in the protein. This is supported by results from permeability studies using mannitol, glycerol and progesterone, where E198 was ineffective at enhancement.

Possible mechanism for enhancement is that denaturation of the protein to a more lipophilic state, renders it more membrane permeable. Urea, sodium dodecyl sulfate (SDS), and guanidinium are agents which are known to disrupt the structure of proteins. SDS is most commonly used in gel electrophoresis to estimate the sizes of polypeptide chains, it unfolds and binds most proteins, resulting in complexes that have a constant density of negative charge along the chain. Polypeptide chains unfolded in this manner appear as elongated flexible chains with a constant diameter and a length proportional to the number of amino acid residues present. At the concentration chosen, which was below the cmc, the detergent decreased the permeability coefficient of growth hormone. Guanidinium and urea decrease protein stability by preferentially interacting with protein surfaces, especially those nonpolar surfaces that tend to be in the interiors of folded conformations. From their structure it is seen that they possess an obvious potential for hydrogen bonding and at high concentrations denaturants disrupt the usual aqueous hydrogen bond network. From this, it is concluded that they interact with both nonpolar and polar surfaces more favorably than does water (Creighton, 1993). High concentrations (≥ 4–5 M), of urea or guanidinium will completely denature the protein structure, however, concentrations of 1–2 M have been shown to stabilize the molten globule state (Mitaku et al., 1991; Hagihara et al., 1993). It was not known what effects these two agents would have on tissue permeability, at these concentrations. Urea is used pharmaceutically as a keratolytic agent, but given systemically will act as an osmotic diuretic (Harvey, 1985). Guanidine hy-

drochloride increases the sensitivity of skeletal muscle to acetylcholine, and therefore is occasionally used in cases of myasthenia gravis (ibid.). Again, no information is available on permeability effects of these two agents, so concentrations were chosen so as to balance the desired effect of protein denaturation with any possible toxicity to the intestinal tissue. However, urea at substantial concentrations, 0.1 M and 5 M, significantly decreased the permeability of the duodenal tissue to ^{125}I-hGH. Guanidine and SDS, at concentrations of 4.5 mM and 1 mM, respectively, also decreased growth hormone permeability. These results lead to the general conclusion that enhancers likely do not operate by denaturing growth hormone.

3.4.1. Paracellular versus transcellular enhancement

Using radiolabeled test probes in permeability studies involving enhancers is two-fold in the information it reveals. Depending on the transport pathway of the marker, changes in its transport characteristics would give insight in to the mechanism by which enhancers enhanced the permeation of human growth hormone. Additionally, any such changes in permeability might be used to assess tissue damage. This approach is commonly used in permeability (Nadai et al., 1975; Anderberg et al., 1992) and absorption studies (Peters et al., 1987; Swenson et al., 1994).

Transport across the epithelium occurs primarily via two pathways, transcellular and paracellular (Sachs et al., 1973; Schultz and Frizzel, 1976). The transcellular route involves partitioning into, diffusion through, and partitioning out of the lipid bilayer, or a series of alternating partition and diffusion steps. The latter would involve partitioning through the apical membrane, followed by diffusion in the cytosol, or partitioning into cellular compartments, and lastly, partitioning through the basal membrane. It would seem likely that because of the energetic barriers involved in numerous partitioning steps and the alternating nature of hydrophobic and hydrophilic environments, that diffusion through the hydrophobic portion of the bilayer, around the cell proper, would be a more efficient and energetically favorable path. This is not to imply however, that all mechanisms of transcellular transport are passive in nature. Many compounds, sugars and amino acids in particular, are transported against their electrochemical gradient and hence require energy.

The paracellular path is anatomically the intercellular space, controlled by the tight junctions and dependent on the electrochemical gradient of the transporting molecule, on hydrostatic and osmotic pressures, and surface properties of the membrane. Tight junction permeability has been shown to depend on a number of factors including: (a) degree of maturation of the epithelia (Humbert et al., 1976); (b) response to physiological requirements (Tice et al., 1975); (c) change in environmental conditions, e.g. ionic strength and osmolarity (Urakabe et al., 1970; Wade et al., 1973); (d) presence of drugs, vitamins and hormones (Elias and Friend, 1976).

It is generally accepted that the route by which lipophilic drugs penetrate epithelial tissues is transcellular, while hydrophilic drugs pass through the paracellular space. Therefore, compounds that effect the degree of 'openness' of the tight junctions or water flux, will effect permeation rates of paracellular probes, and those which effect membrane properties such as bilayer fluidity will likewise effect permeation rates of transcellular markers.

Since neither E198 nor E277 significantly affected the permeability of ^{3}H-mannitol or ^{14}C-glycerol, it is concluded that the enhancers do not enhance transport via the paracellular path. It was not surprising that this would be ruled out as the means by which E198 enhanced human growth hormone permeability, because of the large molecular weight (21 500) of the protein. One could not envision the tight junctions opening wide enough to accommodate a compound of that size. It was determined from ^{14}C-progesterone permeability that the enhancers also did not appreciably affect the transcellular path either. Additionally proteinoids did not damage intestinal tissue sufficiently to enhance permeability.

References

Anderberg, E.K., Nystrom, C., Artursson, P., 1992. Epithelial transport of drugs in cell culture. VII. Effects of pharmaceutical surfactant excipients and bile acids on transepithelial permeability in monolayers of human intestinal epithelial (Caco-2) cells. J. Pharm. Sci. 81, 879–887.

Creighton, T.E., 1993. Proteins: Structures and Molecular Properties, second ed. Freeman, New York.

Elias, P.M., Friend, D.S., 1976. Vitamin A-induced mucous metaplasia: an in vitro system for modulating tight and gap junction differentiation. J. Cell Biol. 68, 173–188.

Gardner, M.L., 1988. Gastrointestinal absorption of intact proteins. Ann. Rev. Nutrition 8, 329–350.

Gonella, P.A., Walker, W.A., 1987. Macromolecular absorption in the gastrointestinal tract. Adv. Drug Deliv. Rev. 1, 235–248.

Grass, G.M., 1985. Mechanisms of corneal drug penetration, Ph.D. Thesis, University of Wisconsin, Madison.

Hagihara, Y., Aimoto, S., Fink, A.L., Goto, Y., 1993. Guanidine hydrochloride-induced folding of proteins. J. Mol. Biol. 231, 180–184.

Harvey, S.C., 1985. Topical drugs. In: Gennaro, A.R. (Ed.), Remington's pharmaceutical sciences. Mack Publishing, Easton, PA, pp. 773–791.

Humbert, F., Montesano, R., Perrelet, A., Orci, L., 1976. Junctions in developing human and rat kidney: a freeze-fracture study. J. Ultrastruct. Res. 56, 202–214.

Jantzen, G.M., 1992. Lysine/sodium cotransport system as a corneal penetration enhancer, M.S. Thesis, University of Wisconsin, Madison, WI.

Lee, V.H.L., Dodda-Kashi, S., Grass, G.M., Rubas, W., 1991. Oral route of peptide and protein drug delivery. In: Lee, V.H.L. (Ed.), Peptide and protein drug delivery. Marcel Dekker, New York, pp. 691–738.

Leone-Bay, A., Ho, K., Agarwal, R., et al., 1995. N-Acylated and amino acids as novel oral delivery agents for proteins. J.Med.Chem. 38 (21), 4263–4269.

Liaw, J., Rojanasakul, Y., Robinson, J.R., 1992. The effect of drug charge type and charge density on corneal transport. Int. J. Pharm. 88, 111–124.

Madara, J.L., Pappenheimer, J.R., 1987. Structural basis for physiological regulation of paracellular pathways in intestinal epithelial. J. Membr. Biol. 100, 149–164.

Marcon-Genty, D., Tome, D., Kheroua, O., Dumontier, A.M., Heyman, M., Desejeux, J.F., 1989. Transport of beta-lactoglobulin across rabbit ileum in vitro. Am. J. Physiol. 256, G943–G948.

Masuda, Y., Yoshikawa, H., Takada, K., Muranishi, S., 1986. The mode of enhanced enteral absorption of macromolecules by lipid-surfactant mixed micelles I. J. Pharm.-Dyn. 9, 793–798.

Milstein, S.J., et al., 1998. Partially unfolded proteins efficiently penetrate cell membranes-Implications for oral drug delivery. J. Controlled Release 53, 259–267.

Mitaku, S., Ishido, S., Hirano, Y., Itoh, H., Kataoka, R., Saitô, N., 1991. Hydrophobic core of molten-globule state of bovine carbonic anhydrase B. Biophys. Chem. 40, 217–222.

Nadai, R., Kume, M., Tatematsu, A., Sezaki, H., 1975. Drug-induced histological changes and its changes and its consequences on the permeability of the small intestinal mucosa. II. Chem. Pharm. Bull. 23.

Peters, G.E., Hutchinson, L.E.F., Hyde, R., McMartin, C., Metcalfe, S.B., 1987. Effects of sodium 5-methoxysalicylate on macromolecule absorption and mucosal morphology in a vascularly perfused rat gut preparation in vivo. J. Pharm. Sci. 76, 857–861.

Pusztai, A., 1989. Transport of proteins through the membrane of the adult gastro-intestinal tract-a potential for drug delivery? Adv. Drug Delivery Rev. 3, 215–228.

Rojanasakul, Y., Robinson, J.R., 1989. Transport mechanisms of the cornea: characterization of barrier permselectivity. Int. J. Pharm. 55, 163–172.

Rubas, W., Villagran, J., Cromwell, M., McLeod, A., Wassenberg, J., Mrsny, R., 1995a. Correlation of solute flux across Caco-2 monolayers and colonic tissue in vitro. *S.T.P.* Pharma Sci. 5, 93–97.

Rubas, W., Jezyk, N., Grass, G.M., 1995b. Mechanism of dextran transport across rabbit intestinal tissue and a human colon cell-line (CaCo-2). J. Drug Target. 3, 15–21.

Sachs, G., Spenney, J.G., Shoemaker, R.L., Goodall, M.C., 1973. Conductance pathways in epithelial tissues. Exp. Eye Res. 16, 241–249.

Schichiri, M., Etani, N., Kawamori, R., Karasake, K., Okada, A., Shigeta, Y., Abe, H., 1973. Absorption of insulin from perfused rabbit small intestine in vitro. Diabetes 22, 459–465.

Schoenwald, R.D., Haung, H.S., 1983. Corneal penetration of β-blocking agents. I. Physicochemical factors. J. Pharm. Sci. 72, 1266–1270.

Schultz, S.G., Frizzel, R.A., 1976. Ionic permeability of epithelial tissues. Biochim. Biophy. Acta 443, 181–189.

Swenson, E.S., Milisen, W.B., Curatolo, W., 1994. Intestinal permeability enhancement: efficacy, acute local toxicity, and reversibility. Pharm. Res. 11, 1132–1142.

Tice, L.W., Wollaman, S.H., Carter, R.C., 1975. Changes in tight junctions of thyroid epithelium with changes in thyroid activity. J. Cell Biol. 66, 657–663.

Urakabe, S., Handler, J.D., Orloff, J., 1970. Effect of hypertonicity on permeability properties of the toad bladder. Am. J. Physiol. 218, 1179–1187.

Wade, J.B., Revel, J.P., DiScala, V.A., 1973. Effect of osmotic gradients on intercellular junctions of the toad bladder. Am. J. Physiol. 224, 407–415.