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Absorption of recombinant methionyl-human growth hormone (Met-hGH) from rat nasal mucosa

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Summary

The absorption of recombinant methionyl human growth hormone (Met-hGH) from the nasal mucosa into the systemic circulation was studied in anesthetized rats. Met-hGH was administered intranasally (i.n.), intramuscularly (i.m.) and intravenously (i.v.) to determine the relative and absolute bioavailability of an intranasal Met-hGH formulation. In the absence of detergent enhancers, the absolute bioavailability of met hGH was < 1%. Hypotonicity enhanced absorption slightly. The absolute bioavailability of Met-hGH increased markedly in the presence of the non-ionic surfactant, polyoxyethylene 9-lauryl ether (laureth-9). The histological changes in the nasal mucosa of rats treated with 1% laureth-9 were severe, however, and included complete removal of the nasal epithelium in places. The bile salt sodium glycocholate was also evaluated for its permeation enhancing abilities. At 0.5% glycocholate there were few noticeable histological changes relative to controls and the absolute bioavailability of Met-hGH was approximately 7–8%. While absorption of Met-hGH from the nasal epithelium is demonstrated, the effects of permeation enhancing detergents on the delicate nasal mucosa must be better understood before the intranasal route of administration may be considered suitable for delivering Met-hGH to the systemic circulation.

Introduction

Human growth hormone is currently administered by subcutaneous or intramuscular injection, usually 50 μg/kg daily, to children diagnosed as growth hormone deficient. Because growth hormone therapy is continued for several years, a non-parenteral route of administration would be preferable to the current method of administration. Oral delivery of polypeptides is complicated by the presence of hydrolyzing digestive enzymes in the gastrointestinal tract. Intranasal (i.n.) delivery of polypeptides is attractive for several reasons; the nasal mucosa appears to have less proteolytic activity than the gastrointestinal tract (Parr, 1983), first-pass hepatic metabolism is bypassed, and depending on molecular weight, absorption into the systemic circulation can occur relatively rapidly. I.n. administration may result in more consistent absorption of a drug which has variable bioavailability by the oral route, as is the case for propranolol (Hussain et al., 1979). The surface area of the highly vascularized nasal mucosa is extensive due to subdivisions of the nasal passages into turbinates, sinuses, and the

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numerous microvilli on mucosal cells (Parr, 1983). Mechanisms by which drugs are absorbed through the nasal mucosa have not been determined; several have been proposed, e.g. passive diffusion, facilitated and active transport (Parr, 1983) and paracellular transport through intracellular channels with water influx (Morimoto et al., 1985). In a paper discussing the nasal absorption rates of drugs having very different partition coefficients, Huang et al. (1985) found little difference in absorption rates. The mechanism of absorption may depend on the chemical nature of the administered drug and formulation, i.e. addition of absorption enhancers.

For certain drugs, the plasma concentration–time profile after i.n. administration is similar to that seen after intravenous (i.v.) dosing (Hussain et al., 1980a; Parr, 1983). Intranasal formulations having poor bioavailability have been improved by the addition of surfactants such as bile salts and non-ionic polyoxyethylene ethers (Hirai et al., 1978, 1981a and b; and Moses et al., 1983). Possible mechanisms by which these enhancers may exert their effects include direct alteration of the mucosa, i.e. increasing permeability by changing the membrane structure (Hirata, 1979), inhibition of proteolytic enzymes (Hirai et al., 1981b), formation of aqueous pores (Gordon et al., 1985), and solubilization of the drug in the formulation vehicle (Hirai et al., 1981a). McMartin et al. (1987) concluded that the most probable route of membrane transport of peptides and proteins is through intercellular junctions, and that surface-active adjuvants which increase absorption may do so by temporarily converting hydrophobic contacts between junctional proteins into hydrophilic pores.

The purpose of the present study was to determine the effects of detergent enhancers on the nasal absorption characteristics of recombinant methionyl human growth hormone (Met-hGH) (mol. wt. 22,000).

Materials and methods

Animal model

Unfasted male Sprague–Dawley rats (Charles River, Wilmington, MA) weighing between 300 and 350 g were used for the experiments. The studies were done with groups of 6 animals, having weights within 20% of the mean weight. The animals were anesthetized with a combination of ketamine HCl (Vetaler, Parke-Davis) 33 mg/kg, acepromazine (PromAce, Aveco) 0.75 mg/kg and zylazine (Rompun, Haver-Lockhart) 4 mg/kg.

The surgical procedure used to prepare the animals for studying nasal mucosal absorption of drugs was modified from that described by Hirai et al. (1981c). The sole surgical intervention was to intubate the trachea, to maintain ventilation.

The anesthetized animals were dosed i.n. by inserting a pipet tip filled with 70 μl of test solution into the external nares. The animals were positioned on their backs on a heating pad for the duration of the experiment. Blood samples (approx. 0.3 ml) were collected by orbital bleeds at timed intervals up to 6 hours after dosing.

Met-hGH studies

Met-hGH (Protropin, human growth hormone), Genentech Inc., S. San Francisco, CA) produced by recombinant DNA methods described by Goeddel et al. (1979) was obtained as a lyophilized powder. The lyophilized Met-hGH was reconstituted in 5 mM phosphate buffer containing 16 mg/ml mannitol and enhancer was added to the formulation at either 0.5 or 1% (w/w). The effect of osmolarity was also examined. In these studies, lyophilized hGH was dissolved in distilled water (DW) or DW containing sucrose or glucose to make solutions at 300, 600 or 1500 mOsm. Met-hGH was also administered without enhancer in the mannitol phosphate buffer at 1, 2 and 3 mg/kg. Sodium glycocholate, sodium deoxycholate, and polyoxyethylene 9-lauryl ether (laureth-9), were obtained from Sigma Chem. Co., St. Louis, MO. The critical micelle concentrations (CMC) of these detergents at 0–0.5 M NaCl are approximately 7, 4 and 0.1 mM, respectively (Neugebauer, 1987). The pH of the nasal formulations with Met-hGH was 7.4.

Plasma Met-hGH concentrations after i.n. administration of Met-hGH formulations with and without permeation enhancers were compared to those following intramuscular (i.m.) and i.v. administration of Met-hGH (no enhancer) to de-

termine the relative and absolute bioavailability of the various i.n. Met-hGH formulations. Met-hGH was administered at 0.1, 0.3, and 0.5 mg/kg i.m. and at 0.2 mg/kg i.v. The i.v. and i.m. injections were made via the jugular vein and hind limb, respectively, in a volume of 0.1 ml.

Histology studies

The effects of the surfactants in the Met-hGH formulations on nasal mucosa were studied in rats prepared as previously described, but used for histological evaluation only. The animals were anesthetized, dosed i.n. then subsequently sacrificed at 30 min, 1, 3, 6 and 24 h after dosing. Anesthesia was maintained until sacrifice time, except in the case of the animals to be killed at 24 h, where no surgery was performed (no tracheal intubation) and they were allowed to recover after 6 h of anesthesia. The animals were sacrificed by decapitation, the nasal region of the skull excised, fixed in 10% phosphate-buffered formalin and put in decalcifying solution (Easy Cut, American Histology Reagent Co., Stockton, CA). The nasal region was embedded in paraffin and cut in 4–6 μm sections distal and proximal to the cranium. The sections were stained with hematoxylin and eosin, to see changes in mucus membrane structure or stained with Alcian blue-PAS to stain mucin-containing goblet cells (Sheehan and Hrapchak, 1980). Sections were examined without prior knowledge of treatment, and changes were scored semiquantitatively.

Assay and statistical analysis

The rat plasma samples were diluted with horse serum and analyzed by an immunoradiometric assay (IRMA) for hGH (Hybritech, La Jolla, CA) having a range of 1–100 ng/ml. Endogenous rat growth hormone does not cross-react in the IRMA, nor does the IRMA detect fragments of human growth hormone.

Data points for plasma Met-hGH concentrations represent the mean value of 6 animals. The variation within the group is expressed as the standard deviation. The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal method (Gibaldi and Perrier, 1982). Absorption of Met-hGH was estimated from the area under the curve extrapolated to infinity (McMartin et al., 1987):

$$AUC = AUC_t + C/k$$

where AUC is to total area, AUC_t is the AUC determined during the experimental time, k is the terminal rate constant and C is the concentration at the final time point. Absolute bioavailability is the ratio of AUC after i.n. administration to AUC after i.v. injection, corrected for the difference in doses. Relative bioavailability compares areas determined after i.n. administration to those obtained following i.m. injection (Gibaldi and Perrier, 1982).

Results

Fig. 1 shows the effect of 1% (17 mM) laureth-9 on the absorption of 3 doses of Met-hGH in the anesthetized rat. Peak plasma Met-hGH concentrations were achieved within 10–15 min followed by a rapid decline. In the absence of enhancer only the highest dose of hGH (3 mg/kg)

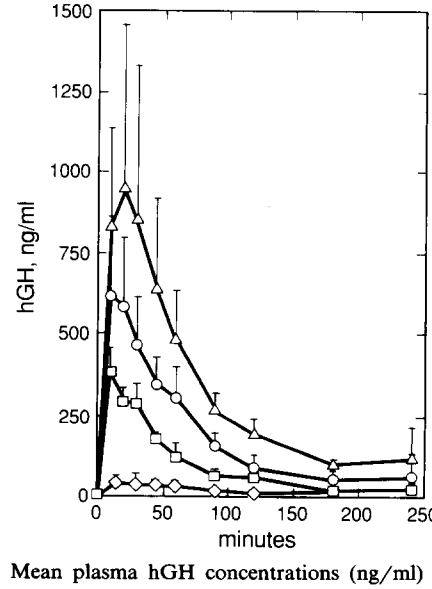

Fig. 1. Mean plasma hGH concentrations (ng/ml) after i.n. administration of Met-hGH with 1% laureth-9 to anesthetized rats at 3 doses. Each point is expressed as the mean ±S.D. of 6 animals. □, 0.2 mg/kg; ○, 0.5 mg/kg; △, 1 mg/kg; ◇, 3 mg/kg; control (no enhancer).

TABLE 1

Area under the curve (AUC) [a] *and absolute bioavailabilities (F)* [b] *of Met-hGH following intranasal, intramuscular, and intravenous administration* [c]

Route	Enhancer	0.1 mg/kg		0.2 mg/kg		0.3 mg/kg		0.5 mg/kg		1 mg/kg		2 mg/kg		3 mg/kg	
		AUC	*F*	*AUC*	*F*	*AUC*	*F*	*AUC*	*F*	*AUC*	*F*	*AUC*	*F*	*AUC*	*F*
i.n.	–									nd		nd		3300 ±800	0.8 ±0.02
i.n.	1% laureth-9			21800 ±3600	79 ±13			44600 ±14000	65 ±20	78000 ±22100	57 ±16				
i.n.	0.5% Na glycocholate			2400 ±300	8 ±2			4600 ±1100	7 ±2						
i.m.	–	3700 ±450	27 ±3			20100 ±2900	48 ±7	31600 ±4100	46 ±6						
i.v.	–			27500 ±1300	100										

[a] Units = ng·min/ml, $AUC = AUC_t + C/k$ where AUC_t = area determined over the experimental time period, k = terminal rate constant, C = concentration at last time of sampling (McMartin et al., 1987)

[b] $F = \frac{AUC_{\text{i.n. or i.m.}}}{AUC_{\text{IV}}} \times \frac{\text{Dose}_{\text{i.v.}}}{\text{Dose}_{\text{i.n. or i.m.}}} \times 100$

[c] Values represent mean bioavailability ± 1 S.D. of 6 rat nd = not detectable

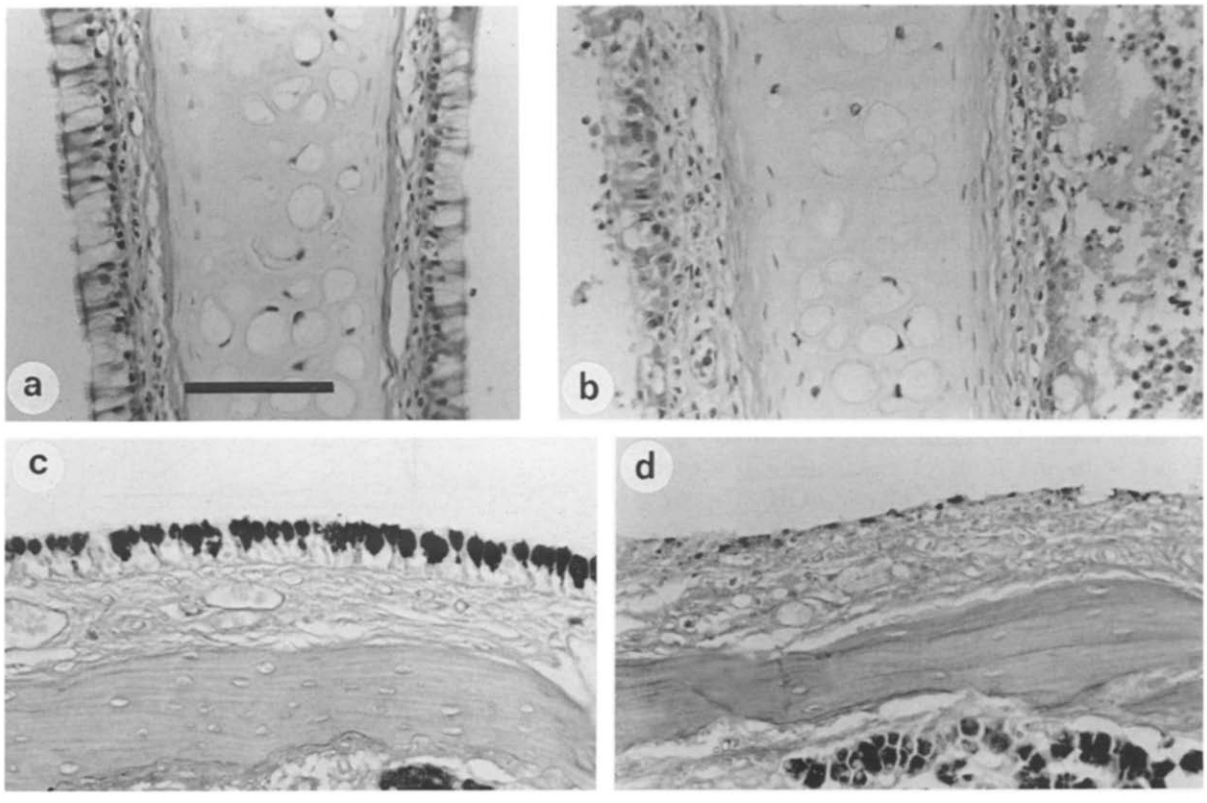

Fig. 2. Nasal mucosal surfaces from a rat treated with met-hGH without a permeation enhancer (a,c) are compared to mucosal surfaces taken from a rat 3 h after exposure to Met-hGH (3 mg/kg) with 1% laureth-9 (b,d). There is focally extensive exudation and epithelial cell loss (b) including loss of goblet cells (d) which normally stain with PAS (c). Scale bar = 100 μm.

showed detectable blood levels (Fig. 1). The absolute bioavailability of hGH in the presence of enhancer was between 60% and 80% (Table 1). Although there was a drop in the mean bioavailability values with an increase in dose, the differences were not significant. In the absence of enhancer the bioavailability of hGH was $< 1\%$. Fig. 2 shows some of the effects of 1% laureth-9 on rat nasal mucosal structure at 3 mg/kg Met-hGH after 3 h exposure. Fig. 2a and c shows control mucosa treated with hGH alone and stained with hematoxylin-eosin and Alcian blue-PAS, respectively. Fig. 2b and d show the destructive effects of 1% laureth-9 on the mucosal lining which included multifocal necrosis, inflammation and exudation, and in some regions, complete removal of the epithelial monolayer (Fig. 2D). Similar histopathology was seen at the earliest (30 min) and latest time points (24 h). The greatest alterations were seen in the back of the nose where administered solutions localized as the animals lay on their backs.

Because of the extensive damage caused by laureth-9, other detergents were sought which could effect hGH absorption without causing mucosal damage. Deoxycholate at 1% (24 mM) caused less extensive damage than laureth-9 but bioavailability was also less (13–23%). Glycocholate at 1% (24 mM) caused even less histopathology but bioavailability dropped further to 11%. Nasal mucosa of rats treated with 0.5% (12 mM) glycocholate were not easily distinguishable, at the light microscopic level, from those dosed with hGH alone. Some had mild, multifocal inflammation and erosion but these indications were also sometimes seen in rats dosed with hGH alone. Fig. 3 shows the plasma concentration versus time curves for these experiments. The shapes of the

profiles were similar to those seen in Fig. 1 although the peak levels were much lower and the absolute bioavailabilities only 7–8% (Table 1).

Fig. 4 shows typical examples of plasma hGH concentrations following i.v., i.m. and i.n. routes of administration. In contrast to the i.v. dose, which was rapidly cleared ($t_{1/2} = 20$ min), the i.m. dose produced sustained plasma concentrations which peaked at about 60 min and gradually declined over the next several hours. I.n. dosing with enhancer gave a plasma profile more similar to the i.v. route than the i.m. with a rapid peak followed by a gradual decline.

Table 1 summarizes the results shown in Fig. 1, 3 and 4 in the form of calculated *AUC*s and absolute bioavailabilities of met-hGH. The *AUC* of a 0.2 mg/kg i.v. dose was chosen for calculations of *F* because it gave an *AUC* value that was reasonably close to the i.m. and i.n. *AUC*s. The results show that there was a roughly proportional increase in *AUC* with dose and that *F* did not change significantly with dose.

The effect of osmolarity on nasal hGH absorption at a dose of 3 mg/kg in the absence of

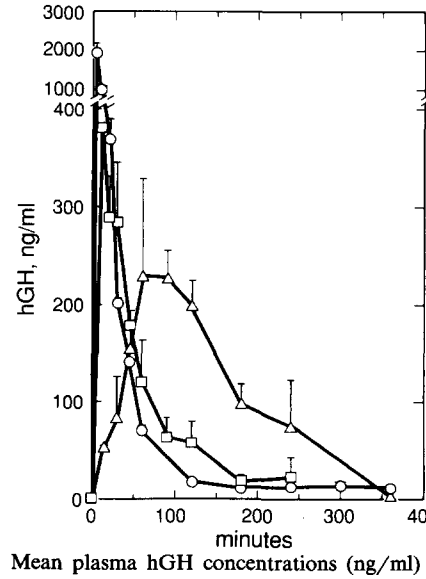

Fig. 4. Mean plasma hGH concentrations (ng/ml) after i.n., i.m., and i.v. administration of Met-hGH. Each point is expressed as the mean ± S.D. for 4 animals for i.v.; 6 animals for i.m. and i.n. □, 0.2 mg/kg with 1% laureth-9, i.n.; ○, 0.2 mg/kg, i.v.; △, 0.5 mg/kg, i.m.

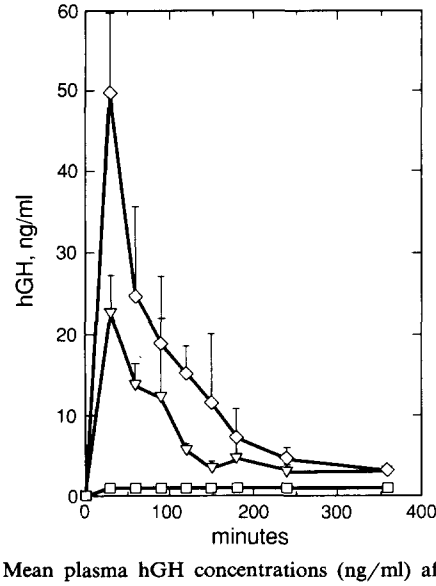

Fig. 3. Mean plasma hGH concentrations (ng/ml) after i.n. administration of Met-hGH with 0.5% sodium glycocholate to anesthetized rats at two doses. Each point is expressed as the mean ± S.D. of 6 animals. ◇, 0.5 mg/kg; (▽, 0.2 mg/kg; □, 1 mg/kg control (no enhancer).

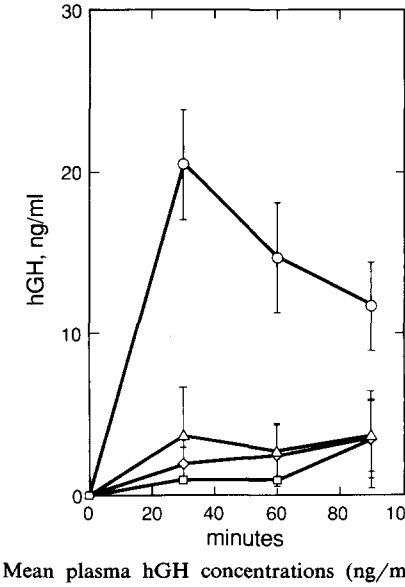

Fig. 5. Mean plasma hGH concentrations (ng/ml) after in administration of 3 mg/kg Met-hGH with and without sucrose. Each point is expressed as the mean ± S.D. for 6 animals. ○, distilled water; □, 300 mOs sucrose; △, 600 mOs sucrose; ◇, 1500 mOs sucrose.

enhancers was also examined. Fig. 5 shows that in the absence of sugar and salt, hGH absorption was enhanced, whereas absorption was markedly reduced in the presence of isotonic or hypertonic sucrose solutions. Similar results were seen with glucose (data not shown). Note that the profile seen with the hypotonic solution in Fig. 5 is similar to that seen with the control hGH dose in Fig. 1 which was also a relatively hypotonic solution (90 mOs).

Discussion

The object of intranasal drug delivery is to achieve therapeutic drug levels systemically in the absence of damage to the nasal mucosa. The nasal mucosa is a poorly understood, heterogeneous monolayer of at least 6 cell types (Monteiro-Riviere and Popp, 1984) some of whose functions include antigen sampling and antibody production (Enerback et al., 1986; Bergmann et al., 1986), mucus production (Thaete et al. 1981), mucus movement (Menco, 1984), and odor detection (Menco and Farbman, 1985). Beneath the nasal mucosa cells, specialized glands produce additional mucins and other unknown substances which are secreted onto the mucosal surface (Thaete et al., 1981). A constantly moving blanket of mucus serves as a barrier and trap for particles in inspired air and is probably a site of antibody binding to viral and microbial antigens (Hounsell and Feizi, 1982). For a macromolecule like growth hormone (mol. wt. ~ 22,000) to be absorbed intranasally it must be able to diffuse through the mucus blanket and then either pass between the mucosal epithelial cells (paracellular transport) which are normally connected by tight junctions impermeable to macromolecules, or pass though the epithelial cells by a process known as transcytosis (Mostov and Simister, 1985).

The results of this study suggest that in the absence of enhancers and in the absence of pathology, e.g. rhinitis, the nasal mucosa is essentially impermeable to therapeutic doses of growth hormone. Similar conclusions have been reached with intranasal insulin (Hirai et al., 1981b and c; Gordon et al., 1985). Only at the highest dose of growth hormone (3 mg/kg) was a small amount of absorption (< 1%) seen in the absence of enhancers. In the rat model used in this study, there was no air flow through the nares and the animals were anesthetized. The animals were positioned on their backs, so that there was probably pooling of the dose in the rear of the nasal cavity. It could thus be argued that clearance mechanisms were inhibited and the opportunity for growth hormone absorption was greater. Absorption of Met-hGH administered intranasally was compared in anesthetized and awake animals (unpublished data), which did show greater absorption in the surgically prepared anesthetized animal. The very poor hGH absorption that occurred in the absence of enhancing agents suggests that, indeed, systemic macromolecule absorption in the rat nose is minimal.

Since the absorption of hGH peaked within the first hour, the stresses associated with decreased total blood volume from the serial blood sampling and anesthesia were minimal. The model permitted precise dosing and good dose retention. In the absence of enhancers hGH absorption was minimal. Thus, the anesthetized rat model (Hirai et al., 1981c) is an appropriate model for studying enhancer effects on nasal absorption of macromolecules such as hGH.

This study also showed that detergents markedly enhanced nasal growth hormone absorption. Detergents may enhance intranasal absorption of proteins by dispersing the mucus barrier (Marriott et al., 1983), arresting ciliary movement (Duchateau et al., 1986), inhibiting nasal proteases (Stratford and Lee, 1986; Hirai et al., 1981c), dispersing protein aggregates (Gordon et al., 1985), or by disrupting the cellular permeability barrier (Hirai et al., 1981c). Although detergents can lower the viscosity of mucus gels, they also readily stimulate the discharge of mucus from goblet cells (Goerg et al., 1982); therefore, any decrease in the viscosity of the mucus caused by intranasal detergents could be counteracted by an increase in mucus mass. The inhibition of nasal proteases (Hussain et al., 1985) by detergents could enhance protein absorption but the problem of epithelial permeability remains. Bile salts are thought to disperse microcrystals and aggregates that occur

in commercial insulin preparations, thereby increasing the insulin monomer concentrations at the absorptive surface (Gordon et al., 1985) but growth hormone preparations do not appear to contain significant amounts of microcrystals or aggregates (J. Oeswein, personal communication). The most likely mechanism by which detergents enhanced intranasal hGH absorption in this study was by disrupting the cellular permeability barrier. The ability of detergents to solubilize membrane lipids is well known (Lichtenbert et al., 1983; Coleman et al, 1980) and obviously the amount of laureth-9 used in this study was sufficient to solubilize the entire mucosal layer in certain places. Glycocholate at 0.5% did not appear to disrupt the continuity of the epithelial lining (as viewed in paraffin-embedded sections by light microscopy) but did enhance Met-hGH absorption. This less destructive action of glycocholate can be explained by its weaker lipid-solubilizing capacity relative to the other two detergents (Hirai et al., 1981b and c; Gordon et al., 1985).

Although studies of the effects of bile salts on the ultrastructure and function of nasal mucosa have been limited (Hirai et al., 1981c), there have been a number of studies with gastrointestinal epithelium. Goerg et al. (1982) perfused rat colon with 8 mM deoxycholate and observed marked alteration of the mucosal surface by scanning and transmission electron microscopy. Ballooned cells were observed over most of the surface. The height of the cells seemed to be decreased. The microvilli were shorter, reduced in number, or even absent. The mitochondria were swollen and the endoplasmic reticulum vacuolated. In spite of these injuries, gaps in the epithelium were never found and the continuity of the mucosa was maintained. The changes were reversed by reperfusion with buffer alone. Georg et al. (1983) concluded from additional studies that the increased mucosal permeability to fluid caused by bile salts in the absence of mucosal disruption could only be explained by an increase in paracellular permeability. Fagundes-Neto et al. (1981) examined the effect of perfusing a variety of bile salts (5 mM) on jejunal absorption of horseradish peroxidase (HRP) (mol. wt. 40,000). HRP penetrated into the intercellular spaces with all of the bile salts. Preparations perfused with deoxycholate or cholate showed damage to organelles and with deoxycholate many cells stained diffusely for HRP consistent with damage to plasma membrane integrity. Again, in spite of these changes, disruption of overall mucosal integrity was not observed. In a related study, glycocholate (12 mM) removed significant amounts of acetylcholinesterase and membrane phospholipids from erythrocytes prior to cell lysis (Coleman and Holdsworth, 1976). These studies, conducted at bile salt concentrations equivalent to those used in the present study, illustrate that before outright cell destruction occurs, there can be a variety of detergent effects on epithelium which may enhance mucosal permeability. Foremost among these effects may be the opening up of space between cells. The danger of increased permeability to macromolecules is an increase in the absorption of antigens, viruses, and microbial toxins (Goerg et al., 1983; Teichberg et al., 1983).

Hyperosmotic solutions disrupt the blood–brain barrier and allow passage of macromolecules from the blood into the brain. Presumably the reversible opening that occurs is mediated by osmotically induced shrinkage of individual cerebrovascular endothelial cells and consequent widening of tight junctions (Robinson et al., 1987). In this study, hyperosmotic solutions did not enhance intranasal absorption of Met-hGH. Some enhancement of absorption occurred with a hypotonic solution although the levels achieved were small compared to those seen with detergents. Hypotonic solutions may cause osmotic bursting of some epithelial cells, thereby disrupting the barrier.

The hope has been that, if properly formulated, detergents can enhance absorption without causing irreversible damage to the mucosa. There is qualitative evidence that submicellar concentrations (0.5 mM) of deoxycholate can enhance HRP absorption in the perfused rat jejunum (Teichbert et al., 1983) without showing any ultrastructural alterations in the epithelial cells; however, this enhancement may be quantitatively small. Gordon et al. (1985) showed that at 2.5 mM deoxycholate (CMC is 3 mM) intranasal absorption of insulin was minimal; maximal absorption occurred only at deoxycholate concentrations above 12 mM

which are known to alter mucosal integrity. It appears therefore that some transient mucosal injury is required for intranasal protein absorption. It is clear from the work of Hirai et al. (1981b and c) and others (Coleman et al., 1980), and from this study that the trihydroxy bile salts like glycocholate are the least membrane damaging of the intranasal detergent enhancers. It is questionable, however, whether the nasal mucosa could withstand the long-term, possibly multiple daily administration of a detergent-containing formulation. Tolerance of such a formulation would be integral to an intranasal Met-hGH therapy regimen.

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