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Intestinal permeation and metabolism of a model peptide (leuprolide) and mechanisms of permeation enhancement by non-ionic surfactants

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Abstract

The intestinal permeability of leuprolide, a model nonapeptide, was determined using an in vitro model involving everted intestinal sacs of rat. Degradation of leuprolide by intestinal proteolytic enzymes was suppressed with a mixture of protease inhibitors which was shown to completely inhibit degradation in separate experiments using intestinal homogenates. This allowed the true permeability coefficient of leuprolide to be determined. The enhancement of this intestinal permeability by the Igepal CO® homologue series of surfactants was investigated using members of this series with hydrophilic-lipophilic balance values (HLB) ranging between 12 and 19. Enhancement showed a non-monotonic dependence on HLB with a maximum at HLB ≈ 15, while at both lower and higher HLB values an enhancement decrease was observed. Surfactant uptake into the tissue showed a similar non-monotonic dependence on HLB. On the other hand, red blood cell lysis and lecithin solubilization potencies of the Igepal CO showed a monotonic decrease with increasing HLB within the HLB range studied. Hence, it is suggested that the process of permeation enhancement should be dissociated from that of membrane solubilization in terms of the mechanism of surfactant-membrane interaction. While membrane disruption, for example, in the case of hemolysis, seems to be correlated with phospholipid solubilization it does not appear to be a prerequisite for permeation enhancement. Permeability increase of the plasma membrane may conceivably be accomplished by surfactant incorporation into the bilayer causing highly permeable disorder points and/or an increased membrane fluidity.

Keywords: Peptide; Surfactant; Intestinal permeation; Enhancer; Metabolism absorption model

1. Introduction

The limited gastrointestinal absorption typically exhibited by peptide drugs is in general due to the combination of (a) their enzymatic degradation by proteolytic enzymes in the gastrointestinal tract and (b) intrinsically low permeability of

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the gastrointestinal mucosal membrane to peptides. These two factors represent independent processes taking place in parallel and their relative significance may vary depending on the peptide in question. Therefore, quantifying the contribution of each of them in limiting oral peptide absorption is essential for developing oral delivery strategies for individual peptides.

Dipeptides and selected tripeptides were shown to be taken up intact by the intestinal mucosa through the peptide carrier system of the enterocytes (Adibi and Kim, 1981). Peptides with four or more amino acids are not substrates for this carrier system (Adibi and Kim, 1981) and have usually shown no detectable intestinal absorption, being hydrolysed outside and/or on the surface of the cells by luminal and brush-border peptidases (Smithson and Gray, 1977; Matuszewska et al., 1988). However, a small amount of intestinal transport of intact nonapeptides was observed when chemically modified analogues of natural peptides were employed to increase their enzymatic stability (Lundin and Vilhardt, 1986; Vilhardt and Lundin, 1986) or when peptidase inhibitors were used (Takaori et al., 1986). It was suggested that this transport was taking place by passive diffusion. Although the extent of absorption was in general very small, pharmacologic activity could be measured following oral administration (Vilhardt and Bie, 1984; Saffran et al., 1988).

The possibility of using surfactants for absorption enhancement has long been recognized. The effect of surfactants on drug absorption depends on the physicochemical properties of both the drug and the surfactant and is generally considered to be the net result of the following factors which have in part opposite effects and may operate simultaneously (Gibaldi and Feldman, 1970; Amidon et al., 1982; Florence et al., 1984): (a) interaction with the mucosal membrane of the absorptive epithelium causing perturbation of the membrane structure or solubilization of membrane segments, both resulting in an increase in membrane permeability; (b) increase in the dissolution rate and/or aqueous solubility of drugs administered in solid form due to improved wettability and/or solubilization of barely soluble drugs by surfactant micelles; (c) incorporation of drug molecules into micelles resulting in decreased thermodynamic activity and decreased diffusivity of the drug in solution; (d) reduction of the transport resistance of the diffusion boundary layer in drug saturated solutions because of drug being ferried across the boundary layer by the micelles; and (e) biochemical or pharmacologic activity.

Surfactants were shown to enhance the absorption of peptide drugs by various routes of administration (Hirai et al., 1981; Okada et al., 1982; Longenecker et al., 1987). Since peptides are rather large, water-soluble molecules exhibiting low membrane permeability, this absorption enhancement is primarily achieved through interaction of the surfactants with the mucosal membrane causing a permeability increase. This membrane interaction of surfactants, however, may also give rise to local toxicity due to membrane damage.

In the present work, the intestinal absorption of leuprolide, a nonapeptide LH-RH analogue used here as a model compound, was studied with an in vitro model using everted intestinal sacs of rat. A mixture of protease inhibitors was used to suppress enzymatic degradation of leuprolide in the intestine in order to determine its true intestinal permeability. The activity of a homologue series of non-ionic surfactants (Igepal CO®) as intestinal permeation enhancers of leuprolide was evaluated. Emphasis was placed on elucidating the mechanism(s) by which the surfactants interact with the epithelial membrane and exert their enhancement effect. This work focuses particularly on the lipid bilayer domain of the cell membrane as the predominant site of surfactant activity, other types of interaction, i.e., with the protein fraction or the tight junctions, thought to be of lesser significance. Membrane activity of the Igepal CO was further assessed by means of hemolysis and lecithin solubilization studies. Since permeation enhancement is often associated with local membrane toxicity, mechanistic studies of the membrane-surfactant interaction should provide an insight into the benefit/adverse reaction profile of surfactants and potentially help optimize their effect.

2. Materials and methods

2.1. Chemicals

Leuprolide is a nonapeptide analogue of the human luteinizing hormone releasing hormone (LH-RH) with the structure des-Gly^{10},[D-Leu^{6}]-LH-RH-ethylamide. Its complete amino acid sequence is pGlu-His-Trp-Ser-Tyr-(D-)Leu-Leu-Arg-Pro-NHEt, its molecular weight is 1209, and it has a net positive charge at neutral pH. It was used in its acetate form as obtained from Sigma Chemical Co. (St. Louis, MO, USA, 1989 catalog no. L5009). The following peptidase inhibitors were used: sodium *p*-hydroxymercuribenzoate (Na-PHMB) (Sigma), 1,10-phenanthroline monohydrate ACS reagent (Sigma), and phenylmethylsulfonyl fluoride (PMSF) ultra-pure grade (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA).

The Igepal CO® homologue series of surfactants (GAF Chemicals Corp., Wayne, NJ, USA) were used as received. Igepal CO are nonylphenoxypoly(ethyleneoxy)ethanols with the following general formula: CH_3-$(CH_2$-$)_8C_6H_4$-$O(-CH_2CH_2O)_n$-H. The index n represents the average molar ratio of ethylene oxide to nonylphenol moieties. The following members of this homologue series were used; they are denoted by their CO-code number, followed by the corresponding n value and hydrophilic-lipophilic balance value (HLB) – CO-610: $n = 7.5$, HLB = 12.2; CO-630: $n = 9$, HLB = 13; CO-660: $n = 10$, HLB = 13.2; CO-710: $n = 10.5$, HLB = 13.6; CO-730: $n = 15$, HLB = 15; CO-850: $n = 20$, HLB = 16; CO-880: $n = 30$, HLB = 17.2; CO-890: $n = 40$, HLB = 17.8; CO-990: $n = 100$, HLB = 19. These surfactants do not ionize in water and do not undergo acid or alkali hydrolysis in aqueous solutions.

Ethyl carbamate (urethane) (Mallinckrodt Inc., Paris, KY, USA) was used for anesthesia as a 0.5 g/ml solution in deionized water. For euthanasia the T-61 Euthanasia Solution (Hoechst-Roussel, Sommerville, NJ, USA) was used.

2.2. Animals

Adult male Sprague-Dawley rats (Simonsen Labs, Gilroy, CA, USA) with a body weight between 250 and 400 g were used.

2.3. Enzymatic degradation

Enzymatic degradation was studied in intestinal homogenates. Rats were anesthetized with a 1 mg per g body weight i.p. bolus injection of urethane. A midline abdominal incision was made and an approx. 30 cm long segment of the small intestine starting a few centimeters below the duodenal-jejunal junction was removed and placed in ice-cold phosphate-buffered saline (PBS). The rats were subsequently killed with an intracardiac injection of 0.3 ml T-61 euthanasia solution. The intestinal segment was cut into roughly 3 cm long pieces and the luminal contents were thoroughly rinsed off with PBS. All pieces were transferred into a centrifuge tube containing 15 ml of fresh PBS kept in an ice bath and were homogenized with a 'polytron-type' biohomogenizer (Biospec Products, Inc., Bartlesville, OK, USA) for 3.5 min at high speed. The homogenate was centrifuged for 5 min at 2000 $\times g$ to remove coarse fragments and the supernatant was split into 2 ml fractions. Peptidase inhibitors were added from stock solutions to each fraction and were allowed to equilibrate for 20 min at room temperature. Subsequently, 2 ml of leuprolide solution in PBS were added to each homogenate fraction and the mixtures were incubated at 37°C in a shaking water bath. In the final incubation solution, leuprolide concentration was approximately equal to 50 μg/ml and inhibitor concentrations were as follows: PHMB, 1 mM; PMSF, 5 mM; 1,10-phenanthroline, 0.5 mM. The final tissue concentration corresponded approximately to an intestinal length of 1 cm per ml of incubation solution. Samples of 0.5 ml were withdrawn at predetermined time points from the incubations and were added to 1 ml of methanol. The mixtures were centrifuged for 3 min at 12000 $\times g$ and leuprolide was assayed in the supernatants by HPLC.

2.4. Intestinal permeation

Permeation studies were carried out using everted intestinal sacs of rat (Wilson and Wiseman, 1954). An approx. 50 cm long segment of small intestine was removed from rats as de-

scribed under section 2.3 and placed in a dissection trough containing tissue culture medium 199 (Sigma), which was pre-saturated with a mixture of $O_2/CO_2 = 95:5$ (USP grade, United States Medical, Denver, CO, USA) and kept in an ice bath. This intestinal segment was rinsed with culture medium to remove luminal contents and was gently everted over a 2 mm wide glass rod. The everted intestine was filled with a sufficient amount of fresh culture medium to give a physiologic dilation and was ligated at both ends with silk surgical sutures. Sacs were made by tying pairs of sutures at distances of 2 cm from each other along the everted intestine and separating the formed sacs using scissors. Throughout this preparation the intestine was kept immersed in ice-cold culture medium. The sacs had a length of 2 cm and an outer diameter of 0.54 cm which, assuming cylindrical shape, gave a surface area of 3.4 cm^2 per sac.

From every rat 18 sacs were prepared and divided into six groups of three sacs each, and each group was suspended in a separate glass vial. The vials contained the mucosal solution consisting, at this stage, of 6 ml culture medium in which the inhibitors had been dissolved at the concentrations given under section 2.3. The sacs were equilibrated with the inhibitors for 20 min at room temperature and subsequently stock solutions of an Igepal CO surfactant and of leuprolide, both in culture medium, were added to each vial, this marking the beginning of the permeation experiment. The final mucosal solution had a volume of 8 ml, a leuprolide concentration of approx. 500 μg/ml and a surfactant concentration of 10 mg/ml, and was maintained at equilibrium with an $O_2/CO_2 = 95:5$ gas mixture at 37°C throughout the experiment. To achieve sufficient agitation of the mucosal solution with minimal mechanical damage to tissue, the glass vials were rotated at 585 rpm. For this purpose, they were positioned onto rotating bases which were connected with each other and with a motor by a belt and pulley system. Preliminary experiments had shown that no diffusion boundary layer effect on the transport rate of rather hydrophilic compounds could be detected at this rotation speed.

To assess the intestinal permeability of leuprolide, the total amount of drug taken up by the sacs including the amount present in the serosal fluid was determined. This tissue uptake reflects the drug amount permeating the intestinal epithelium, since the surface of the sacs exposed to the mucosal solution is entirely covered by epithelial cells. This method was adopted following preliminary experiments which had shown that after about 100 min of incubation the amount of leuprolide entering the serosal fluid was less than 1/10 of that found in the tissue, suggesting that, at the applied concentrations, the tissue acts as a sink allowing only a small amount of drug to leak into the serosal side.

Tissue uptake was determined at 10 and 30 min of incubation, using at each time point all three sacs of a given vial. The sacs were simultaneously withdrawn from the mucosal solution, rinsed briefly in culture medium and each one was homogenized separately in 1.5 ml of culture medium with the biohomogenizer. 0.5 ml of the homogenate was added to 1 ml of methanol, the mixture was centrifuged for 3 min at $12000 \times g$ and the amounts of leuprolide and Igepal CO in the supernatant were determined by HPLC. Intestinal permeability coefficients of leuprolide were calculated using Eq. 1:

$$P = \frac{Q_{30\,\mathrm{min}} - Q_{10\,\mathrm{min}}}{S(\overline{C}_D - \overline{Q}/V)\Delta t} \quad (1)$$

where P is the permeability coefficient, $Q_{10\,\mathrm{min}}$ and $Q_{30\,\mathrm{min}}$ represent the amounts of drug uptake by the tissue at 10 and 30 min, respectively, representing averages of three sacs, S is the surface area of a sac, $\overline{C}_D$ denotes the average drug concentration in the donor (mucosal) solution in the time interval between 10 and 30 min, $\overline{Q}$ is the average drug content of tissue in the same time interval, V represents the volume of tissue of a sac which, based on tissue weight, was found to be equal to 0.23 ml and Δt is equal to 20 min.

Plots of Q vs time always yielded a significant positive y-axis intercept, suggesting that rapid adsorption of drug on the adhering mucus and/or on the tissue surface was taking place during the first few minutes of incubation. From the y-axis

intercept, denoted here $Q_{t=0}$, an apparent volume of adsorption, V_{ads}, was calculated as follows:

$$V_{ads} = \frac{Q_{t=0}}{C_{D,t=0}} \quad (2)$$

where $C_{D,t=0}$ is the drug concentration in the mucosal solution at time zero.

In every experiment involving one rat, two vials, out of a total of six, contained no surfactant and the corresponding sacs were used to measure the intestinal permeability coefficient of leuprolide with no enzymatic degradation taking place. This also served as a baseline value for the evaluation of the enhancer effects of the surfactants.

2.5. HPLC assay

Chromatography was performed on a Waters system (Milford, MA, USA) with a 600 multisolvent delivery system, a 712 WISP, a lambda-max model 481 variable-wavelength UV detector and an 840 chromatography data station. A Whatman reversed-phase column, partisphere C-18 (110 mm × 4.6 mm, 5 μm), was used and solvents were purchased from Burdick and Jackson (Muskegon, MI, USA). When leuprolide was assayed alone, MeOH/0.25 M CH_3COONH_4(aq.) = 55:45 was used as the mobile phase in isocratic mode. For simultaneous assay of leuprolide and of an Igepal CO, mobile phase composition was varied in a gradient mode: solvent A: MeOH/0.25 M CH_3COONH_4(aq.) = 55:45; solvent B: 100% tetrahydrofuran; start: 100% A; 0–20 min: linear increase of B from 0 to 30%; 20–21 min: linear decrease of B from 30 to 0%; 21–30 min: equilibration with 100% A. Both leuprolide and Igepal CO were detected at 280 nm. The retention time of leuprolide was around 5 min and that of Igepal CO varied from 18 to 22 min depending on their HLB. The Igepal CO gave rather broad peaks because they were not pure substances, their n values representing averages of the actual distributions of hydrophilic/lipophilic moiety molar ratios.

2.6. Hemolysis

Packed human erythrocytes, 32 days old, were obtained from a blood bank (LDS Hospital, Salt Lake City, UT, USA). The cells were washed two to three times, each with approx. 10-times their volume of ice-cold PBS and a 10% v/v stock cell suspension in PBS was prepared and kept in an ice bath. Serial dilutions of individual Igepal CO in PBS were mixed with the stock cell suspension to give a final cell concentration of approx. 1% v/v and were incubated at room temperature for 5 min under periodic agitation. After incubation, the mixtures were centrifuged at 12000 × g for 3 min and the released hemoglobin was assayed in the supernatant by UV spectrometry at 541.3 nm (Lambda 4B UV/Vis spectrophotometer, Perkin Elmer, Norwalk, CT, USA).

The amount of hemoglobin released from the erythrocytes was used to quantify the hemolytic activity of the surfactants which was expressed on a percent scale using Eq. 3:

$$\%\,\text{hemolysis} = \frac{A - A_{blank}}{A_{100\%} - A_{blank}} \times 100 \quad (3)$$

where A is the UV absorbance of hemoglobin released in the presence of surfactants, A_{blank} represents the UV absorbance obtained by incubating red cells in PBS in the absence of surfactants and accounts for spontaneous cell lysis and background effects, and $A_{100\%}$ is the UV absorbance obtained in the case of 100% hemoglobin release, which was achieved with an osmotic shock by diluting the stock cell suspension with deionized water to a final cell concentration of 1% v/v. A_{blank} was always less that 1% of $A_{100\%}$ and surfactant absorption at 541.3 nm was negligible. The surfactant concentration, EC_{50}, required to induce a 50% hemolysis was obtained from the hemolysis vs concentration curve by linear interpolation.

2.7. Lecithin solubilization

Solutions of Igepal CO with a concentration of 5 mg/ml were prepared in PBS. Increasing amounts of preweighed lyophilized egg lecithin

(Avanti Polar Lipids, Alabaster, AL, USA) were added to the Igepal CO solutions at room temperature and stirred frequently. The maximal amount of lecithin solubilized was determined by visual evaluation of the transparency of the final solutions.

3. Results and discussion

3.1. Metabolism

Fig. 1 shows the results of the leuprolide degradation studies in rat intestinal homogenates. When no inhibitors were added, 60% degradation was observed within 150 min, demonstrating that leuprolide is susceptible to enzymatic hydrolysis by intestinal peptidases. No individual inhibitor could fully suppress degradation, PMSF and 1,10-phenanthroline showing a weaker effect than PHMB. Increasing the inhibitors' concentrations did not significantly improve these effects (results not shown). Since each of these inhibitors shows specificity for a different type of enzyme, the differences in their effectiveness probably reflect differences in the abundance and metabolic activity of the corresponding enzymes. 1,10-Phenanthroline is an inhibitor of metalloenzymes, which include brush border aminopeptidases and endopeptidases, and PHMB is an inhibitor of cysteine proteases, which include cytoplasmic and lysosomal proteases. These enzyme groups appear, as expected, to be chiefly responsible for leuprolide degradation in the intestinal homogenates; whether lysosomal enzymes, however, attack leuprolide by leaching into the soluble phase of the homogenates was not investigated under the present conditions. PMSF is an inhibitor of serine proteases, which include trypsin and chymotrypsin; these pancreatic enzymes seem, therefore, to be present in the homogenates, even though the luminal contents were rinsed off prior to homogenization.

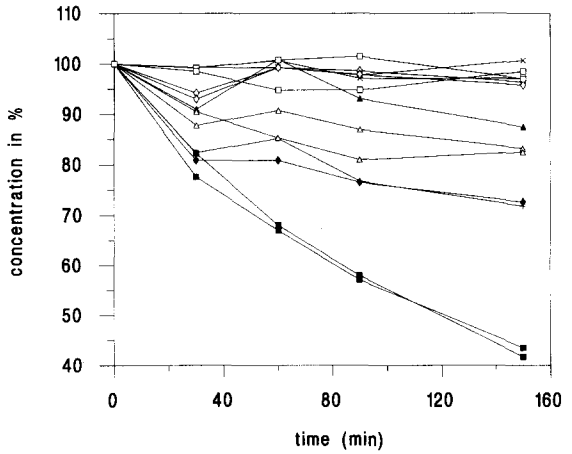

Fig. 1. Leuprolide concentration expressed in % of the starting (zero time) concentration as a function of incubation time with intestinal homogenates in the presence of single peptidase inhibitors and inhibitor combinations. (■) No inhibitor added; (♦) PMSF; (▲) PHMB; (+) 1,10-phenanthroline; (×) PMSF plus PHMB; (△) PMSF plus 1,10-phenanthroline; (◇) PHMB plus 1,10-phenanthroline; (□) PMSF plus PHMB plus 1,10-phenanthroline.

The combinations PMSF plus PHMB and PMSF plus PHMB plus 1,10-phenanthroline completely inhibited leuprolide hydrolysis and the latter was selected to be used in the permeation experiments. The concentrations of inhibitors in the permeation experiments were the same as those in the homogenates, and the amount of tissue in terms of intestinal sac length per milliliter of mucosal solution was comparable to the tissue concentration in the homogenates. Moreover, it was reasonable to assume that the inhibitors access all compartments of the intact tissue which are accessible to leuprolide, thus abolishing degradation in the intestinal sacs as effectively as in the homogenates. This methodology provided, therefore, the means to eliminate the confounding effect of metabolism in permeation experiments and to determine the true intestinal permeability of leuprolide.

3.2. Intestinal permeation

Under conditions of complete inhibition of enzymatic degradation, considerable intestinal permeation of leuprolide was measured resulting in a permeability coefficient of 1.5×10^{-5} cm/s when no Igepal CO surfactants were present (Fig. 2). This demonstrated that leuprolide can be intestinally absorbed when no metabolism takes place, this being a noteworthy result considering

the large size and the net charge of this compound. It should be noted, however, that the true permeability coefficient value may be lower than that measured here, since the peptidase inhibitors may be causing some perturbation of the cell plasma membrane; intestinal transport of intact peptide, however, seems evident. The apparent volume of adsorption, V_{ads}, corresponding to the rapid rise of tissue drug content during the early minutes ($\ll$ 10 min) of incubation was equal to 22.5 μl/sac with a standard deviation of 16.7 μl/sac ($n = 15$).

Fig. 2 shows leuprolide permeability coefficients, P, determined in the presence of members of the Igepal CO homologue series of surfactants. The effect of these surfactants on P was found to depend on their HLB value in a non-monotonic fashion. The maximal permeation enhancement was achieved at HLB $\approx$ 15, this effect decreasing and eventually disappearing for HLB values both higher and lower than 15. Although the overall enhancement is modest reaching at maximum 2-fold, its HLB dependence is clearly demonstrated. Similar patterns of permeation enhancement by homologue series of surfactants have been reported by Hirai et al. (1981) and Florence et al. (1984). No clear effect of the surfactants on V_{ads} was detected.

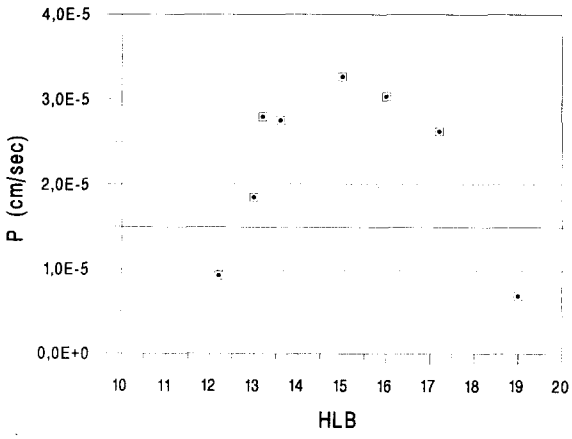

Fig. 2. Intestinal permeability coefficient of leuprolide in the presence of Igepal CO surfactants as a function of surfactant HLB, obtained under complete inhibition of enzymatic degradation. Solid and dashed lines give the numerical value ± standard deviation of the permeability coefficient in the absence of surfactants with $n = 15$.

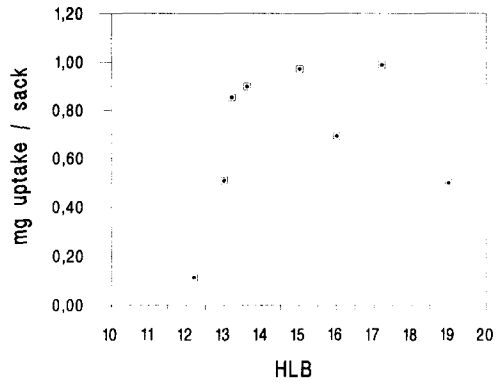

Fig. 3. Amount of Igepal CO taken up by intestinal sacs within 30 min of incubation vs surfactant HLB.

3.3. Mechanisms of permeation enhancement

In order to gain a better understanding of the interaction of the Igepal CO with the cell membrane and the role of this interaction in the non-monotonic relationship between enhanced P values and HLB (i) surfactant uptake by the sacs was quantified simultaneously with the leuprolide permeation measurements, and (ii) the hemolytic potency and (iii) lecithin solubilization properties of these surfactants were determined.

The amount of the Igepal CO taken up by sacs exhibited a non-monotonic dependence on HLB (Fig. 3). This non-monotonic relationship showed a striking resemblance to that between the P values of leuprolide and HLB (Fig. 2). This might be interpreted as meaning that a causal link exists between the amount of surfactant entering the tissue and the permeation enhancement effect exerted by the surfactant. The results on hemolysis and lecithin solubilization discussed below, however, appear to contradict such a hypothesis.

The surfactant concentration (EC_{50}) causing 50% hemolysis was found to increase, i.e., hemolytic potency decreased, as HLB increased (Fig. 4). For HLB < 15, Igepal CO were strong hemolyzers, their EC_{50} concentrations lying slightly above the corresponding CMCs. This and the fact that the hemolyzed red blood cell prepa-

rations were macroscopically and microscopically clear suggested that hemolysis was taking place by solubilization of the plasma membrane by Igepal CO micelles. For HLB > 15, Igepal CO caused no measurable hemolysis at concentrations of at least one order of magnitude higher than their CMC.

The ability of Igepal CO to solubilize cell membrane phospholipids was assessed using lecithin. Fig. 5 shows that the amount of lecithin solubilized by the surfactants decreased as surfactant hydrophilicity increased. For HLB < 15, a solubilization ratio of approx. 1:1 per weight was observed while for HLB > 15 this ratio sharply decreased. Thus, lecithin solubilization was found to correlate well with the hemolytic potency of the Igepal CO, which supports the view that hemolysis is caused by cell membrane solubilization and suggests that the relationship between membrane solubilization potency and HLB of these surfactants should be non-specific with respect to the phospholipid composition of the membrane.

In the permeation experiments with intestinal sacs the concentration of Igepal CO was 10

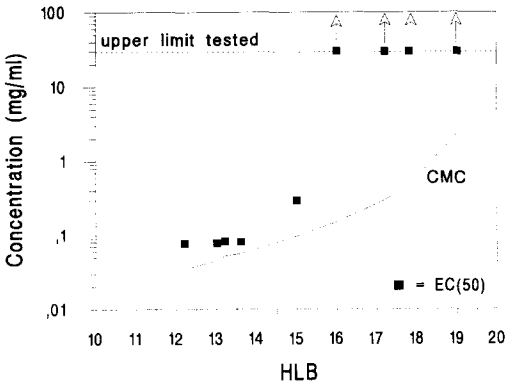

Fig. 4. EC_{50} concentration of Igepal CO surfactants required to induce 50% hemolysis vs surfactant HLB. The solid line shows the critical micellar concentration, CMC, as a function of HLB. This was calculated using the empirical equation $\ln(CMC) = -11.3 + 0.807\ln(n)$, which was obtained from surface tension data, where, n is average molar ratio of ethylene oxide to nonylphenol moieties of the surfactant molecules and CMC is expressed in molar concentration. Arrows (↑) indicate that no measurable hemolysis was observed at the highest tested concentration of 30 mg/ml for these HLB values.

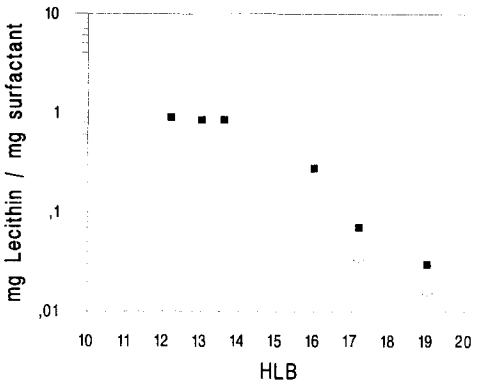

Fig. 5. Amount of lecithin solubilized by 1 mg of Igepal CO surfactants vs HLB. Arrows (↓) indicate that no clear solution was obtained at the reported data point which gives the lowest lecithin amount tested; the maximal lecithin solubilization lies below this point.

mg/ml which was well above their CMC and the estimated surface area of cell membrane exposed to the surfactant solution per unit volume of the solution was of the same order of magnitude as in the hemolysis experiments. Thus, it is inferred based on the hemolysis and the lecithin solubilization results that solubilization of the enterocyte membrane takes place during the leuprolide permeation experiments in the presence of Igepal CO with HLB < 15, while no membrane solubilization should be occurring for HLB > 15.

These results demonstrate that permeation enhancement of leuprolide does not directly correlate with membrane solubilization. While permeation enhancement shows a non-monotonic dependence on HLB, membrane solubilization potency decreases monotonically with increasing HLB. Thus, for HLB values between 12 and 13, the Igepal CO are potent membrane solubilizers, however, they do not show a permeation enhancement effect, while for HLB values between 15 and 17 they produce permeation enhancement without causing membrane solubilization. The latter case is of considerable practical significance since it indicates that, depending on the surfactant structure, permeation enhancement can be dissociated from possible local toxicity effects arising from membrane solubilization.

Permeation enhancement in the range 15 < HLB < 17 may occur by incorporation of surfac-

tant molecules into the membrane bilayer creating sites of disorder and/or increasing membrane fluidity which lead to increased permeability. Perturbation of tight junctions might also be considered as a possible effect of the surfactants in this HLB range. The intensity of these effects decreases with increasing HLB for HLB > 15 probably due to the decreasing lipophilicity and, therefore, the decreasing tendency of the surfactants to partition into the membrane. This is also reflected in the decreasing amount of surfactant taken up by the tissue in this HLB range.

In the range 12 < HLB < 15, membrane solubilization takes place leading to the formation of Igepal CO-phospholipid mixed micelles. A disk-shaped structure with a thickness comparable to that of a phospholipid bilayer has been proposed for bile salt-lecithin mixed micelles (Mazer et al., 1980). Assuming a similar mixed micellar structure for the Igepal CO-phospholipid system, it is likely that leuprolide adsorption takes place on the surface of the phospholipid domain of the mixed micelles. This adsorption would lower the free leuprolide concentration in the mucosal solution, thus reducing the driving force for permeation to an extent that might be able to counterbalance the increased tissue permeability arising from the lysed cell membrane and, therefore, account for the lack of permeation enhancement observed at HLB values between 12 and 13. This adsorption hypothesis is qualitatively consistent with the leuprolide adsorption on the tissue surface observed in the permeation experiments and expressed in terms of the apparent adsorption volume V_{ads}.

The increase in the P values of leuprolide as a function of HLB in the range 12 < HLB < 15 is accompanied by a similar increase in surfactant uptake by the tissue. The latter might also be related to the formation of membrane phospholipid-surfactant mixed micellar systems whose intermicellar monomeric surfactant concentrations increase with increasing surfactant hydrophilicity (in analogy to CMC). It should be noted, however, that, in contrast to the properties of CMC, this intermicellar surfactant concentration is not a constant at a given temperature but varies with the phospholipid/surfactant ratio in the mixed micelles. The incorporation of surfactants in the mixed micelles may possibly result in their depletion to varying degrees from the mucosal solution, this leading to a variable availability for tissue absorption. Clearly, additional research is necessary in order to assess quantitatively the contribution of these phenomena to the self-absorption and permeation enhancement effects of surfactants.

In conclusion, this work shows that, in the studied HLB range, permeation enhancement of the peptide leuprolide by Igepal CO and cell membrane solubilization are not directly correlated. Although the exact mechanisms are not fully understood, it is evident that membrane solubilization is neither required for nor does it guarantee permeation enhancement, and that other factors also play an important role, thereby providing the potential to optimize the effect/toxicity profile of surfactant enhancers.

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