Cyclodextrins as Nasal Absorption Promoters of Insulin: Mechanistic Evaluations

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Received September 10, 1991; accepted February 11, 1992

The safety and effectiveness of cyclodextrins (CD) as nasal absorption promoters of peptide-like macromolecules have been investigated. The relative effectiveness of the cyclodextrins in enhancing insulin nasal absorption was found to be in the descending order of dimethyl-β-cyclodextrin (DMβCD) > α-cyclodextrin (α-CD) > β-cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (HPβCD) > γ-cyclodextrin (γ-CD). A direct relationship linking absorption promotion to nasal membrane protein release is evident, which in turn correlates well with nasal membrane phospholipid release. The magnitude of the membrane damaging effects determined by the membrane protein or phospholipid release may provide an accurate, simple, and useful marker for predicting safety of the absorption enhancers. In order to estimate further the magnitude of damage and specificity of cyclodextrin derivatives in solubilizing nasal membrane components, the enzymatic activities of membrane-bound 5′-nucleotidase (5′-ND) and intracellular lactate dehydrogenase (LDH) in the perfusates were also measured. HPβCD at a 5% concentration was found to result in only minimal removal of epithelial membrane proteins as evidenced by a slight increase in 5′-ND and total absence of LDH activity. On the other hand, 5% DMβCD caused extensive removal of the membrane-bound 5′-ND. Moreover, intracellular LDH activity in the perfusate increased almost linearly with time. The cyclodextrins are also capable of dissociating insulin hexamers into smaller aggregates, and this dissociation depends on cyclodextrin structure and concentration. Enhancement of insulin diffusivity across nasal membrane through dissociation may provide an additional mechanism for cyclodextrin promotion of nasal insulin absorption.

KEY WORDS: cyclodextrins; circular dichroism; insulin dissociation; membrane damage; nasal absorption; 5′-nucleotidase; lactate dehydrogenase; phospholipid release; protein release.

INTRODUCTION

With the continuing development of a significant number of peptide- and protein-based therapeutic agents, noninvasive routes of macromolecular drug absorption are receiving extensive scrutiny. The nasal route especially is receiving considerable attention due to its ease of administration, patient acceptance, and rapid absorption and the avoidance of the first-pass effect (1). Although relatively small and lipophilic compounds are able to traverse the nasal mucosa to a significant extent, hydrophilic and large species (>10 kD) encounter considerable resistance in achieving necessary systemic bioavailability. For instance, insulin, a fairly large peptide (5.8 kD) with 51 amino acids, has been reported to be poorly absorbed into the systemic circulation following intranasal administration (2). In order to facilitate nasal transport, various approaches have been undertaken such as chemical modifications of the molecule, prevention of insulin aggregation, and the use of absorption enhancers (3–8).

Some of the chemical enhancers studied include anionic and cationic surfactants (8), bile salts (9), bile salt–fatty acid mixed micelles (2), fusidic acid derivatives (10–12), and medium-chain fatty acids (13). While significantly improving drug uptake, these enhancers were, in general, found to exert considerable membrane damaging effects as evidenced by protein release (14), ciliostasis (15), and histological examinations (2,16). Such membrane toxicity may preclude further investigations of these agents in clinical trials. Recently cyclodextrins were found able to improve the bioavailability of both steroidal hormones and insulin following nasal administration (17–20). This observation has prompted us to investigate further the mechanisms of absorption enhancement including any membrane damaging effects. Since cyclodextrins are biocompatible polymers composed of naturally occurring D-glucose (dextrose) units, therapeutic application of these compounds has been regarded as relatively safe. Although the underlying mechanisms involving nasal permeation enhancement of insulin by cyclodextrins are not well understood, it may be attributable to any one or more of the following factors: (i) direct membrane disruption effect as evidenced by the extraction of phospholipids and proteins; (ii) complexation of lipophilic penetrants, thereby increasing their aqueous solubility; (iii) inhibition of proteolytic enzyme activity; and (iv) dissociation of insulin oligomers. The objective of this study, therefore, was to delineate some of these possible mechanisms in order to understand better the effectiveness and safety of cyclodextrins as nasal absorption enhancers.

MATERIALS AND METHODS

Chemicals

α-Cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), dimethyl-β-cyclodextrin (DMβCD), trimethyl-β-cyclodextrin (TMβCD), lactate dehydrogenase, and 5′-nucleotidase kits were obtained from Sigma Chemical Co. (St. Louis, MO), whereas hydroxypropyl-β-cyclodextrin (HPβCD) was kindly donated by Pharmatec, Inc. (Alachua, FL). Porcine zinc insulin powder (26.3 IU/mg) was a gift from Eli Lilly and Company (Indianapolis, IN). All other reagents were of analytical grade and used as received.

In Situ Nasal Perfusion Method

The perfusion medium was prepared by dissolving various cyclodextrins in 0.9% saline. Solutions of 1.8 and 5% DMβCD were prepared in order to investigate the effect of CD concentrations on nasal protein and phospholipid release, whereas β-CD was studied at only one concentration of 1.8% due to its limited intrinsic solubility (1.85%) (21). All

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0724-8741/92/0900-1157$06.50/0

other CDs were studied at a 5% level. The concentrations of cyclodextrins are expressed on a percentage (w/v) basis.

The rat *in situ* perfusion technique developed by Hirai (22) and Huang *et al.* (23) was used in this investigation due to the relatively simple nature of the experimental setup and good reproducibility of the method. It allows for multiple sampling of the nasal perfusate, enabling the estimation of time-dependent membrane protein and phospholipid release. Male Sprague–Dawley rats weighing 250 to 350 g were fasted for about 14 to 18 hr prior to use but water was allowed ad libitum. The rats were anesthetized by an intraperitoneal injection of 0.1 ml/100 g body wt of a ketamine (90 mg/ml) and xylazine (10 mg/ml) mixture, followed by an additional 0.1 ml/rat every 30 to 45 min to maintain the anesthetic state. After an incision was made in the neck, the trachea was cannulated with a polyethylene tube (PE-200, Intramedic, Clay Adams, NY) to maintain respiration. Another PE-200 tube was inserted through the esophagus toward the posterior part of the nasal cavity and ligated. The passage of the nasopalatine tract was sealed with an adhesive agent (Instant Jet, Cal Goldberg Models Inc., Chicago, IL) to prevent drainage of the solution from the nasal cavity to the mouth. The cannula served to deliver the solution to the nasal cavity. The perfusion medium was circulated by means of a peristaltic pump and recollected into a reservoir. The temperature of the reservoir was maintained at 37 ± 0.5°C during the course of an experiment. A constant perfusate volume of 5 ml was maintained throughout, with constant stirring. An aliquot (100 μl) was sampled every 15 min for 1.5 hr.

Assay Procedures

Protein Content. The protein contents in the perfusate at various sampling points were measured by the method of Lowry *et al.* (24). Bovine serum albumin (BSA) was used as the standard. A standard curve was constructed in conjunction with the samples in order to minimize errors. Absorbance at 750 nm was measured with a Beckman DU-7 UV/VIS spectrophotometer (Irvine, CA). The presence of various cyclodextrins did not interfere with the assay.

Total Phosphorus. The assay procedure was based on the method described by Bartlett (25) and modified by Feldman *et al.* (26). One hundred microliters of the nasal perfusate was placed in 12-ml conical centrifuge tubes and 0.9 ml of distilled water was added. After the addition of 0.5 ml of 10 *N* sulfuric acid the tubes were kept in an oven set at 150–160°C for a 3-hr digestion. Three drops of 30% hydrogen peroxide were added to each tube thereafter and the tubes were again heated for 3 hr. After the samples were cooled to room temperature, 4.4 ml of distilled water, 0.2 ml of 5% ammonium molybdate, and 0.2 ml of Fiske-SubbaRow reagent (Sigma Chemical Co.) were added and the contents were vortexed for 20 sec. The samples were then heated in boiling water bath for an additional 10 min. The developed color was subsequently read at 830 nm. A standard curve was prepared using potassium phosphate monobasic as the standard and linearity was obtained in the range of 0 to 4 μg of phosphorus content. The presence of various cyclodextrins did not interfere with the assay.

Phospholipid Phosphorus. Phospholipid contents of the samples were measured according to Zilversmit and Davis (27). Proteins and phospholipids were first precipitated with 3 ml of 10% trichloroacetic acid (TCA), which was added in a dropwise manner. The precipitate was then centrifuged at 2000 rpm for 5 min. The supernate was decanted and the pellets were washed with 3 ml of 10% TCA and centrifuged at 2000 rpm for 5 min. Subsequently, 1 ml of distilled water and 0.5 ml of 10 *N* sulfuric acid were added to each tube and the entire phosphorus assay procedure described previously was repeated.

Enzyme Activity Assays. The activity of a nasal membrane-bound marker enzyme, 5′-nucleotidase (5′-ND; EC 3.1.3.5), in the perfusate was analyzed according to a kinetic method reported by Arkesteijn (28). The activity of a nasal epithelial intracellular enzyme, lactate dehydrogenase (LDH; EC 1.1.1.27), was determined by the method of Cabaud and Wroblewski (29).

Circular Dichroism Studies

A CD spectropolarimeter (JASCO Model J600, Japan Spectroscopic Co., Tokyo) was utilized to illustrate the gradual changes associated with insulin dissociation, i.e., change of hexameric form to dimeric and monomeric forms in the presence of cyclodextrins. Zinc insulin solutions containing 0.5 mg/ml zinc insulin and various concentrations of cyclodextrins were scanned from 300 to 250 and from 250 to 200 nm at a scanning speed of 10 nm/min. The temperature was maintained at 37 ± 0.5°C. A 10- and a 1-mm-pathlength quartz curvet (American Scientific Products, McGaw Park, IL) was used, respectively, for the higher and lower wavelength regions to obtain optimum resolution of the spectra. The generated ellipticity values were subsequently converted to molar ellipticities for the entire wavelength range with the help of a computer using the equation $[\theta]_\lambda = \theta_\lambda/(C \cdot l)$, where θ_λ is the observed ellipticity at wavelength λ, C is the decimolar insulin concentration, and l is the pathlength in decimeters.

RESULTS

Effects of Various Cyclodextrins on Rat Nasal Protein and Phosphorus Release

In order to compare the membrane damaging effects, 5% solutions (except for β-CD, which was used at 1.8%) of various cyclodextrins in saline, i.e., α-CD, β-CD, γ-CD, DMβCD, and HPβCD, were perfused through the rat nasal cavity for 90 min. Figure 1 graphically depicts the time course of nasal protein release. The released protein concentrations increased with time during the course of the experiment. Except for DMβCD, which resulted in an initial rapid phase (burst) of protein release at 15 min, followed by a linear phase, all other cyclodextrins extracted nasal proteins in a linear manner over the entire time period. Since dextrose (5%) generated a protein release profile very similar to that of γ-CD, it was not included in Fig. 1 in order to avoid overlapping.

Among the cyclodextrins studied, DMβCD exhibited a uniquely high activity in removing proteins from the rat nasal mucosa. At 90 min, an average protein concentration of 11.41 ± 2.80 mg/ml was observed with DMβCD, a number

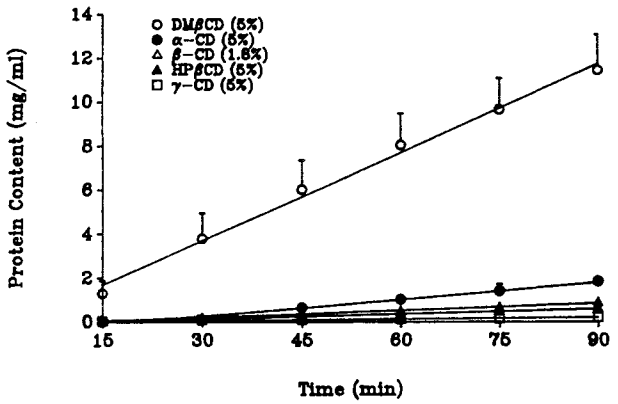

Fig. 1. Nasal protein release profiles in the rat. The perfusate contained various cyclodextrins in saline solution. Values represent means ± SD (n = 3).

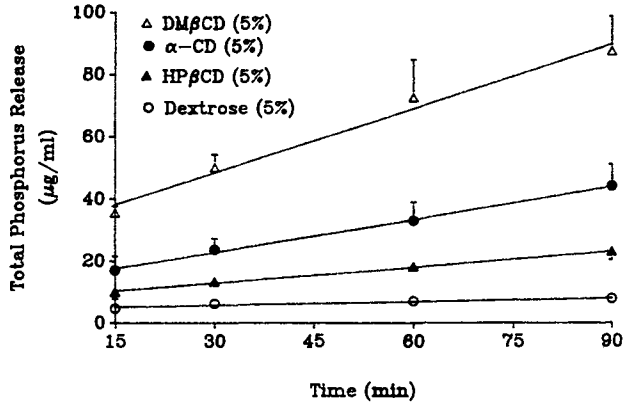

Fig. 2. Release profiles of total phosphorus in the rat nasal perfusates containing 5% dimethyl-β-cyclodextrin, 5% α-cyclodextrin, 5% hydroxypropyl-β-cyclodextrin, and 5% dextrose. Values represent means ± SD (n = 3).

much higher than all other CDs investigated. The order of protein release rates follows, in a descending manner: DMβCD > α-CD > β-CD > HPβCD > γ-CD > dextrose, as listed in Table I.

The release profiles of total phosphorus in the presence of 5% DMβCD, 5% α-CD, and 5% HPβCD are shown in Fig. 2, along with that of 5% dextrose, serving as the control. The total phosphorus accumulation in the perfusate increased linearly with perfusion time, a profile similar to that of protein release. For comparative purposes the release rates of total phosphorus, total phosphorus released, and lipid phosphorus extracted by various cyclodextrins at 90 min are listed in Table I. In agreement with the protein release data, DMβCD also induced very high phosphorus release from the nasal mucosa. Further, the rate of total phosphorus release follows the same rank-order correlation as that of total protein release.

Dependence of Cyclodextrin Concentration and Degree of Methylation

Since 5% DMβCD caused significant removal of membrane components, it was important to determine whether this membrane solubilization property of cyclodextrin was concentration dependent. Therefore a lower concentration of DMβCD (1.8%) was subsequently perfused through the rat nasal cavity. The rates of protein release as well as the cumulative total and lipid phosphorus contents at 90 min were measured and are listed in Table II. A considerably lower rate and extent of release were observed at this concentration compared to 5% DMβCD. Thus a distinctive increasing trend in membrane component release could be identified with increasing levels of DMβCD.

In order to gain a better understanding of the relationship between the degree of methylation and membrane component release properties, 1.8% trimethyl-β-cyclodextrin (TMβCD) was also evaluated and the results are listed in Table II together with those for β-CD and DMβCD for easy comparison. Interestingly, in contrary to its high surface activity, TMβCD exhibited very minimal membrane damage similar to β-CD as evidenced by both protein and phosphorus release.

Nasal Membrane-Bound and Intracellular Enzyme Activities in the Perfusates

In order to understand further the underlying mechanisms by which various cyclodextrins are able to extract nasal proteins, the activities of two marker enzymes in the perfusates were measured. The membrane-bound 5′-nucleotidase (5′-ND) serves as an indicator for the exten-

Table I. Protein Release Rates and Total Phosphorus and Lipid Phosphorus Release in Rat Nasal Perfusate in the Presence of Various Cyclodextrins[a]

Solution	Concentration (%, w/v)	Rate of protein release (μg/ml/min)[b]	Rate of total phosphorus release (μg/ml/min)[b]	Total phosphorus (μg/ml)[c]	Lipid phosphorus (μg/ml)[c]
DMβCD	5	132.40 ± 23.38	0.734 ± 0.217	87.22 ± 11.36	16.53 ± 1.90
α-CD	5	25.70 ± 6.87	0.354 ± 0.038	43.91 ± 7.02	1.94 ± 0.40
β-CD	1.8	10.67 ± 3.80	—	23.71 ± 5.21	1.34 ± 0.11
HPβCD	5	7.46 ± 1.87	0.171 ± 0.010	12.47 ± 0.58	0.34 ± 0.05
γ-CD	5	2.55 ± 0.05	—	11.37 ± 0.63	0.24 ± 0.08
Dextrose	5	1.09 ± 0.82	0.038 ± 0.019	7.44 ± 1.04	0.07 ± 0.01

[a] Values are means ± SD (n = 3).
[b] Linear regression performed from 15 to 90 min.
[c] Concentrations at 90 min.

Table II. Effect of the Degree of Methylation of β-CD on Nasal Protein and Phospholipid Release

Compound	Conc. (%, w/v)	Degree of methylation (%)	Surface tension (mN/m)[a]	Protein release rate (μg/ml/min)[b]	Total phosphorus (μg/ml)[c]	Lipid phosphorus (μg/ml)[c]
β-CD	1.8	0	71	10.67 ± 3.80	23.71 ± 5.21	1.34 ± 0.11
DMβCD	1.8	30	62	35.27 ± 16.90	57.97 ± 2.97	6.22 ± 1.35
TMβCD	1.8	>90	56	11.76 ± 4.27	25.38 ± 2.72	1.36 ± 0.19

[a] From Ref. 32.
[b] Linear regression performed from 15 to 90 min.
[c] Concentrations at 90 min.

siveness of nasal membrane damage, while the activity of the intracellular enzyme, lactate dehydrogenase (LDH), provides additional evidence for the leaking effect of cytosolic constituents, which might be caused by extensive membrane protein and phospholipid removal and perhaps even cell lysis.

Figure 3 illustrates the activities of 5′-ND in the nasal perfusates in the presence of DMβCD and HPβCD. Five percent HPβCD wash of the rat nasal cavity resulted in a gradual yet minimal increase in 5′-ND activity, reaching only 11.1 ± 3.5 U/L (means ± SD; n = 3) at 90 min. In contrary, 5% DMβCD caused considerably more 5′-ND release from the nasal mucosa such that the activity of 5′-ND was found to be significant even at the end of 15-min perfusion. At 90 min, an average 5′-ND activity of 190.5 ± 45.2 U/L was detected.

Figure 4 demonstrates the release of an intracellular enzyme, LDH, as a function of perfusion time. The incorporation of 5% HPβCD in the medium did not elicit any measurable LDH release even at 90 min. DMβCD, on the contrary, at the same perfusate concentration caused a drastic release of this marker enzyme. Furthermore, the accumulation of LDH in the perfusate appears to increase linearly with time, reaching 6.12 ± 1.47 KU/ml at 90 min.

Circular Dichroism Studies

The circular dichroism spectra of porcine zinc insulin (0.5 mg/ml) in the absence and presence of various concentrations of DMβCD are depicted in Figs. 5A and B. Figure 5A represents the CD spectra in the wavelength range from 300 to 250 nm, while Fig. 5B was obtained within the far-UV wavelengths of 250 to 200 nm. The negative maximum at 274 nm is assigned to the contribution of the B23–28 aromatic residues of insulin in the form of the antiparallel β structure (30). The main Cotton effects at 221 and 211 nm were assigned in large part to β structure in the form of dimers and the α-helical structure in the form of monomers, respectively. Therefore, the attenuation of the maxima at 274 and 221 nm indicates the dissociation of insulin hexamers and dimers, respectively. As shown in Figs. 5A and B, the presence of DMβCD considerably attenuated the absorptive intensities at the above maxima. Similarly, the effects of α-CD, β-CD, γ-CD, and HPβCD on insulin hexamer dissociation were also monitored using the circular dichroism method. The molar ellipticities at 274 and 221 nm are summarized in Table III. The relative effects of various cyclodextrins in causing insulin hexamer dissociation were found to follow the descending order of DMβCD (15%) > DMβCD (10%) > DMβCD (5%) > DMβCD (1.8%) > α-CD (5%) > HPβCD (5%) > γ-CD (5%) > β-CD (1.8%).

DISCUSSION

The differential effects of unsubstituted cyclodextrins (α-, β-, and γ-CD) on erythrocyte membrane component release were reported previously (31), in which proteins, phospholipids, and cholesterol removal were found to be significant at high cyclodextrin concentrations. While β-CD was primarily responsible for protein and cholesterol removal from biomembranes, α-CD solubilized phospholipids selec-

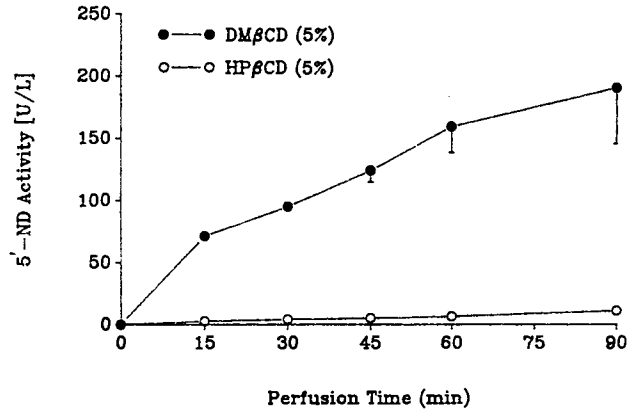

Fig. 3. Release profiles of 5′-nucleotidase from the rat nasal cavity as a function of perfusion time. Data are means ± SD (n = 3).

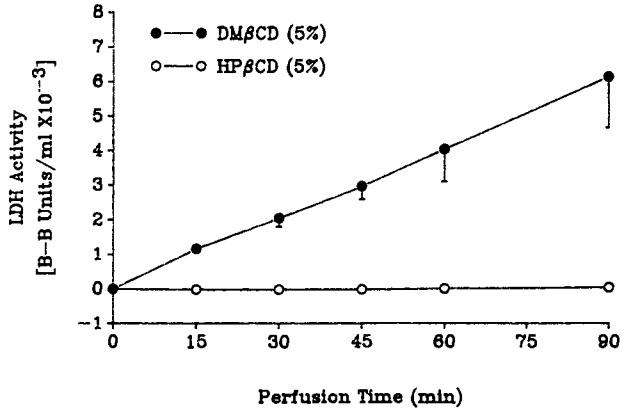

Fig. 4. Activity–time profiles of lactate dehydrogenase in the rat nasal perfusates containing 5% dimethyl-β-cyclodextrin and 5% hydroxypropyl-β-cyclodextrin. Data are means ± SD (n = 3).

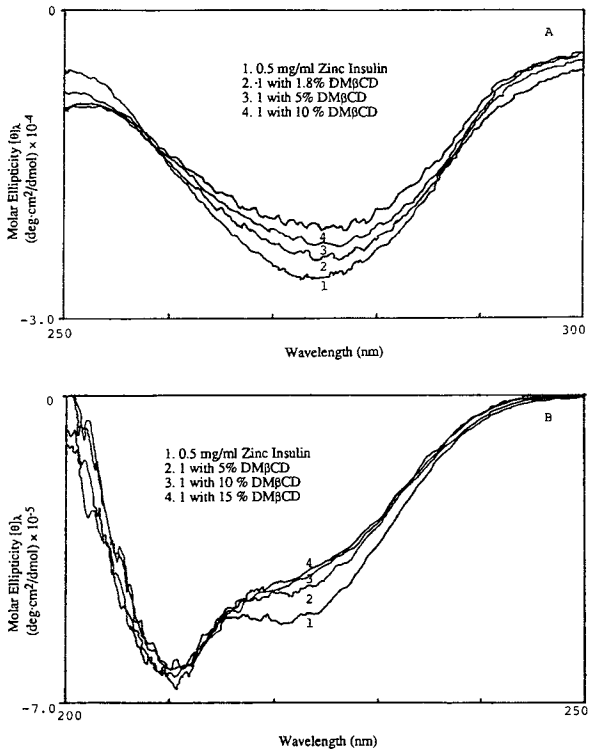

Fig. 5. The circular dichroic spectra of 0.5 mg/ml porcine zinc insulin in the absence and presence of DMβCD. (A) Wavelength range, from 300 to 250 nm; (B) wavelength range, from 250 to 200 nm.

tively. However, results from our protein and phospholipid release experiments revealed similar profiles in both nasal protein and phospholipid extraction. Although cyclodextrins, surfactants, and bile salts possess common properties in inducing membrane component release, the mechanisms of such effect, however, are believed to be different. Surfactants, being amphiphiles, are able to penetrate the lipid structure of the membrane and thereby can disrupt its integrity. Cyclodextrins, on the other hand, form a new lipid-inclusion compartment in the aqueous phase into which membrane phospholipids and cholesterol are extracted. This postulated mechanism was experimentally supported by the transfer of [^{3}H]cholesterol between the erythrocytes and the cyclodextrin complexes (31). According to this mechanism, the extraction of lipids is of primary importance, while the release of proteins from biomembrane is merely the consequence of membrane erosion. In other words, proteins are believed to be shed from the membrane into the aqueous phase after the removal of surrounding phospholipids.

Consistent with this hypothesis, the release of membrane phospholipids in our studies correlated well with the protein release pattern as evidenced by the data listed in Table I. Selective methylation of β-CD at the C2 and C6 primary hydroxyls resulted in abrupt changes in the physicochemical properties of cyclodextrins. DMβCD was found to be highly water soluble and highly surface active (32). Such hydrophilic properties enable DMβCD to possess exceptionally good solubilizing properties and have since been widely used in pharmaceutical preparations. Meanwhile, it was also found to be extremely hemolytic, even at concentrations as low as 1 mg/ml (21). In agreement with the hemolytic effect, nasal membrane component release caused by DMβCD surpassed all other cyclodextrins at the same concentration. Furthermore, surface activity does not appear to be the major cause of such a difference since TMβCD caused much less nasal membrane component release although it was more surface active (32). Therefore, permethylation of the C3 hydroxyl group may inhibit the incorporation of phospholipids into the torus of cyclodextrins.

Although the total protein content in the perfusate serves as an effective indicator of relative toxicity to the nasal membrane, no specific information could be generated concerning the mechanisms of such an effect. The nasal protein release could be the combined result of stimulated mucus secretion, removal of the outer layer of the nasal membrane, or even severe cell lysis. Therefore, we further clarified this issue by measuring two marker enzyme concentrations in the perfusate as a function of time. As evidenced by Figs. 3 and 4, considerably different behavior of 5′-ND and LDH release were produced by HPβCD and DMβCD at the same initial concentration, indicating variable affinities for cyclodextrin derivatives to interact with the nasal epithelia. Incorporation of 5% HPβCD resulted in only marginal removal of 5′-ND from the epithelia, while the intracellular protein leakage represented by LDH was not noticeable. Protein, phospholipid, and enzyme release patterns all suggest that HPβCD is a safe nasal permeation enhancer. Indeed, detailed toxicological studies also showed that HPβCD is well tolerated as a parenteral carrier even at extremely high doses (33). On the other hand, DMβCD at a concentration of 5% caused considerably higher 5′-ND and LDH release. Cumulative activity of 5′-ND indicates gradual removal of the epithelial proteins, to create pores for the cytosolic substituents to leak out. In conclusion, the protein, phospholipid, and enzyme assays together indicate the extremely high membrane solubilizing effect of DMβCD at this concentration.

As a measure of relative tissue irritation, Yoshida *et al.* (21) recently investigated the damaging effects of various cyclodextrins to rabbit muscle tissue. The magnitude of irritation caused by cyclodextrins was found to be in the order of DMβCD > α-CD > HPβCD, β-CD > γ-CD. Therefore, our nasal component release data concur well with the tissue

Table III. Dependence of Insulin Molar Ellipticities at 274 and 221 nm on Cyclodextrin Concentrations[a]

Cyclodextrin conc. (%, w/v)	$[\theta]_\lambda$ at 274 nm, (deg · cm²/dmol) × 10^{-4}	$[\theta]_\lambda$ at 221 nm, (deg · cm²/dmol) × 10^{-5}
0	−2.603	−5.250
α-CD, 5	−2.474	−4.775
β-CD, 1.8	−2.588	−5.220
γ-CD, 5	−2.583	−5.060
HPβCD, 5	−2.481	−4.781
DMβCD, 1.8	−2.471	—
DMβCD, 5	−2.288	−4.627
DMβCD, 10	−2.097	−4.419
DMβCD, 15	—	−4.283

[a] The concentration of zinc insulin was 0.5 mg/ml.

irritation assessment, thus providing an easy and accurate index in predicting the safety for nasal absorption enhancers.

Recently cyclodextrins have been investigated as possible nasal transport promoters for both steroidal hormones and insulin. Hermens *et al.* (17) reported that the bioavailability of 17β-estradiol (E_2) was significantly improved when an E_2–DMβCD formulation was administered intranasally to rabbits and rats. A three- to fourfold increase in bioavailability over that of the E_2 suspension was observed. A significant promoting effect of DMβCD was also noticed by Merkus *et al.* (20). Coadministration of 5% DMβCD resulted in nearly complete nasal insulin absorption in rats, thus making DMβCD one of the most effective nasal absorption enhancers ever found. Nevertheless, DMβCD was also found to cause a severe ciliostatic effect. When 5% DMβCD was applied to the ciliated tissue of chicken embryo trachea, complete ciliostasis was observed within 30 to 40 min. However, this effect appears to be reversible when pure Locke–Ringer solution was used to replace the 5% DMβCD after a 20-min exposure. The relative effectiveness of other cyclodextrins in causing ciliostasis is DMβCD > α-CD > β-CD, γ-CD, and HPβCD. It is very important to note that this sequence bears a close similarity to that of the nasal membrane component release, thus further confirming the reliability of both methods.

Based on all the available experimental observations, proper selection of a cyclodextrin compound plays a key role with regard to absorption enhancement while producing minimal toxic effects. In cases where these two objectives conflict with each other, proper balancing should be carefully considered.

Although direct membrane effect appears to be one of the major modes in promoting nasal drug absorption, other mechanisms have also been postulated (20). Since zinc insulin exists mainly in the form of hexamers in aqueous solution, this sixfold increase in molecular weight over that of the monomeric form renders it poorly permeable across biomembranes (34). Therefore, breakdown of insulin aggregation was believed to be another possible pathway toward enhanced nasal and intestinal insulin absorption (9). We have substantiated this hypothesis, for the first time, by using circular dichroism spectra. Various cyclodextrins are indeed able to dissociate insulin hexamers (Figs. 5A and B and Table III). The dissociation appears to be cyclodextrin structure and concentration dependent and is effective at as low as 1.8% DMβCD.

Interestingly the relative effectiveness of cyclodextrins in dissociating insulin hexamers follows the order of DMβCD > α-CD > HPβCD > γ-CD, all at a 5% (w/v) concentration. Although the underlying mechanism responsible for insulin dissociation is still unclear, partial complexation of the aromatic side chains of insulin molecules and subsequent substitution of the Phe–Tyr hydrogen bonding appear to be the driving force. It is possible that the more effective a particular cyclodextrin is in causing insulin dissociation, the more toxic it will be toward biological membranes. This hypothesis, however, remains to be proven.

In conclusion, depending on the chemical structure and concentration, cyclodextrins exert a direct effect on the nasal mucosa as evidenced by both protein and phospholipid release data and enzyme assays, a phenomenon which may play an important role in promoting nasal drug absorption. Nevertheless, the extent of such an effect also deserves further consideration for *in vivo* situations. A small amount of cyclodextrins applied to the nasal mucosa, dilution by the mucus, and constant nasal mucociliary clearance may substantially alleviate the risk toward nasal membrane damage. Indeed, nasal formulations of estradiol containing 2% DMβCD, administered to oophorectomized women twice daily over a period of 3 to 6 months, were well tolerated (19).

Our circular dichroism study, on the other hand, also confirmed a second contributing mechanism responsible for enhanced *in vivo* insulin bioavailability, i.e., through dissociation of insulin hexamers.

ACKNOWLEDGMENTS

This investigation was supported in part by a Merck Faculty Development Award (A.K.M.) and in part by a Young Investigator Award from the American Association of Pharmaceutical Scientists (A.K.M.). Z.S. is a David Ross Research Fellow.

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