Cyclodextrins as Mucosal Absorption Promoters of Insulin. II. Effects of β-Cyclodextrin Derivatives on α-Chymotryptic Degradation and Enteral Absorption of Insulin in Rats

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The relative effectiveness of two β-cyclodextrin derivatives, i.e., dimethyl-β-cyclodextrin (DMβCD) and hydroxypropyl-β-cyclodextrin (HPβCD), in enhancing enteral absorption of insulin was evaluated in the lower jejunal/upper ileal segments of the rat by means of an *in situ* closed loop method. The incorporation of 10% (w/v) DMβCD to a 0.5 mg/ml porcine-zinc insulin solution dramatically increased insulin bioavailability from a negligible value (~0.06%) to 5.63%, when administered enterally at a dose of 20 U/kg. However, addition of 10% (w/v) HPβCD did not improve enteral insulin uptake significantly with a bioavailability of only 0.07%. Similarly, the pharmacodynamic relative efficacy values obtained after the enteral administration of 20 U/kg insulin, 20 U/kg insulin with 10% HPβCD, and 20 U/kg insulin with 10% DMβCD were 0.24%, 0.26%, and 1.75%, respectively. Biodegradation studies of 0.5 mg/ml insulin hexamers by 0.5 μM α-chymotrypsin revealed no inhibitory effect on the enzymatic activity by the two cyclodextrins. On the contrary, the apparent first-order rate constant increased significantly in the presence of 10% DMβCD, suggesting insulin oligomer dissociation by DMβCD. Histopathological examination of the rat intestine was performed to detect tissue damage following enteral administration of the β-cyclodextrin derivatives. Light microscopic inspection indicated no observable tissue damage, thereby arguing direct membrane fluidization as the primary mechanism for enhanced insulin uptake. This study indicates the feasibility of using cyclodextrins as mucosal absorption promoters of proteins and peptide drugs.

KEY WORDS: biodegradation; stability; α-chymotrypsin; cyclodextrins; enteral absorption; histology; insulin.

INTRODUCTION

For noninvasive delivery of insulin, a polypeptide with a molecular weight of 5.7 kD, the oral pathway remains attractive because of direct input of mesenterically absorbed dose to the liver via the portal vein, similar to normal physiologic release and uptake of insulin from the pancreas. However, insulin bioavailability is limited by oligomer formation above 0.1 μM concentration in aqueous solution (1); degradation by luminal and cellular peptidases (2,3); and hydrophilicity restricting partitioning across biological membrane barriers (4,5).

In order to improve enteral insulin bioavailability, protease inhibitors, targeted enteral delivery, and facilitated transport by absorption enhancers have been tested (6,7). The application of cyclodextrin derivatives as mucosal drug absorption promoters has been recognized recently (8–13). Physicochemical properties of cyclodextrins enhancing the formulation of macromolecules include solubilization by encapsulation of hydrophobic amino acid side chains to minimize aggregation and polymerization and low permeability through the membrane bilayer thus affording high tissue compatibility.

The relative effectiveness of various cyclodextrins and their derivatives in promoting insulin absorption across the nasal, pulmonary, and rectal mucosa has been studied (8–13). This article characterizes the mechanism and efficacy of cyclodextrins as mucosal insulin absorption enhancers. Two β-cyclodextrin derivatives, i.e., dimethyl-β-cyclodextrin (DMβCD) and hydroxypropyl-β-cyclodextrin (HPβCD), were examined with respect to their effects on insulin degradation by α-chymotrypsin, enteral absorption, and membrane effects. Probable mechanisms of insulin absorption enhancement are further elucidated.

MATERIALS AND METHODS

Materials

Crystalline porcine-zinc insulin (lot #504JR8, potency 26.3 U/mg) was donated by Eli Lilly and Company (Indianapolis, IN). Lyophilized α-chymotrypsin prepared from bovine pancreas (56 units/mg protein) was purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile (HPLC grade) was obtained from Baxter Health care Corporation (Muskegon, MI). Phosphoric acid and triethylamine were obtained from Fisher Scientific (Fairlawn, NJ). Trifluoroacetic acid (TFA), tris(hydroxymethyl)-aminomethane (Tris), and dimethyl-β-cyclodextrin (DMβCD) were procured from Sigma Chemical Co. Hydroxypropyl-β-cyclodextrin (HPβCD) was donated by Pharmatec, Inc. (Alachua, FL). Deionized double-distilled water was used throughout the study. All other chemicals were of analytical reagent grade and were used as received.

HPLC Analysis of Insulin

Insulin analysis was performed on a computer controlled gradient high-pressure liquid chromatographic (HPLC) system (Rainin Instruments, Woburn, MA) equipped with a variable-wavelength ultraviolet/visible detector (Knauer, Germany). The gradient system used in this study consisted of mobile phase A, triethylammonium phosphate (TEAP) solution prepared by adjusting the pH of 0.25 N phosphoric acid to 2.25 with triethylamine, and mobile phase B, 100% acetonitrile. The gradient system was programmed by increasing the proportion of mobile phase B from 26% to 35% over 16 min. Twenty microliters of the sample was injected onto a Rainin reversed-phase C8 Microsorb column (250 × 4.6 mm) connected to a C8 precolumn. The gradient mobile phase was run at a flow rate of 1 ml/min. The ultraviolet/visible detector was set at 220 nm;

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the recorded signal was analyzed with an electronic integrator (model 3390 A, Hewlett-Packard Co., Avondale, PA). The chromatographic method as described previously (14) provides baseline separation of insulin from its enzymic degradation products.

α-Chymotryptic Degradation of Porcine-Zinc Insulin

Ten milliliters of 0.5 mg/ml porcine-zinc insulin solution was prepared in a buffer composed of 100 mM Tris and 1 mM $CaCl_2$ adjusted to pH 8.0 at which the enzyme assumes high catalytic activity. The solution was pre-equilibrated at 37°C for 15 min. Just prior to the addition of the enzyme, the solution was vortexed for 2 seconds and a 100 μl sample was immediately taken as the zero time sample. Then 50 μl of enzyme stock solution was added to the insulin solution to generate a final enzyme concentration of 0.5 μM. Aliquots (100 μl) were withdrawn at 1, 2, 5, 10, 15, and 20 min and immediately added to 0.9 ml of 0.2% TFA solution to arrest the reaction. The samples were subsequently stored in a freezer at −20°C until HPLC analyses were performed. Studies were performed in triplicate. This procedure was used throughout the enzymatic degradation study. The cyclodextrin derivative was added to the Tris buffer solution and sonicated for 5 minutes at room temperature prior to the addition of enzyme.

Preparation of Insulin Solutions for Enteral Administration

Crystalline porcine-zinc insulin was dissolved in a few drops of 0.1 N HCl in order to facilitate its solubilization. Diluted phosphate buffered saline, 0.01 M, at pH 7.4 was then added to generate a final insulin concentration of 0.5 mg/ml. The solution was made just prior to use and the cyclodextrin (10%, w/v) was then added. The pH of the final solution was again measured and adjusted to pH 7.4 if necessary.

Enteral Absorption with Closed Loop Technique

Male Sprague-Dawley rats weighing 175–250 g were fasted for 16–20 hours prior to an experiment. Water was allowed *ad libitum*. The animals were anesthetized by an intraperitoneal injection of a mixture of 90 mg/kg ketamine and 10 mg/kg xylazine. One-third to one-half of the original dose was administered every 45–60 minutes thereafter to maintain anesthesia/analgesia. The core body temperature was maintained close to 37°C by placing the animal on a platform above a 40°C water bath with a 100-watt light bulb and a reflector above.

Cannulation of the right external jugular vein was performed by inserting a 3-inch piece of Silastic® tubing, 0.047 inch O.D. (Dow Corning, Midland, MI). A collar made from a 1-cm piece of PE 200 polyethylene tubing (Becton Dickinson, Parsippany, NJ) was attached to the outer end of the Silastic® tubing. Before insertion, the cannula was filled with saline containing 2 U/ml heparin. Microdissecting scissors were used to cut a small opening in the jugular vein, and one tip of a micro-dissecting forceps, extra delicate, was inserted through the hole to guide the cannula towards the heart. Surgical thread underneath the vein was tied around the collar of the cannula to secure it. A 23-gauge needle with the bevel removed was inserted into the cannula and was attached to a heparinized 1-ml plastic syringe for the removal of blood samples. The sampling times were 0, 15, 30, 60, 90, 120, 150, 180, 210, and 240 minutes following insulin administration.

A mid-abdominal incision was made to expose the small intestine. The distal jejunum/proximal ileum segment with a length of 15 cm, beginning 16 cm above the cecum, was used in this study because a previous report from this laboratory has shown this segment to have higher insulin permeability (5). The segment was washed by perfusing pre-warmed (37°C) normal saline through the lumen via a peristaltic pump (Model 1203, Harvard Apparatus, Millis, MA) to remove any residual gut contents. A total of 30 ml saline was circulated at a rate of 3 ml/min. The segment was then carefully ligated both above and below the incisions to prevent any fluid loss. The distal end of the segment was ligated and appropriate insulin solution (approximately 0.3 ml) was instilled to generate a final insulin dose of 20 U/kg. The concentration of insulin solutions employed was 0.5 mg/ml, or 13.15 U/ml. Finally, the proximal end of the intestinal segment was quickly ligated to form a closed sac which was carefully returned back to its original position inside the peritoneal cavity.

Measurement of Blood Glucose and Plasma Insulin

Blood samples were immediately examined for glucose levels using Chemstrip bG® reagent test strips (Boehringer Mannheim Diagnostics, Indianapolis, IN) with an AccuChek II® blood glucose monitor (Boehringer Mannheim Diagnostics). The sample size consisted approximately of 30 μl whole blood. The precision of the assay was found to be within ±3% and measurable glucose levels ranged within 10 to 500 mg/dl.

The collected whole blood was transferred into a heparinized Natelson® capillary tube (Scientific Products, McGaw Park, IL). It was then centrifuged in a Damon/IEC® CRU-5000 centrifuge for 15 min at 2,500 rpm. The plasma was collected and insulin concentration assayed by a radioimmunoassay procedure using Coat-A-Count® kits purchased from Diagnostic Products Corporation (Los Angeles, CA).

Pharmacokinetics and Pharmacodynamics

Plasma insulin concentrations were normalized by subtracting the endogenous insulin level at time zero. The areas under the plasma insulin concentration curves (AUCs), areas under the first moment curves (AUMCs), and mean absorption times (MATs) were then calculated using Rstrip® software ver. 4.02 (MicroMath Scientific Software, Salt Lake City, Utah) by extrapolating time to infinity. Intravenous injection of 0.2 U/kg insulin was performed through the rat tail vein and blood samples were collected at 0, 0.5, 2, 4, 6, 10, 15, 20, and 30 min post administration. The absolute bioavailability (F) following enteral administration of insulin in the absence and presence of cyclodextrins was estimated using the following equation:

$$F = \frac{AUC_{0\text{-}\infty\ \text{enternal}}}{AUC_{0\text{-}\infty\ \text{i.v.}}} \times \frac{Dose_{\text{i.v.}}}{Dose_{\text{enteral}}} \quad (1)$$

Table I. Pharmacokinetic Parameters of Insulin Administered Intravenously and Enterally[a]

Route of administration	Formulation	Insulin dose (U/kg)	$AUC_{0-\infty}$ (μU/ml · hr)	C_{max} (μU/ml)	t_{max} (hr)	F (%)	AUMC (μU/ml · hr^2)	MAT (hr)	n
Intravenous	Insulin in saline	0.2	45 ± 3	—	—	100	1.5 ± 0.1	0	4
Enteral	Insulin in PBS	20	3 ± 2	—[c]	—[c]	0.06 ± 0.05[b]	—[c]	—[c]	3
Enteral	Insulin in PBS + 10% HPβCD	20	3 ± 2	—[c]	—[c]	0.07 ± 0.06[b]	—[c]	—[c]	3
Enteral	Insulin in PBS + 10% DMβCD	20	255 ± 65	149 ± 51	1.5	5.63 ± 1.44	515.7 ± 120.3	1.99	5

[a] Value represents mean ± SE; AUC, area under the curve of insulin concentration versus time; C_{max}, peak insulin concentration; t_{max}, peak time; AUMC, area under the first moment curve; MAT, mean absorption time; F, absolute bioavailability; n, number of determinations.
[b] Not statistically significant from 0.
[c] Values are not significant to be calculated.

From MRT calculations of insulin following intravenous and enteral administration, the mean absorption times associated with intestinal insulin absorption were obtained using the following equation:

$$MAT = MRT_{enteral} - MRT_{iv} \quad (2)$$

The areas above the blood glucose-time curves (AACs) were calculated by the linear trapezoidal method as reported by Touitou and Rubinstein (7). The percent maximum blood glucose depression (100 − percent minimum glucose level) was chosen as the second parameter for pharmacodynamic evaluation. Relative efficacy (E) was calculated according to the method of Aungst et al. (15) using a previously established $AAC_{0\text{-}4\ hr}$ versus log (i.v. dose) relationship (16), i.e., $AAC_{0\text{-}4\ hr}$ = 228.9 log (i.v. dose) + 355.9.

$$E(\%) = \frac{\text{IV dose to produce equivalent hypoglycemic response}}{\text{Actual enteral insulin dose}} \times 100 \quad (3)$$

Histological Studies

Histopathological examination of the rat intestinal tract was performed according to the method of Schilling (17). Dilute phosphate buffered solutions with and without cyclodextrin derivatives were instilled into the ligated distal jejunum/proximal ileum. Insulin was not included in the solution in order to simplify the system and render a clear interpretation of the possible damaging effect caused by the cyclodextrin. After a 240-minute exposure interval, the animal was sacrificed by cervical dislocation. The upper part of the segment, about 1 inch in length, was carefully excised with a pair of delicate scissors and sliced open. The excised segment was then pinned onto a piece of cardboard and placed in 60 ml of 10% neutral formalin for fixation. Five-micrometer sections were cut on a microtome and subsequently stained by conventional hematoxylin and eosin method for microscopic examination.

RESULTS AND DISCUSSION

Enteral Absorption of Porcine-zinc Insulin in Rats

The enteral absorption of hexameric insulin is low and site-dependent, the optimal region being the lower portion of the intestinal tract (5). Intestinal absorption, performed with the closed loop technique, demonstrated significantly

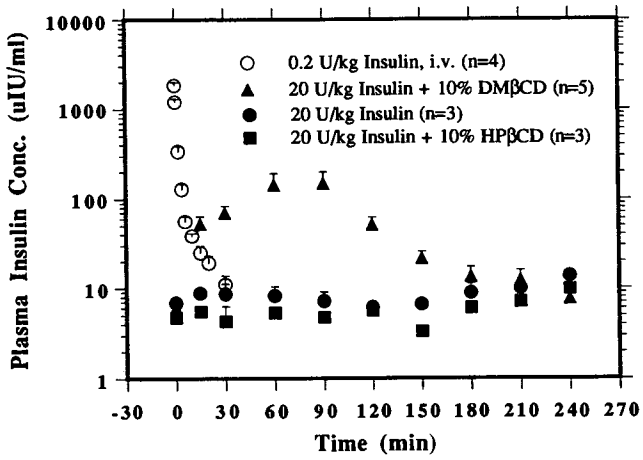

Figure 1. Plots of plasma insulin concentrations versus time following intravenous administration of 0.2 U/kg insulin, enteral administration of 20 U/kg insulin alone, enteral administration of 20 U/kg insulin with 10% (w/v) HPβCD, and enteral administration of 20 U/kg insulin with 10% (w/v) DMβCD. Values denote means ± SE.

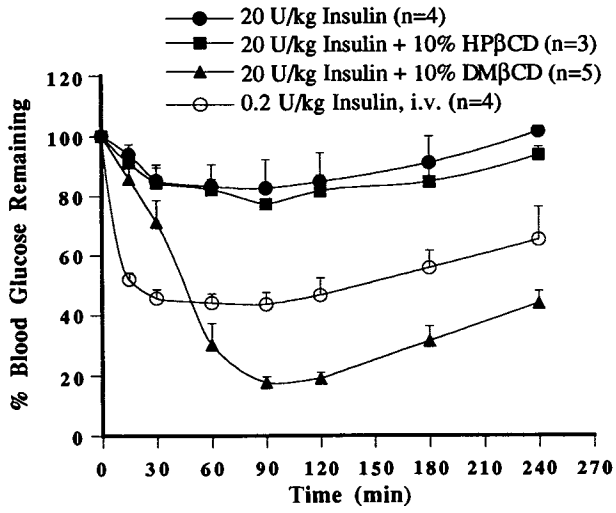

Figure 2. Pharmacodynamic response following enteral administration of 20 U/kg porcine-zinc insulin in 0.01 M phosphate buffered saline in the absence and presence of 10% β-cyclodextrin derivatives. Data from intravenous administration of 0.2 U/kg insulin have also been included. Values represent means ± SE.

greater hypoglycemic response from insulin administered to the distal jejunum/proximal ileum segment as compared to the duodenum/proximal jejunum segment (18).

In order to investigate the effects of cyclodextrins on intestinal insulin absorption, the closed loop technique was again utilized, and insulin solutions were delivered to the distal jejunum/proximal ileum segment of the rat with and without the presence of cyclodextrins. Fig. 1 illustrates the plasma insulin concentration profiles plotted as a function of time. Three aqueous formulations were individually administered to anesthetized nondiabetic rats, i.e., 0.5 mg/ml insulin (I), I with 10% (w/v) HPβCD, and I with 10% DMβCD all at an insulin dose of 20 U/kg. When insulin alone was administered enterally to rats, the plasma insulin concentration did not increase significantly during the course of an experiment. Incorporation of 10% HPβCD also failed to improve systemic insulin absorption. The presence of 10% DMβCD, however, strongly promoted absorption as evidenced by high immunoreactive insulin in plasma which peaked near 148 μU/ml at 90 min. Table I summarizes the pharmacokinetic parameters following enteral administration of the three formulations together with that of intravenous administration at a dose of 0.2 U/kg.

It needs to be pointed out that although the absorption enhancing potency of DMβCD appears promising, the absolute bioavailability of insulin is still low and quite variable, with a coefficient of variation of 57%. High dosing variability fosters a formidable challenge in the successful development of an oral insulin delivery system.

The corresponding hypoglycemic effects were monitored simultaneously (Fig. 2). Enteral administration of 20 U/kg insulin hexamers to the distal jejunum/upper ileum segment of the rat resulted in minimal glucose-lowering effect with a bG_{max} of 17%. Addition of 10% HPβCD improved bG_{max} only to 23%. On the other hand, 10% DMβCD significantly improved insulin hypoglycemic effect to a 82% glucose reduction at 90 min post administration. The pharmacodynamic parameters following enteral administration of the three formulations are listed in Table II, together with that of intravenous injection of 0.2 U/kg insulin.

The superiority of DMβCD to other cyclodextrins as nasal and rectal insulin absorptions has been recently reported (9,12,13). Merkus et al. (9) found 5% DMβCD to be extremely effective as an insulin absorption enhancer in rats with almost complete insulin uptake. Meanwhile, 5% HPβCD merely increased nasal insulin bioavailability to 1.2%. Watanabe and co-workers also reported a significantly increased AUC in rabbits after incorporating 30 mg DMβCD in their nasal insulin formulation (13). The addition of the same amount of HPβCD, on the other hand, only increased the AUC slightly.

α-Chymotryptic Degradation of Insulin

The mechanism and degradation kinetics of insulin by trypsin and α-chymotrypsin were previously reported (2). Alpha-chymotrypsin was the primary proteolytic enzyme responsible for initial cleavage and unfolding of insulin globular structure, exposing the molecule to subsequent attack by brush border and enterocytic enzymes. The rate of degradation depended on the degree of insulin association. Quantitative mathematical relationships between rate constants of enzymic degradation and degree of insulin dissociation were established previously (14). Since different cyclodextrins were shown to exhibit varying capabilities in dissociating insulin hexamers by circular dischroism studies (8), their effects on α-chymotrypsin-mediated insulin degradation were investigated.

When 10% HPβCD was incorporated into insulin hexamer solution, the kinetic profile of enzymatic degradation was not altered significantly (Fig. 3). However, the incorporation of 10% DMβCD greatly facilitated α-chymotryptic degradation of insulin. Further, all profiles appear to follow apparent first order kinetics throughout the degradation process. This trend suggests the lack of any enzyme inhibition by the cyclodextrins. When fitted into first-order kinetics by least squared regression, the observed rate constants (k_{obs}) for no cyclodextrin (control), HPβCD, and DMβCD were 0.038 ± 0.004, 0.038 ± 0.002, and 0.110 ± 0.008 min^{-1} (mean ± SD, n = 3), respectively. This observation indicates that HPβCD did not significantly alter insulin degradation characteristics while DMβCD greatly promoted insulin biodegradation.

Lack of enzyme inhibitory effect by HPβCD and DMβCD tends to suggest the catalytic site difference between a serine protease and an aminopeptidase which was shown to be inhibited by cyclodextrins (11). The facilitated enzymatic degradation of insulin by the presence of 10% DMβCD, on the other hand, indicates the ability of DMβCD to dissociate insulin hexamers. The extent of insulin dissociation, nevertheless, is rather incomplete since a 6-fold increase in the first-order rate constant should be observed upon complete monomerization (14). The mechanism by which cyclodextrins minimize protein aggregation is postulated to be the molecular encapsulation of exposed hydrophobic amino acid side chains (19). In the process, between several dozen to several hundred cyclodextrin molecules may attach to the exposed amino acids creating a volumi-

Table II. Pharmacodynamic Parameters Related to the Hypoglycemic Effects of Insulin Administered Intravenously and Enterally[a]

Route of administration	Formulation	Insulin dose (U/kg)	$AAC_{0-4\ hr}$ (% · hr)	bG_{max} (% gluc.)	t_{max} (hr)	E (%)	n
Intravenous	Insulin in saline	0.2	194 ± 22	56	1.5	100	4
Enteral	Insulin in PBS	20	44 ± 27	17	1.5	0.24 ± 0.06	4
Enteral	Insulin in PBS + 10% HPβCD	20	60 ± 13	23	1.5	0.26 ± 0.03	3
Enteral	Insulin in PBS + 10% DMβCD	20	248 ± 14	82	1.5	1.75 ± 0.26	5

[a] Values represent means ± SE; AAC, area above the curve of percentage glucose remaining versus time; bG_{max} = 100 − minimum percentage glucose level at t_{max}; E, relative efficacy; n, number of determinations.

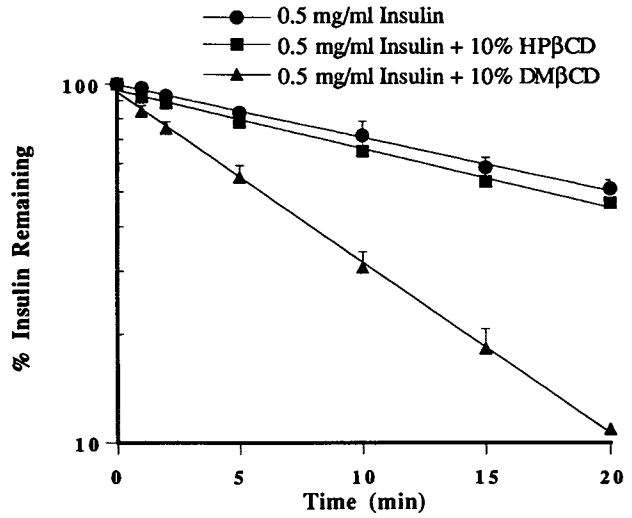

Figure 3. Degradation of porcine-zinc insulin by alpha-chymotrypsin in the absence and presence of 10% β-cyclodextrin derivatives. Values denote means ± SD of three determinations.

nous hydrate shell which in turn deagregates, solubilizes, and eventually denatures the dissolved protein molecule (20). As suggested in a previous report (14), the circular dichroic method and the enzymatic kinetic approach indeed mutually corroborate with each other in supporting the theory of insulin oligomer dissociation.

Histopathological Study

Microscopic evaluation of intestinal cross sections is used for assessing cell damage associated with exposure to absorption adjuvants. Four parameters were found to be good indicators for mucosal damage, i.e., desquamation of the epithelial cells, surface debris, pycnotic nuclei in the lamina propria, and mitotic figures (17).

Microscopic examination was performed on H/E stained cross sections from the rat distal jejunum/proximal ileum segments which were treated with all three formulations for 4 hours. Comparison of the parameters did not reveal any apparent differences existing among the three groups. As shown in Fig. 4, treatment with 10% DMβCD appears to preserve the overall cellular integrity of the intestinal epithelium without any observable disruption of the villus. The only observable effect under high magnification is that the villus revealed some occurrence of epithelial cell shedding, although to a very slight extent.

Therefore, the mucosal toxicity of both HPβCD and DMβCD appears to be low and no observable destruction to the overall tissue integrity was observed, in agreement with the previous report (21). This behavior is quite different with other types of surfactants probably because of the inability of cyclodextrins to actively penetrate the membrane bilayers (22).

The mechanisms accounting for cyclodextrin-promoted insulin absorption have not been fully explored. However, several possible contributions may co-exist such as dissociation of insulin oligomers leading to smaller diffusing penetrants (8), inhibition of proteolytic enzyme activity thereby retarding presystemic degradation (11), complexation of cyclodextrins with interstitial Ca^{2+} ion to widen the tight junction (11), and even bilayer solubilization effect causing membrane disruption and improved transcellular insulin diffusion.

In conclusion, DMβCD but not HPβCD appears to improve enteral insulin absorption in rats significantly which may be contributed in part to its ability to partially dissociate insulin oligomers. No inhibitory effect on α-chymotryptic activity was observed by both cyclodextrins at 10% concentration. In addition, microscopic examination of the exposed intestinal section indicated high tolerance of the intestinal mucosa to the two cyclodextrin derivatives further favoring their use intestinally.

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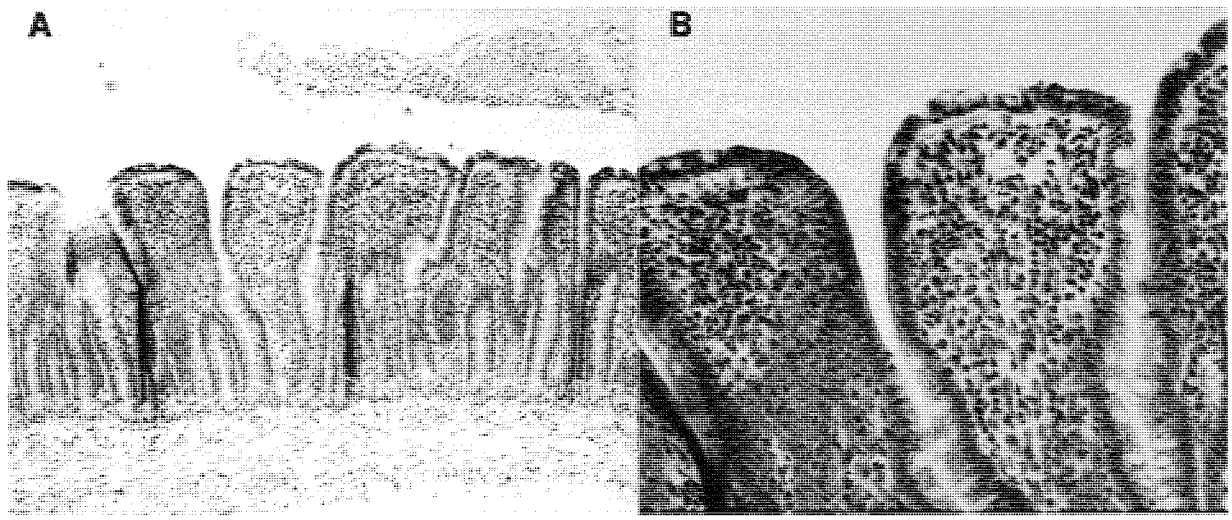

Figure 4. Typical light photomicrographs of rat small intestine treated with 10% DMβCD in 0.01 M phosphate buffered saline solution. Exposure time was 4 hours followed by fixation in 10% neutral formalin, embedded in liquid paraffin, microtomed to 5-μm sections, and stained by conventional H/E. A, original magnification ×88; B, original magnification ×224.

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