Differential Effects of Anionic, Cationic, Nonionic, and Physiologic Surfactants on the Dissociation, α-Chymotryptic Degradation, and Enteral Absorption of Insulin Hexamers

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Various surfactants were investigated to compare their effects on insulin dissociation, α-chymotryptic degradation, and rat enteral absorption. With a circular dichroism technique, sodium dodecyl sulfate (SDS) at a 5 m*M* concentration was found to completely dissociate procine-zinc insulin hexamers (0.5 mg/ml) into monomers. The catalytic activity of α-chymotrypsin (0.5 μ*M*) was also abolished by 5 m*M* SDS. When insulin was injected into the distal jejunum/proximal ileum segment of the rat, 5 m*M* SDS greatly enhanced its pharmacological availability, from a negligible value to 2.8%. Being a cationic surfactant, hexadecyl trimethylammonium bromide (CTAB) also efficiently dissociated insulin hexamers at concentrations of 1–5 m*M*. However, extensive charge–charge interaction was observed below a CTAB concentration of 0.6 m*M*, leading to insulin precipitation at a molar CTAB:insulin ratio of 1:1 to 2:1. An α-chymotryptic degradation study also revealed near-complete dissociation of insulin hexamers at 1 m*M* CTAB. Above 1 m*M*, however, CTAB acted as an enzyme inhibitor, most likely by means of charge repulsion. Enteral absorption studies showed a much lower pharmacological availability, only 0.29%. Nonionic surfactants such as Tween 80 and polyoxyethylene 9 lauryl ether were ineffective in dissociating insulin hexamers. Tween 80, at 5 m*M*, neither significantly altered the α-chymotryptic degradation pattern nor enhanced the enteral absorption of insulin. The relative effectiveness of different species of bile salts on insulin hexamer dissociation appeared to be similar. Sodium glycocholate at a 30 m*M* concentration also significantly increased insulin pharmacological availability, to 2.3%. A morphological study did not reveal any significant alteration of the rat intestinal mucosal integrity after exposure to 5 m*M* SDS for 30 min. The results further emphasize the importance of the degree of insulin aggregation on its enteral transport.

KEY WORDS: bile salts; α-chymotrypsin; degradation; enteral absorption; hexadecyl trimethylammonium bromide; insulin dissociation; polyoxyethylene 9 lauryl ether; sodium lauryl sulfate; surfactants; Tween 80.

INTRODUCTION

The primary route for the administration of most biologically active macromolecules involves parenteral injection. The inconvenience and poor patient compliance associated with this route of administration have prompted recent investigation of alternative pathways such as oral, nasal, ocular, buccal, rectal, pulmonary, and transdermal routes (1).

Noninvasive delivery of insulin, as a model protein drug, has attracted considerable attention. The oral route remains the most attractive choice because of its wide acceptance for chronic drug administration. More importantly, mesenteric absorption also delivers insulin directly to the liver via the portal vein exactly as would occur physiologically. Nevertheless, development of a successful oral insulin dosage form with adequate bioavailability (10–20%) has encountered difficulties. Three intrinsic properties of insulin impair its oral effectiveness, i.e., (i) oligomer formation above a 0.1 μ*M* concentration in aqueous solution (2); (ii) extensive cleavage by various luminal and cellular enzymes such as pepsin, trypsin, α-chymotrypsin, carboxypeptidases, and aminopeptidases (3–6); and (iii) hydrophilic characteristics hindering effective partitioning across biological membrane barriers (7,8). In order to overcome these difficulties, various approaches have been investigated such as incorporation of protease inhibitors, targeted enteral delivery, and facilitated transport by absorption enhancers (9–14).

Different types of absorption enhancers have been extensively studied, including cationic, anionic, nonionic, and physiologic surfactants (15). The relative effectiveness of these adjuvants was postulated to be a combination of their differing abilities in dissociating insulin oligomers, protecting insulin degradation by proteolytic enzymes, and increasing mucosal membrane permeability (6,14,16,17).

In this study, the differential effects of these surfactants on insulin dissociation, degradation by α-chymotrypsin, and enteral absorption are systematically evaluated and compared. Probable mechanisms of insulin absorption enhancement are further elucidated. Finally, the feasibility of oral insulin delivery is discussed.

MATERIALS AND METHODS

Materials

Crystalline porcine-zinc insulin (Lot No. 504JR8; potency, 26.3 U/mg) was kindly donated by Eli Lilly and Company (Indianapolis, IN). Lyophilized α-chymotrypsin prepared from bovine pancrease (56 U/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), sodium glycocholate (NaGC), sodium taurocholate (NaTC), sodium cholate (NaC), sodium deoxycholate (NaDC), sodium dodecyl sulfate (SDS), hexadecyl trimethylammonium bromide (CTAB), Tween 80, polyoxyethylene 9 lauryl ether (PLE), and tris(hydroxymethyl)aminomethane (Tris) were procured from Sigma Chemical Co. Deionized double-distilled water was used throughout the study. All other chemicals were of analytical reagent grade and were used as received.

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Circular Dichroism Studies

A CD spectropolarimeter (JASCO Model J600, Japan Spectoscopic 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 various surfactants. Zinc insulin solutions in 0.01 *M* phosphate-buffered saline solution, pH 7.4, containing 0.5 mg/ml zinc insulin and 0 to 5 m*M* SDS or CTAB, 0 to 10 m*M* Tween 80 or PLE, and 10 m*M* NaGC, NaTC, NaC, or NaDC were scanned from 300 to 250 and from 250 nm to 200 nm at a scanning speed of 5 nm/min, respectively. The temperature was controlled at 37°C. Ten- and one-millimeter-pathlength quartz cuvettes (American Scientific Products, McGaw Park, IL) were used, respectively, for the higher- and lower-wavelength regions to obtain optimum resolution of the CD 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).

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, West 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 22 to 40% within 32 min. Twenty microliters of the sample was injected onto a Rainin reversed-phase C-8 Microsorb column (250 × 4.6 mm) connected to a C-8 precolumn. The gradient mobile phase was run at a flow rate of 1 ml/min. The ultraviolet/visible detector was set at 220 nm; the recorded signal was analyzed with an electronic integrator (Model 3390 A, Hewlett-Packard Co., Avondale, PA). The chromatographic method as described previously (16) provides baseline separation of insulin from its enzymic degradation products.

α-Chymotryptic Degradation of Porcine-Zinc Insulin

Ten milliliters of a 0.5 mg/ml porcine-zinc insulin solution was prepared in a buffer composed of 100 m*M* Tris and 1 m*M* $CaCl_2$ adjusted to pH 8.0. The solution was preequilibrated at 37°C for 15 min. Just prior to the addition of the enzyme, the solution was vortexed for 2 sec 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 μ*M*. Aliquots (100 μL) were withdrawn at 1, 2, 5, and 10 min and immediately added to 0.9 ml of a 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. In the case of micellar solutions, the surfactant was added initially to the Tris buffer solution and sonicated for 5 min at room temperature prior to the addition of insulin.

Preparation of Insulin Solutions for Enteral Administration

Crystalline porcine-zinc insulin was initially 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 0.127 mg/mL. The solution was made just before use and the surfactant was then added. The pH of the final solution was again measured with a pH meter and adjusted to pH 7.4 if necessary.

Enteral Absorption with the Closed-Loop Technique

Male Sprague–Dawley rats weighing 175–250 g were fasted for 16–20 hr 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 min 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-W light bulb and a reflector above.

Cannulation of the right external jugular vein was performed by inserting a 3-in. piece of Silastic tubing, 0.047-in. 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 10 U/ml heparin. Microdissecting scissors were used to cut a small opening in the jugular vein, and one tip of a microdissecting forceps, extra delicate, was inserted through the hole to guide the insertion of the cannula toward the heart. Surgical thread underneath the vein was tied around the collar of the cannula to secure it. A 23-G needle with the sharp tip 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, and 180 min after insulin administration.

A midabdominal 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, as a previous report from this laboratory has shown this segment to have higher insulin permeability (18). The segment was washed by perfusing prewarmed (37°C) normal saline into the intestine 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 the appropriate solution (approximately 0.3 ml) was instilled. The concentration of insulin solutions employed was 0.127 mg/mL, or 3.33 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 place inside the peritoneal cavity.

Blood Glucose Measurement

Blood samples were immediately tested 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 measureable glucose levels ranged within 10 to 500 mg/dL.

The areas above the blood glucose–time curves (AACs) were calculated by the linear trapzoidal method as reported by Touitou and Rubinstein (11). The percentage maximum blood glucose depression (100 − percentage minimum glucose level) was chosen as the second parameter for pharmacodynamic evaluation. Pharmacological availability (f) was calculated according to Ritschel and Ritschel (19) with the following equation:

$$f = \frac{AAC_{0\text{–}180\ \text{min enteral}}}{AAC_{0\text{–}180\ \text{min i.v.}}} \times \frac{Dose_{\text{i.v.}}}{Dose_{\text{enteral}}}$$

Histological Studies

Histopathological examination of the rat intestinal tract was performed according to the method of Schilling (14). Diluted phosphate-buffered solutions with and without 5 m*M* SDS 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 surfactant. After a 30-min exposure interval, the animal was sacrificed by cervical dislocation. The instilled solution was withdrawn out of the intestinal lumen with the help of a 23-G needle attached to a 1-mL syringe. The recovered solutions were subjected to protein measurement for the estimation of total protein release. The upper part of the segment, about 1 in. 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% formalin for fixation. Six-micrometer sections were cut on a microtome and subsequently stained with conventional hematoxylin and eosin method for microscopic examination.

RESULTS

Dissociation of Insulin Hexamers in the Presence of Surfactants

In the presence of zinc ions insulin molecules self-associate, forming primarily hexamers which contain a threefold axis of dimers. The zinc ions are coordinated through the imidazole groups of B10 or sometimes B5 histidine residues. Meanwhile, an insulin dimer is formed by hydrophobic interactions and four hydrogen bonds arranged as a β-sheet structure between the two antiparallel COOH terminal strands of the B chain, while the insulin monomer itself also coils as α-helices involving both A and B chains arranged in a distorted form. The dissociation of hexameric insulin can thus be characterized by circular dichroism spectroscopy (20–22).

Figures 1A and B depict typical CD spectra of zinc insulin at a concentration of 0.5 mg/ml. The negative maximum at 276 nm is assigned to the contribution of the B23–B28 aromatic residues in the form of the antiparallel β structure. Attenuation of this band could, therefore, be correlated with deaggregation, while strengthening of this band could be associated with enhanced association of monomers. In the far-UV region, there are two major Cotton effects, resulting in negative maxima at 222 and 209 nm, respectively. The 222-nm band was assigned in large part to the β structure, which is a predominant feature of the dimer. The 209-nm band was proposed to be largely attributed to the α-helical structure, which is a characteristic feature of the monomer (20).

When SDS was incorporated in the solution, significant attenuation of the 276-nm band was observed (Fig. 1A). Below the critical micellar concentration (CMC; ~1 m*M*), little change in the CD spectrum was noted. However, above CMC the molar ellipticity ($[\theta]_\lambda$) values decreased rapidly. At a 5 m*M* SDS concentration the curve became almost flat. The change in $[\theta]_\lambda$ strongly suggests dissociation of insulin hexamers in the presence of SDS above its CMC. Figure 1B illustrates similar spectral changes in the far-UV region. The addition of 1 and 5 m*M* SDS significantly attenuated the negative maximum at 222 nm, indicating gradual dissociation of insulin dimers. Further, the intensity of the maximum at 209 nm continued to increase in a stepwise manner with the increase in SDS concentration from 1 to 5 m*M*. Strengthening of this band correlates positively with the formation of insulin monomers. Above a 5 m*M* concentration, SDS did not exhibit any additional effect in attenuating or strengthening the three major bands. These results together suggested that anionic surfactants such as SDS are capable of effectively dissociating insulin oligomers above their CMC. Storage of SDS (5 m*M*)–insulin solution at room temperature for several months also did not result in any insulin precipitation. Thus, long-term insulin polymerization in aqueous solution appears to be greatly reduced in the presence of SDS.

In addition, a widely used cationic surfactant, CTAB, was used in this investigation in order to compare the effects of electric charge on insulin dissociation. When CTAB was added to insulin solution, precipitation occurred immediately. Figure 2 depicts the percentage of insulin in aqueous solution as a function of the CTAB concentration. Both zinc insulin and sodium insulin exhibited similar behavior, suggesting that the precipitation was not due to zinc ion chelation with other anions. At a CTAB concentration of 0.1–0.2 m*M*, almost 95% of the insulin was precipitated out, where the ratio of CTAB to insulin is approximately 1:1 to 2:1. With the p*I* of insulin near 5.4, insulin molecules are negatively charged at pH 7.4. The stoichiometry of CTAB to insulin neutralization thereby suggests that 1 to 2 negative charges developed on each insulin molecule. Above 0.2 m*M*, charge inversion gradually took place, leading to solubilization of insulin precipitates by CTAB micelles. Above a 0.6 m*M* CTAB concentration, the solution became optically clear again, indicating complete charge inversion. Because of the observed charge–charge interaction between CTAB and insulin molecules, CD spectra were taken above a 1 m*M* CTAB concentration. The molar ellipticity values at 276, 222, and 209 nm were recorded and the ellipticity percentages remaining were calculated and plotted as a function of

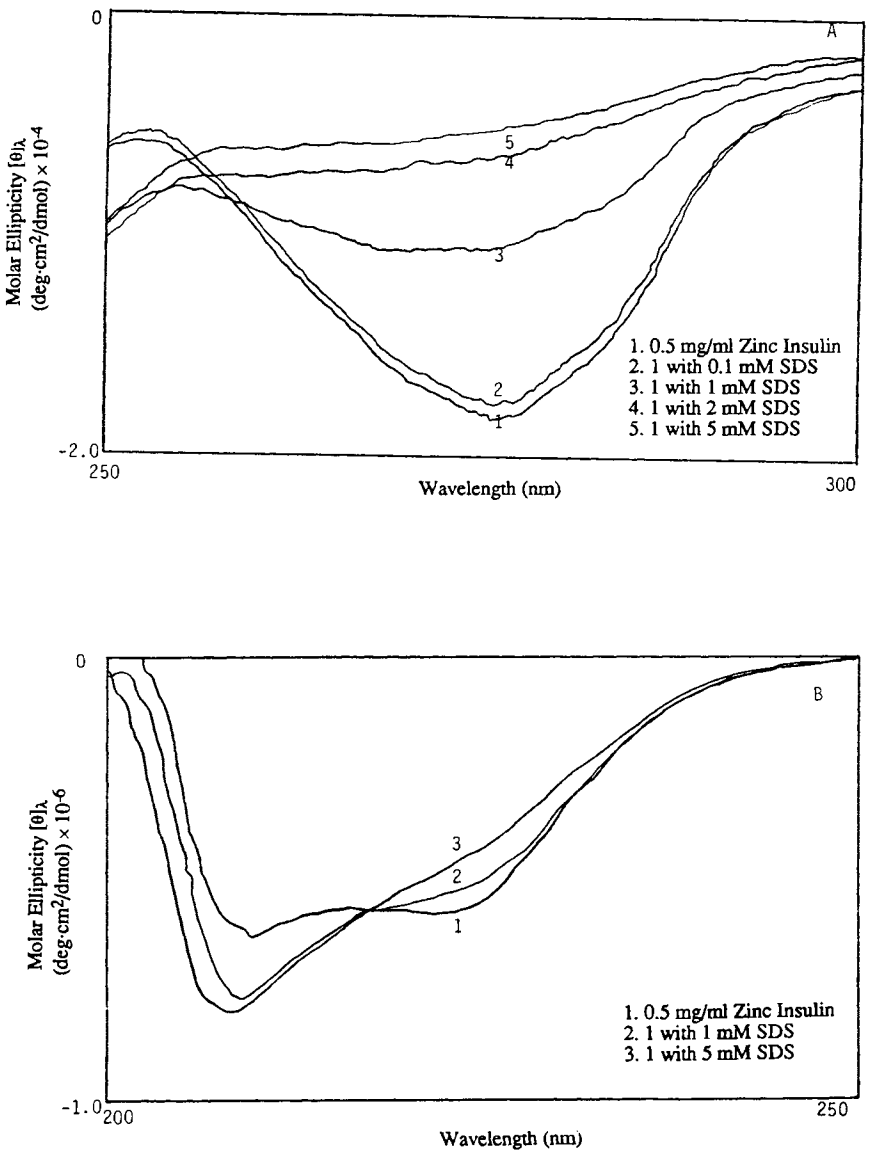

Fig. 1. Circular dichroism spectra of 0.5 mg/ml porcine-zinc insulin solutions containing various concentrations of sodium dodecyl sulfate (SDS). (A) Wavelength range from 250 to 300 nm; (B) Far-UV region from 200 to 250 nm.

surfactant concentration, as shown in Figs. 3A–C, and it appears that CTAB exhibits similar dissociating effects on insulin oligomers as SDS.

Tween 80 and polyoxyethylene 9 lauryl ether (PLE), both being nonionic surfactants, were also studied. Unlike SDS and CTAB, these two surfactants did not cause any measurable change in dissociation–association equilibria of insulin hexamers. Figure 3A illustrates the percentage $[\theta]_{276\ nm}$ plotted against the concentrations of Tween 80 and PLE, along with those of SDS and CTAB. No apparent dissociation of insulin was also observed with Tween 80 even at a surfactant concentration of 10 m*M*.

Different species of bile salts were previously found to have different hydrophobicity and exhibit different insulin enhancing effects. Gordon *et al.* (23) have demonstrated that the adjuvant potency for nasal insulin absorption by bile salts correlates well with the increasing hydrophobicity of

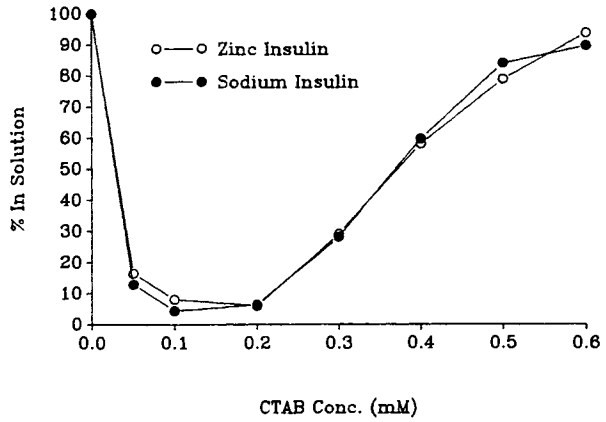

Fig. 2. Percentage insulin in solution expressed as a function of CTAB concentration. The initial concentration of insulin was 0.5 mg/mL before adding CTAB.

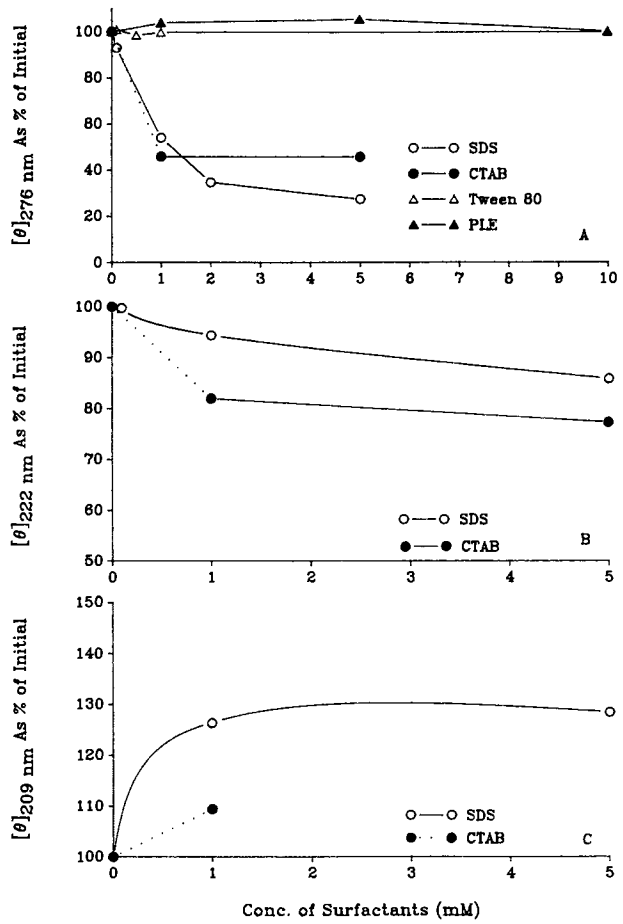

Fig. 3. Change of molar ellipticities at (A) 276 nm, (B) 222 nm, and (C) 209 nm following the incorporation of anionic, cationic, and nonionic surfactants. Dotted lines represent the concentration region of charge–charge interaction leading to insulin precipitation.

the steroid nucleus. We had, therefore, decided to investigate any possible correlation between the insulin dissociating effect of the bile salts and their differing enhancing effectiveness. The CD spectra of 0.5 mg/ml zinc insulin in the presence of four bile salts, i.e., NaGC, NaTC, NaC, and NaDC, were subsequently acquired. The result suggests that although all of the four bile salts exhibited considerable insulin dissociating power, no significant differences exist among them.

α-Chymotryptic Degradation of Insulin

The degradation kinetics of insulin by trypsin and α-chymotrypsin was previously reported from this laboratory (4). α-Chymotrypsin was found to be the primary proteolytic enzyme responsible for initial cleavage and unfolding of the insulin globular structure, exposing the molecule to subsequent attack by brush border and enterocytic enzymes. The rate of degradation was also found to be dependent on insulin aggregation. Quantitative mathematical relationships between rate constants of enzymic degradation and insulin dissociation were established (16,22). Since different surfactants were shown to exhibit varying capabilities in dissociating insulin hexamers, it is desirable to investigate further their effects on α-chymotrypsin-mediated insulin degradation.

When 5 m*M* SDS was incorporated into the insulin hexamer solution, the initial rate of enzymic degradation was found to be greatly facilitated, as shown in Fig. 4. However, this trend slowed down substantially after the 1-min sample, and no further decline in insulin concentration was observed after 5 min. This profile is suggestive of possible enzyme denaturation in the presence of SDS, resulting in complete loss of activity in approximately 5 min. In order to substantiate this hypothesis, 5 m*M* SDS was also preincubated with α-chymotrypsin solution for 15 min. Reaction was then initiated in the same manner. Pretreatment of the enzyme indeed destroyed the catalytic activity of α-chymotrypsin as shown in Fig. 4.

Since neither Tween 80 nor PLE indicated any measurable effect on the insulin dissociation process, Tween 80 was then selected to examine its effect on enzymic degradation characteristics of insulin. Insulin degradation profile in the presence of 5 m*M* Tween 80 is also shown in Fig. 4, together with that of the control experiment. Incorporation of 5 m*M* Tween 80 did not significantly change the degradation pattern. When fitted by the apparent first-order kinetics, the observed rate constant was found to be 0.04238 ± 0.00294 min^{-1} (mean ± SD; $n = 3$). No statistically significant difference was observed when compared with the rate constant of the control (0.03822 ± 0.00389 min^{-1}). This observation indicates that 5 m*M* Tween 80 did not significantly alter insulin degradation characteristics.

Because of the charge–charge interaction between insulin and CTAB molecules, the influence of CTAB on enzymic insulin degradation was investigated in a more careful manner. Concentrations of 1, 2, 5, 10, and 15 m*M* CTAB were studied and the results are shown in Figs. 5A and B. When 1 m*M* CTAB was included, the degradation rate of insulin was significantly increased as shown in Fig. 5A. Further, the data could be fitted by apparent first-order kinetics. Increases in CTAB concentration, however, gradually decreased the rate and extent of insulin disappearance. Figure 5B illustrates the apparent first-order rate constants as a function of CTAB concentration. Increasing CTAB concentration had a decelerating effect on insulin degradation. At 15 m*M* CTAB, an average rate constant of 0.03726 ± 0.00645 min^{-1} was observed, which was statistically not significant

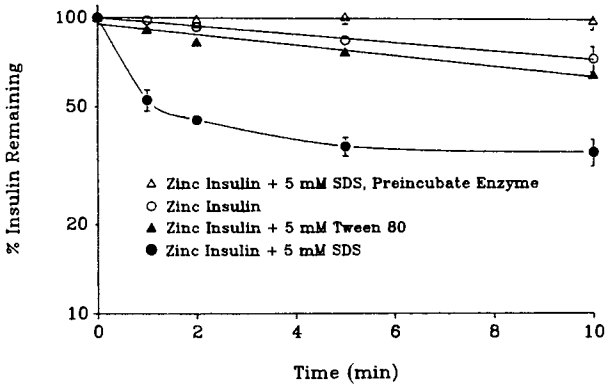

Fig. 4. Semilogarithmic plots of porcine-zinc insulin biodegradation by α-chymotrypsin in the presence of SDS or Tween 80.

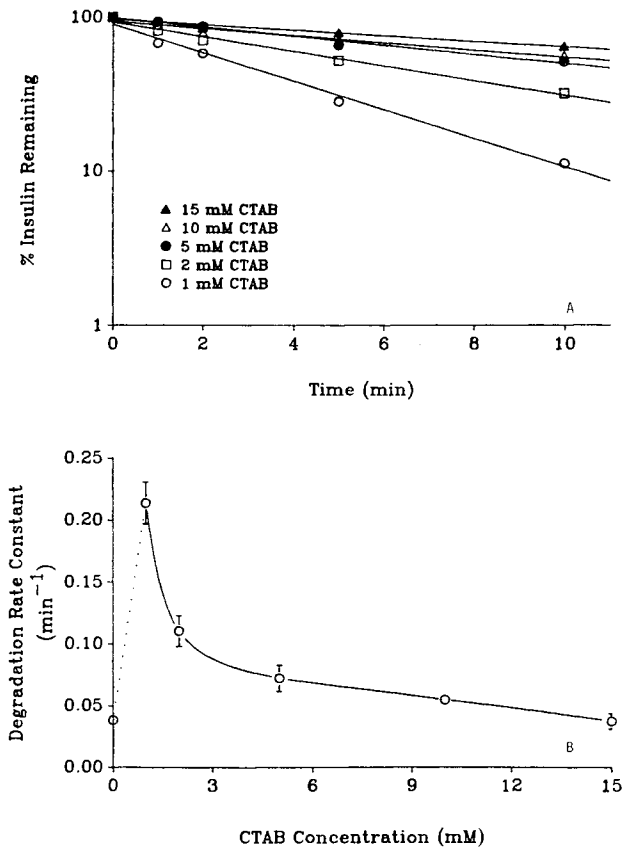

Fig. 5. (A) Semilogarithmic plots of porcine-zinc insulin biodegradation by α-chymotrypsin in the presence of various concentrations of CTAB. Bars are not shown for clarity. (B) The effect of CTAB concentration on the apparent first-order rate constants of insulin biodegradation. The dotted segment represents the region of charge–charge interaction leading to insulin precipitation. Values denote means ± SD (n = 3).

from the control. More importantly, at 1 mM CTAB the apparent first-order rate constant reached 0.2138 ± 0.017 min^{-1}, 5.6-fold higher than that of the control. This number is close to the theoretical value of 6 when complete dissociation of insulin hexamers to monomers occurs (16). Moreover, pretreatment of α-chymotrypsin with 10 mM CTAB did not result in any alteration of the insulin degradation profile (data not shown), thus excluding the possible effect of CTAB on enzyme activity.

Enteral Absorption of Zinc-Porcine Insulin in Rats

Previous results from this laboratory indicated that the optimal region for insulin absorption resides in the lower portion of the intestinal tract (8). Intestinal absorption with the closed-loop technique also demonstrated a significantly greater hypoglycemic response from insulin administered to the distal jejunum/proximal ileum segment compared to the duodenum/proximal jejunum segment (18). Nevertheless, hexameric insulin was poorly absorbed from the intestinal tract, partly as a result of its large size and intrinsic physicochemical properties, resulting in a bioavailability of less than 0.2%.

In order to investigate the effects of insulin deaggregation on its intestinal absorption, the previously established closed-loop technique was used, and insulin solutions were delivered to the distal jejunum/proximal ileum segment of the rat. Four surfactants were individually incorporated, i.e., 5 mM SDS, 5 mM CTAB, 30 mM NaGC, and 5 mM Tween 80. At these concentrations all surfactants except Tween 80 completely dissociate insulin hexamers into monomers. The hypoglycemic effects of 5 U/kg insulin in the presence of these surfactants are shown in Fig. 6. Tween 80, which was ineffective in insulin dissociation, did not promote any measurable glucose-lowering effect, again indicating the poor absorptivity of insulin hexamers in the intestinal tract. Incorporation of 5 mM CTAB, on the other hand, exhibited a significant hypoglycemic effect, with maximum glucose reduction at 60 min postadministration. Nevertheless, a maximum glucose reduction of only 16.5% and a pharmacological availability of 0.29% were obtained (Table I).

When 30 mM NaGC was used as the adjuvant, a much stronger hypoglycemic effect was observed. A maximum glucose reduction of 40% resulted at 60 min. The pharmacological availability also reached 2.3%. The incorporation of 5 mM SDS elicited a similar effect with a slightly higher pharmacological availability of 2.8%. SDS, however, caused a faster initial drop in the blood glucose level, reaching a maximum reduction of 58% at 30 min.

Histopathological Study

Since the average life span of the rat intestinal epithelial cells is approximately 20 hr, microscopic evaluation of intestinal cross sections has been widely used as an easy and practical method for assessing any damaging effect associated with acute exposure to absorption adjuvants. Four parameters were previously chosen to serve as the appropriate indicators for mucosal damage, i.e., desquamation of the epithelial cells, surface debris, pycnotic nuclei in the lamina propria, and mitotic figures (14). Photomicrographs (not shown) were taken from H/E-stained cross sections of both the control (0.01 M PBS) and the SDS (5 mM)-treated rat

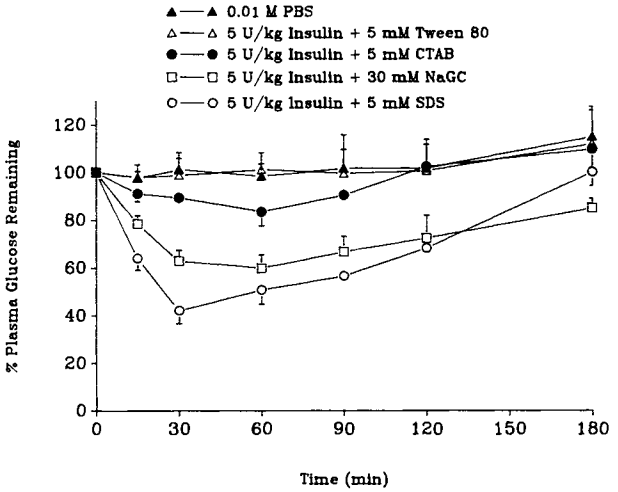

Fig. 6. Blood glucose levels (means ± SE) following *in situ* administration of blank 0.01 M PBS or 5 U/kg porcine-zinc insulin with various surfactants. All solutions were delivered into a 15-cm ligated segment of the rat distal jejunum/proximal ileum (n = 4–6).

Table I. Some Pharmacodynamic Parameters Related to the Hypoglycemic Effects of Porcine-Zinc Insulin in Rats in the Presence of Various Surfactants Administered to the Distal Jejunum/Proximal Ileum[a]

Surfactant	Concentration (m*M*)	Insulin dose (U/kg)	$AAC_{0-180\ min}$ ± SE (% · min)[b]	bG_{max} (% glucose reduction)[c]	t_{max} (min)	f ± SE (%)[d]
Tween 80	5	5	63.0 ± 1266.0[e]	—	—	—
TCAB	5	5	652.7 ± 211.4	16.5	60	0.29 ± 0.10
NaGC	30	5	5052.0 ± 893.2	40.2	60	2.27 ± 0.40
SDS	5	5	6154.5 ± 341.4	57.9	30	2.77 ± 0.15

[a] Number of determinations (n) = 4–6.
[b] The $AAC_{0-180\ min}$ following i.v. administration of 0.2 U/kg porcine-zinc insulin was obtained from a previous report (18).
[c] The maximum percentage of blood glucose depression (100 − percentage minimum glucose level).
[d] Pharmacological availability.
[e] Not significantly different from 0.

distal jejunum/proximal ileum. PBS treatment did not noticeably alter the integrity of the morphological structure, indicating the reliability of the preset experimental procedure (ligation and washing). Photomicrographs of SDS-treated samples also revealed a well-preserved overall structural integrity. In no circumstances was separation of villi from muscularis mucosa or sign of hemorrhage observed. Nevertheless, close inspection of the sections revealed the occurrence of surface debris, although to a very slight extent. The protein concentrations in the recovered 0.01 *M* PBS control and SDS samples at 30 min were found to be 5.97 ± 1.52 and 5.90 ± 1.36 mg/mL (mean ± SD; n = 3), respectively, thus further indicating no significant difference between treatments with the two solutions.

DISCUSSION

Four groups of surfactants were studied with respect to their effects on insulin dissociation, α-chymotryptic degradation, and rat enteral absorption. The ability of different groups of surfactants to dissociate insulin oligomers was found to differ significantly. In general, anionic surfactants and bile salts are able to dissociate completely hexameric insulin to monomers. Nevertheless, their relative efficacy was significantly different. While 5 m*M* SDS was able to break down 0.5 mg/ml insulin hexamers, a 30 m*M* concentration of NaGC was needed (16). Cationic surfactants such as CTAB interacted with insulin aggregates, resulting in almost complete precipitation of insulin at a CTAB-to-insulin ratio of 1:1 to 2:1. Excess surfactant then resolubilized the precipitate, leading to charge inversion of insulin–CTAB mixed micelles. Nevertheless, 1 to 5 m*M* CTAB still resulted in complete dissociation of hexameric insulin (0.5 mg/mL). In contrast, nonionic surfactants such as Tween 80 and PLE failed to produce any measurable effects on insulin dissociation. This result suggests that an ionic functional group in the surfactant molecule is needed to cause insulin hexamer dissociation. Therefore, absorption enhancement by nonionic surfactants does not appear to involve insulin dissociation. Although nonionic surfactants are unable to dissociate insulin aggregates, extensive extraction of biological membrane components was previously reported for intranasal administration (6). Alteration of the membrane integrity, therefore, is likely to constitute a major mechanism for nonionic surfactant-mediated absorption enhancement.

The hydrophobicity of the bile salt steroid nucleus correlated linearly with the extent of nasal insulin absorption enhancement (23). However, our circular dichroism studies indicated that the effect of insulin dissociation by different bile salts follows approximately the same order of magnitude. Hydrophilic bile salts caused more extensive insulin dissociation than hydrophobic counterparts. Hence, the stronger enhancing effect of more hydrophobic bile salts cannot be attributed solely to their solubilization efficiency. Considering that NaDC is able to penetrate into the nasal membrane more efficiently than NaGC (17), the channel-type reverse micelle mechanism proposed by Gordon *et al.* (23) concerning bile salt-mediated insulin transport is suggested.

Trypsin and α-chymotrypsin are the most abundant proteolytic enzymes in the upper intestinal tract (24). α-Chymotrypsin was previously shown to possess extensive serine protease activity, resulting in rapid insulin biodegradation. Destruction of intact insulin at the site of administration was considered to be another important reason responsible for low oral insulin bioavailability. Recent studies indeed revealed that incorporation of enzyme inhibitors such as aprotinin and Bowman–Birk inhibitor significantly improved the efficacy of orally administered insulin microspheres (9,10). Hirai *et al.* (6) also demonstrated that NaGC significantly inhibited the enzymic hydrolysis of insulin by leucine aminopeptidase. However, our α-chymotryptic degradation results revealed different inhibition patterns depending on the ionic state of the surfactants. SDS, being a strong anionic protein denaturant, completely denatured α-chymotrypsin at a 5 m*M* concentration within 5 min. The cationic surfactant, CTAB, exhibited a different behavior. One milimolar CTAB, which is sufficient to dissociate completely 0.5 mg/mL insulin hexamers, did not inhibit the enzyme activity to any significant extent. Increases in CTAB concentration, however, gradually slowed down the enzymic activity. Since the p*I* of α-chymotrypsin is approximately 8.8 (25), the enzyme is therefore positively charged at pH 8. The positively charged insulin–CTAB mixed micelles could be dispersed away from the enzyme catalytic site as a result of simple charge–charge repulsion. An excess amount of cationic surfactants, therefore, acts as a α-chymotrypsin inhibitor, probably by a different mechanism.

The effect of NaGC on α-chymotryptic degradation of insulin was previously reported from this laboratory (16).

The presence of 0–50 m*M* NaGC did not significantly alter the catalytic activity of α-chymotrypsin, which emphasizes the unique transport enhancing role of such physiologic surfactants. On the other hand, a nonionic surfactant, 5 m*M* Tween 80, acted as an inert component. Its inability to inhibit enzymic activity is also consistent with its inability to dissociate insulin. The different behavior of various surfactants in affecting α-chymotrypin-mediated insulin cleavage suggests that agents such as SDS may serve as better absorption enhancers through both oligomer dissociation and enzyme inhibition mechanisms.

Insulin hexamers are poorly permeable across capillary pores and thereby are nonabsorbable via nonparenteral pathways (26). Recently, Schilling and Mitra (18) reported that the absolute bioavailability of insulin administered as primarily hexameric zinc insulin to the duodenum/proximal jejunum and distal jejunum/proximal ileum was only 0.059 to 0.133%, respectively. Although the exact pathway of insulin transport across biological membranes is still unclear, paracellular diffusion is generally recognized as a major pathway for nonparenteral insulin absorption. The problem of low bioavailability could therefore be due partly to the large aggregation size of insulin, thereby preventing its uptake by the paracellular route.

Hexameric insulin has the shape of a flattened disk approximately 50 Å across and 35 Å thick (27). In contrast, the equivalent pore radius in the small intestine was estimated to be only 7–15 Å (28). Dissociation of insulin hexamers to monomers, however, drastically reduces its molecular dimension, to approximately 12–14 Å (29,30) making this polypeptide easily diffusible across the mucosal tight junctions. Results from our enteral insulin absorption studies indeed strongly supports this hypothesis. Tween 80 has no effect on the dissociation of insulin hexamers and, as a result, produced negligible effect on enteral insulin absorption. On the contrary, negatively charged surfactants such as SDS and NaGC produced strong and comparable effects on enteral insulin absorption at concentrations capable of completely dissociating insulin. However, insulin monomers produced by the addition of CTAB resulted in much lower glucose-lowering effects. The overall pharmacological availability was only one-eighth to one-tenth that produced by SDS and NaGC. This observation suggests the importance of the overall native charge of insulin on its intestinal uptake.

The lack of any morphological damage to the intestinal mucosal cells by acute exposure to SDS is unexpected since SDS is regarded as a toxic adjuvant. A dose of 0.4 mg/rat of 200 g body weight may not compromise the morphological integrity of the distal jejunum/proximal ileum to any significant extent. Unlike the colonic site, the distal jejunum/proximal ileum is able to reabsorb bile salts, leading to the hepatic recycling pathway. Thus, SDS with a molecular weight of only 288 might also be absorbed systemically, further shortening the contact time with the mucosal tissue. The histological effect of 30 m*M* NaGC on the distal jejunum/proximal ileum was previously reported from this laboratory (14). Similarly, NaGC did not cause any observable destruction to the intestinal mucosa, although this concentration is slightly higher than the normal luminal concentration in the fed state (14).

Although histopathological evaluations failed to indicate any significant irritation to the morphological integrity of the rat intestinal mucosa, care must be taken in defining the safety of these absorption enhancers. Effective and successful control of diabetes requires life-long insulin therapy. As a result, chronic tolerance of a diabetic patient to these agents deserves further investigation. Further, an oral pharmacological availability of 2–3% is still insufficient. More research on absorption enhancers must be conducted to enhance the bioavailability of orally administered insulin.

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