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Intestinal mucosal transport of insulin

Robert J. Schilling and Ashim K. Mitra

Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907 (U.S.A.)

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Summary

The development of an oral dosage form rendering adequate bioavailability (10–20%) of insulin would simplify the lives of diabetics and make intensive treatment easier. Provided that a dosage form is developed to selectively release insulin, it is still likely that intestinal membrane transport and metabolism would remain as barriers to oral insulin delivery. Since the cellular morphology and the enzymatic make-up of the brush-border region vary along the length of the intestine, everted gut sac experiments were performed using segments of the duodenum, jejunum and ileum of the rat small intestine to determine the site(s) where insulin absorption would be optimal. No evidence of active transport processes for insulin was observed. Experiments with intact gut sacs showed no significant degradation of insulin exposed to either the mucosal or serosal tissues. Whole tissue homogenates, however, quickly produced significant losses of insulin. According to the measured flux of insulin across the intestinal mucosa, the duodenum showed very little or no absorption of insulin, while the jejunum and the ileum absorbed low, but significantly greater, amounts of insulin. Mixed micelle solutions, used as absorption enhancers, significantly increased the amount of insulin transported across the intestinal mucosa. Further optimization of the insulin/mixed micelle solutions will be necessary to provide optimal absorption of intact insulin.

Introduction

The treatment of Type I diabetic patients (and some Type II diabetics) requires subcutaneous insulin injections, normally once or twice each day. For more intensive treatment, and better metabolic control, diabetics may receive as many as four insulin injections daily. Despite its success, hypodermic injection is not the ideal means for insulin delivery. Local discomfort and inconvenience of multiple daily injections may discourage many diabetics from accepting intensive insulin treatment regimens, and some Type II diabetics may refuse insulin treatment entirely.

The most acceptable method of chronic drug administration is, of course, by the oral route. The development of an oral dosage form providing adequate bioavailability (10–20%) of insulin would revolutionize the treatment of diabetes. Although it may not completely replace parenteral therapy, oral administration would certainly supplement it. Such a development would make intensive treatment easier and would provide better metabolic

Correspondence: A.K. Mitra, Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907, U.S.A.

control, such that diabetic complications which may normally occur late in life may be alleviated or prevented. Various studies have shown that intact insulin can cross the small intestine of rats (Shichiri et al., 1972; Ziv, 1987), rabbits (Shichiri, 1973), and humans (Balsam et al., 1971; Crane and Luntz, 1968), but bioavailability was very poor due to proteolysis and/or the barrier function of the intestinal membrane. Since the cellular structure and the proteolytic enzyme content vary along the length of the gastrointestinal tract, the intestinal mucosal transport and metabolism of insulin can vary significantly in the different segments of the intestine. Thus, to enhance insulin oral bioavailability, the gut segment(s) where insulin permeability is greatest and metabolism is lowest should be determined. Then, selective delivery of insulin to that site may optimize its absorption.

The studies described in this report utilized the everted gut sac technique to effectively compare the relative rates and extents of insulin transport across three different regions of the rat small intestine. These studies also examined the effects of various promoters to enhance insulin transport across the mucosa. Preliminary experiments using bile salt/fatty acid mixed micelles have shown promising results, as described later. The extent of insulin degradation by the brush-border enzymes was also investigated. Based on these findings, in situ perfusions will be performed in the appropriate portions of the rat small intestine to better determine the rate of insulin absorption in diabetic rats.

Materials and Methods

Materials

Purified porcine zinc insulin in crystal form was kindly donated by Eli Lilly and Company (Indianapolis, IN). The buffer used was a modification of Kreb's Ringer phosphate bicarbonate (KRPB) buffer solution, pH 7.4 (Long, 1961), such that it contained 10 mM dextrose and only one third of the calcium chloride recommended. The calcium chloride was reduced principally to eliminate calcium phosphate precipitation. The modified KRPB buffer thus contained: 113.3 mM NaCl, 4.83 mM KCl, 1.214 mM KH_2PO_4, 1.205 mM $MgSO_4$, 16.96 mM $NaHCO_3$, 10.18 mM Na_2HPO_4, 0.645 $CaCl_2$ and 10 mM dextrose. The buffer solutions were prepared with analytical-reagent grade chemicals. FITC-dextran and sodium glycocholate were obtained from Sigma Chemical Company (St. Louis, MO) and linoleic acid was obtained from Aldrich Chemical Company (Milwaukee, WI). The animals used were male, Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 200–300 g. Sodium pentobarbital (Butler, Columbus, OH) was used as the surgical anesthetic.

Everted gut-sac technique

Rats, having been fasted for 16–20 hours, were anesthetized by an i.p. injection of 60 mg/kg sodium pentobarbital. After making a midline incision in the abdomen, the small intestine was cut at two positions, at 3 cm distal to the stomach and at the ileocecal junction. The entire length of the small intestine was then carefully removed and placed immediately in ice-cold saline solution. The duodenum segment was obtained from the first 6–8 cm portion of the intestine closest to the stomach. The next 25–35 cm of intestine was utilized as the jejunal sections, with the first 8–10 cm designated as the proximal jejunum, the middle 8–10 cm (being about the midpoint of the length of the intestine) designated as the medial jejunum, and the last 8–10 cm portion designated as the distal jejunum. The ileum was obtained from the final 8–10 cm of the intestine, just proximal to the cecum.

Fig. 1 depicts a schematic diagram of the everted gut-sac apparatus. The intestinal segments were everted on a thin stainless steel rod, rinsed with ice-cold saline solution, and then secured to the tip of a 1 ml disposable syringe barrel. The other end of the segment was ligated with silk thread after inserting a 2.5 mm glass bead in an effort to keep the sac open. Additionally, a 3.5 g glass weight was tied to the end of the sac to prevent peristaltic muscular contractions, which may otherwise alter the shape and internal volume of the sac. The gut sac was filled with a known volume of the modified KRPB buffer solution and

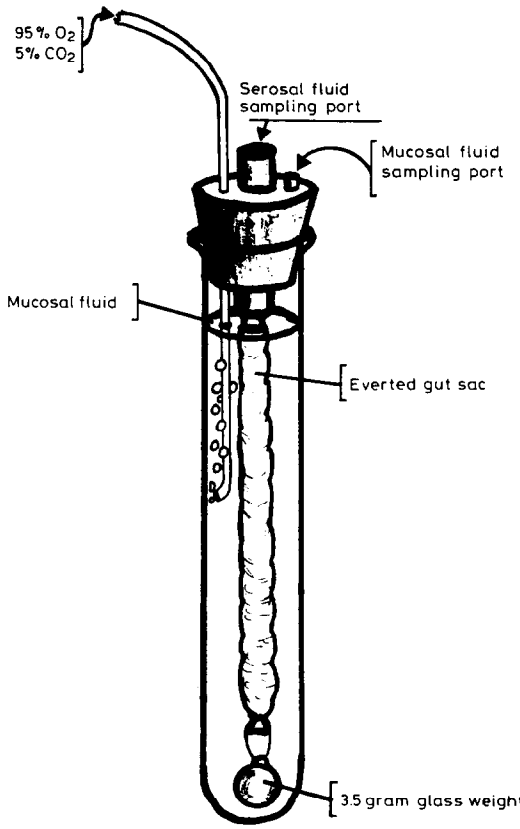

Fig. 1. Everted gut sac set-up. Volume of mucosal fluid was 25 ml, serosal fluid volume was 1.0 to 1.5 ml, and gut sac measured 8 to 10 cm in length.

was then placed inside a test tube containing 25 ml of 37°C test solution continuously bubbled with 95% O_2/5% CO_2. Samples were drawn from both the serosal and the mucosal compartments at appropriate time intervals.

The duration of the gut sac tissue viability was evaluated first by the examination of its gross histology under a light microscope. Tissue samples were taken (a) immediately following its removal from the animal (control); (b) following preparation of the everted gut sac, but prior to the beginning of the experiment; and (c) after running the experiments for 10, 20, 30 and 40 min at 37°C. The samples were fixed in phosphate-buffered formalin (Humason, 1979), embedded in paraffin, sliced to 5 μm, and stained with periodic acid Schiff reagent. The tissue samples from the experiments were then compared with the appropriate controls to observe any changes in the membrane structure.

Additionally, experiments were performed to show that dextrose was actively transported. To examine the integrity of the membrane barrier, the uptake of phenol red was examined in the absence or in the presence of mixed micellar solutions. Phenol red, being charged and very water soluble, may be absorbed slowly by aqueous pores or paracellular routes. The apparent permeability of phenol red was determined in the three intestinal regions with mucosal solutions containing 40 μg/ml (113 μM) phenol red. Likewise, the impermeability of FITC-dextran (20 kDa, 0.75 mg/ml) was examined in the absence or presence of mixed micelles by determining its concentration in the serosal fluid at the end, i.e., 25 min. The FITC-dextran was first purified to remove low molecular mass impurities as suggested by Preston et al. (1987).

Investigation of active transport processes for insulin

An initial concentration of 0.83 μM (5 μg/ml) insulin in modified KRPB buffer solution was placed on both the mucosal and serosal sides of the gut sac. Samples of 50 μl of were withdrawn from both sides at predetermined time points and stored in glass vials at 3°C for a time period not exceeding 24 h. The sample volume was replaced with 50 μl of fresh 0.83 μM insulin solution. The duodenum, medial jejunum and ileum sections were studied in triplicate. If the serosal insulin concentration increased with time, then active transport processes would be indicated, otherwise the concentration of insulin in each fluid would remain essentially unchanged. To demonstrate the biochemical integrity of the experimental gut membrane, experiments were performed which showed glucose was transported against a concentration gradient from the mucosal to the serosal fluid (data not shown).

Measurement of the apparent permeability of insulin in various intestinal regions

Preliminary experiments with 16.7, 41.7, 83.3 and 833.0 μM insulin in modified KRPB buffer solution as the mucosal solution and blank KRPB buffer solution as the serosal fluid were carried

out. Moreover, insulin permeability across the duodenum, medial jejunum, and ileum was examined in four rats at 37°C and in five rats at 17°C, all using 83.3 μM (0.5 mg/ml) insulin as the mucosal solution, and blank KRPB buffer solution as the serosal fluid. Samples of 50 μl the serosal and mucosal fluids were withdrawn at appropriate times and stored in glass vials at 3°C for a period of less than 24 h before they were subjected to HPLC analysis.

The apparent permeability of insulin across the intestinal mucosa was determined from flux. Flux is defined in Eqn 1,

$$J = \frac{dM}{S\,dt} = \frac{DK}{h}([C]_d - [C]_r) \quad (1)$$

where, M is the amount of insulin crossing the membrane; S the apparent surface area of the gut sac, determined by measuring the external dimensions of the sac; D the diffusion coefficient of insulin; K the partition coefficient for insulin (between membrane and solution); h, the thickness of the membrane barrier; $[C]_d$ the donor concentration of insulin (mucosal side); and $[C]_r$, the receptor concentration of insulin (serosal side). Since the insulin uptake was very low ($< 0.1\%$ over the time period of the study) and $[C]_d$ remained essentially constant during the experiments, then at all times $[C]_d \gg [C]_r$. Eqn 1 can thus be simplified to:

$$J = \frac{dM}{S\,dt} = \frac{DK}{h}[C]_d \quad (2)$$

Substituting the apparent permeability coefficient (P_{app}) for DK/h,

$$\frac{dM}{S\,dt} = P_{app}[C]_d \quad (3)$$

By plotting $M/S[C]_d$ versus time, the data can be normalized with respect to the diffusional area of the gut sacs and the donor insulin concentrations. The slope of this plot, as determined by linear regression, is then the apparent permeability, as represented by Eqn 4:

$$P_{app} = \frac{1}{S[C]_d}\frac{dM}{dt} \quad (4)$$

It is important to remember that the apparent permeability determined in everted gut sac experiments can provide meaningful comparisons, but it will be lower than the permeability which would be observed in vivo, where the diffusional and metabolic barriers do not include the serosal tissue. With the duodenal, jejunal and ileal sections from the same animal having been studied, the apparent permeability of insulin across the different intestinal regions could be compared statistically using a paired Student's t-test. Experiments were also performed under identical conditions to determine whether there was any significant degradation of insulin in the bulk solution by the intestinal mucosa (i.e., the brush-border enzymes) and whether any correlation existed between mucosal insulin degradation and insulin absorption. Finally, to verify whether any insulin loss from the mucosal solution was due to enzymatic degradation, intestinal segments were heated at 95°C for 30 min prior to exposing them to the insulin solution so as to degrade any enzymes present in the tissue.

Investigation of insulin absorption enhancement

In an attempt to enhance the absorption of insulin, bile salt/fatty acid mixed micelle solutions were employed to alter the membrane fluidity. Insulin and sodium glycocholate were dissolved in modified KRPB buffer solution, then linoleic acid was added. This mixture was sonicated at 37°C in a Branson 3200 ultrasonicator (Shelton, CT) for 8–10 min, yielding a clear final solution containing 40 mM sodium glycocholate and 15 mM linoleic acid in the form of mixed micelles, with 83.3 μM insulin. The amount of insulin transported across the duodenum, jejunum and ileum in the presence of mixed micelles was then compared to that observed in the absence of any adjuvant.

Analytical techniques

The samples were analyzed for insulin concentration by a high-performance liquid chromatographic method. Injections were made directly onto a reversed-phase C_8, 5 μm 50 × 4.6 mm Microsorb column (Rainin Instruments, Woburn, MA). Chromatography was carried out

using a Rainin single-pump liquid chromatographic system equipped with a variable wavelength ultraviolet detector (Knauer, Berlin) set at 220 nm. The mobile phase comprised of triethylammonium phosphate, pH 2.25 (TEAP 2.25) with 26.5% acetonitrile and the flow rate was 0.9 ml/min. TEAP 2.25 was prepared by adjusting 0.25 N phosphoric acid to pH 2.25 with triethylamine. The minimum detectable insulin concentration was about 1 μg/ml.

Phenol red samples were diluted and analyzed using a Beckman DU-7 spectrophotometer (Irvine, CA) set at 558.5 nm. FITC-dextran samples were diluted and analyzed with a SLM-8000 fluorescence detector (SLM Instruments, Urbana, IL), with excitation and emission wavelengths set at 490 and 516 nm, respectively.

Results and Discussion

Everted gut sac technique

Insulin is a relatively hydrophilic molecule and therefore has a low partition coefficient at pH 7.4 (Banks and Kastin, 1985). Results from our experiments also found insulin to have a negligible partition coefficient close to zero using an aqueous phase of various pH values, i.e., pH 3.5, pH 6.0, pH 7.4 or pH 9.0 (data not shown). It is important to know how long the intestinal mucosal barrier remains intact under the conditions of the everted gut sac experimental set-up. When the intestinal mucosal cells start deteriorating, and aqueous pathways open up, hydrophilic molecules may more easily pass through the enlarged intercellular junctions. Upon examination of the gross histology at ×100 and ×430 magnification, the intestinal mucosa appears very normal up through 20 min of incubation at 37 °C, though some edema is shown to develop by 20 min. The tissue samples at 30 and 40 min, however, showed marked edema and/or cell lysis. Therefore, under these experimental conditions, it appears that the gut sac is viable for at least 20 min. These observations agree well with studies reported by Osiecka et al. (1985).

Permeability experiments with phenol red showed apparent permeabilities on the order of (1–1.5) $\times 10^{-5}$ cm/s (Table 1). The rate of absorption was constant for the first 20–30 min, after which time the flux was observed to abruptly increase, probably as a result of the loss of membrane integrity. In studies with 20 kDa FITC-dextran, apparent permeability was found to be much lower, on the order of 1×10^{-8} cm/s, and at 25 min the serosal concentration of FITC-dextran was only 0.01–0.02% that of the mucosal fluid. Additionally, it appears that the mixed micellar solutions did not significantly disrupt the intestinal membrane, since little change was observed histologically, nor was there any significant change in the uptake of phenol red or FITC-dextran. Considering the histology studies and the results from the phenol red and FITC-dextran experiments, everted gut sac studies were thus limited to less than 25 min to ensure that the membrane integrity is maintained throughout the experiment.

TABLE 1

Comparison of the apparent permeabilities observed in everted rat small intestine with modified KRPB buffer

Compound	M_r	Apparent permeability (cm/s) Mean ± SE	
		$P_{app} \times 10^5$	
Phenol red ($n = 5$)	354	duodenum	1.10 ± 0.26
		medial jejunum	1.22 ± 0.21
		distal jejunum	1.55 ± 0.32
		ileum	1.54 ± 0.29
		$P_{app} \times 10^7$	
Insulin ($n = 3$–5)	5800	duodenum	0.78 ± 0.54
		medial jejunum	4.97 ± 1.51
		distal jejunum	7.80 ± 1.54
		ileum	6.82 ± 1.87
		$P_{app} \times 10^8$	
FITC-dextran ($n = 1$)	20000	duodenum	0.61
		distal jejunum	2.40

Active transport of insulin

Active transport mechanisms for a number of substrates, including small peptides, are found throughout the small intestine of rats and other animals (Adibi and Kim, 1981). While it would seem unlikely that a carrier would exist in adult rats for molecules as large as insulin, experiments

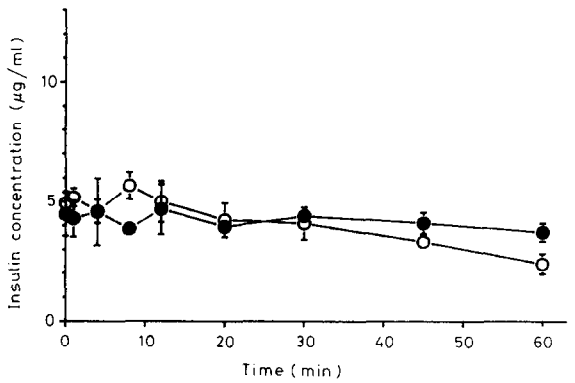

Fig. 2. Experiments examining for presence/absence of active transport of insulin in the ileum, mean ± S.E., $n = 3$. At start of experiment, 0.833 μM insulin in modified KRPB buffer solution, pH 7.4, 37°C was placed on both mucosal and serosal sides of gut sac. No statistically significant increase in insulin concentration was seen in the serosal fluid. ●, serosal side; ○, mucosal side.

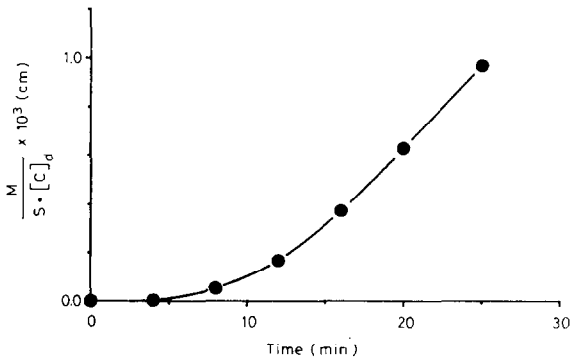

Fig. 3. Example of passive diffusion of insulin into ileal everted gut sac, with experiment starting with 833 μM insulin in modified KRPB buffer solution, pH 7.4, 37°C as mucosal fluid, and serosal fluid was blank. M, μg of insulin absorbed; S, apparent surface area of gut sac; $[C]_d$, donor (mucosal) insulin concentration.

were performed nonetheless to determine whether an active transport system for insulin could be present. At the start of these experiments, 5 μg/ml insulin solutions were placed on both sides of the gut sac membrane. Fig. 2 exhibits the results from such an experiment performed with the ileal segment. For all intestinal regions, comparisons of the mucosal and serosal insulin concentrations by the paired t-test did not show any significant differences. Therefore no evidence of active transport mechanisms for insulin could be observed in the duodenum, jejunum or ileum. Similar experiments carried out using higher insulin concentrations (1.67, 16.7, 41.7 and 83.3 μM) also showed no evidence of active transport for insulin.

Nonfacilitated passive diffusion of insulin

Preliminary experiments were performed with four different insulin concentrations using medial jejunal gut sacs. Since the apparent permeability coefficients were very similar regardless of the insulin concentration used, an insulin concentration of 83.3 μM was chosen as the starting mucosal insulin concentration. This concentration produced sufficient transport of insulin during the first 20 min to enable accurate quantitation. The rate of insulin uptake was constant for the first 20–30 min, from which the apparent permeability could be determined (Table 1). Longer experiments showed a large increase in absorption of insulin between 30–60 min which correlates well with the loss of membrane integrity. Some experiments were also performed using 833 μM insulin solutions, an example of which has been presented in Fig. 3.

Paired t-tests were employed to compare the apparent permeability coefficients of insulin across each intestinal region. Although the overall amount of insulin transported was low (< 0.2%), significant differences in absorption were found among different regions. Fig. 4 depicts results from experiments conducted at 17°C. The permeability

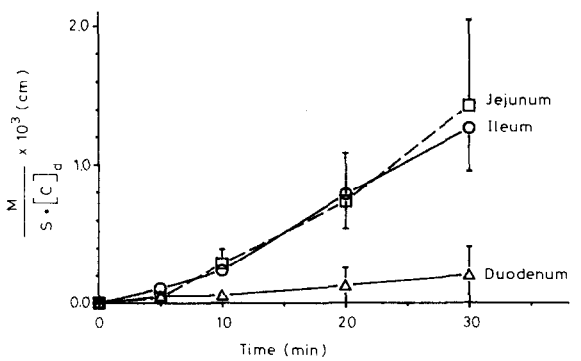

Fig. 4. Comparison of insulin absorbed into the everted gut sac among three regions of the small intestine from each of four animals at 17°C. At start of the experiments mucosal fluid was 83.3 μM insulin in modified KRPB buffer solution, pH 7.4, and serosal fluid was blank.

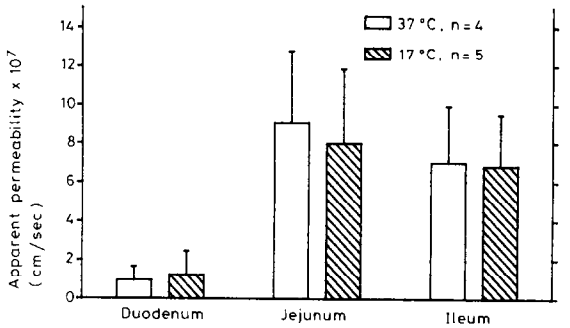

Fig. 5. Apparent permeability (mean ± S.E.) of insulin across the three intestinal regions at 17 and 37 °C. At start of each experiment, the mucosal fluid was 83.3 μM insulin in modified KRPB buffer solution, pH 7.4, and serosal fluid was blank.

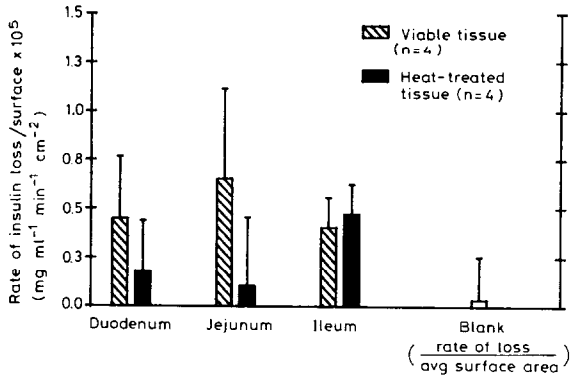

Fig. 6. Rate of insulin loss per unit area (mean ± S.E.) from mucosal solutions.

coefficients of insulin across the ileum and jejunum were very similar, however little or no insulin was absorbed in the duodenum. Virtually identical results were obtained at 37 °C (Fig. 5), with P_{app} in the jejunum (9.08×10^{-7} cm/s) being nearly the same as that for the ileum (7.01×10^{-7} cm/s), but each of these being significantly greater than the P_{app} for the duodenum (9.39×10^{-8} cm/s), as presented in Table 2. The permeability in each intestinal region showed no difference between experiments performed at 17 and 37 °C, providing additional evidence that no active transport processes for insulin were present. It is also important to point out that later experiments where more samples were collected during the course of the experiment produced the same P_{app} for insulin in each region of the intestine as was determined by earlier experiments with fewer time points.

TABLE 2

Statistical analysis of apparent permeability of insulin through everted gut sacs

Nondirectional, paired Student's *t*-test ($n = 4$)	
Test, P_{app} in:	Significance
Duodenum vs jejunum	$P = 0.12$
Duodenum vs ileum	$P = 0.08$
Jejunum vs ileum	NS ($P = 0.70$)

NS, not significantly different.
Mucosal fluid: 83.3 μM (0.5 mg/ml) insulin in modified KRPB buffer, 37 °C; serosal fluid at start of experiment: blank modified KRPB buffer.

In these experiments, the final serosal concentration of insulin at 20 min was less than 0.1–0.2% that of the starting mucosal insulin solution. The extent to which insulin degradation occurs was then addressed. Experiments to confirm the stability of insulin in the mucosal fluid of the everted gut sac setup revealed that at least 90–95% of the initial insulin concentration was still present at 30 min, regardless of whether the duodenum, jejunum or ileum was used. Similarly, little or no insulin loss was observed in experiments exposing insulin to the serosal tissue. Comparing the rates of loss of insulin from the mucosal fluid, normalized by the apparent surface area of the gut sac, showed no significant differences among the three intestinal regions (Fig. 6). Testing by one-way ANOVA of the rates of mucosal insulin loss per unit area of gut sac in viable tissue, heat treated tissue and blank solution (where the rate was divided by the average surface area of all gut sacs) also showed no significant differences at the 95% confidence level. Mucosal insulin loss in each intestinal region was compared to that observed in the heat-treated intestinal experiments, and again, no significant differences were observed (Table 3). Thus, while only small quantities of insulin appear in the serosal fluid, insulin metabolism at the brush border must be very minimal, if present at all. Differences in the apparent permeability among the three intestinal regions therefore are not the result of differences in insulin degradation in the bulk mucosal fluid. In addition, the low

TABLE 3

Results of one-tailed t-test for differences in the rates of insulin loss/surface area (n = 4)

Insulin loss per unit surface area ($mg \cdot ml^{-1} \cdot min^{-1} \cdot cm^{-2}$) in:		Insulin loss per unit surface area ($mg \cdot ml^{-1} \cdot min^{-1} \cdot cm^{-2}$) in:	*P*
Viable duodenum	vs	heat-treated duodenum	0.268
Viable jejunum	vs	heat-treated jejunum	0.189
Viable ileum	vs	heat-treated ileum	0.384

None of the differences are significant.

apparent permeability observed in the everted gut sac is not due to significant degradation of the insulin molecule in the bulk mucosal or serosal solutions, but more likely it is due to the barrier presented by the intestinal membrane.

On the other hand, disruption of cells in whole tissue intestinal homogenates subjects insulin to enzymes and lysozymes apparently not encountered in intact tissue, since homogenates of the duodenum and of the jejunum showed near complete loss of insulin within 30 min (data not shown). Luminal enzymes, i.e., trypsin and chymotrypsin, have also been shown to rapidly degrade insulin. Concerning the brush-border enzymes, various reports have shown that brush-border peptidases, such as the predominant aminooligopeptidases, are present at the aqueous/membrane interface and are capable of acting upon only small peptides containing eight or fewer amino acid residues (Adibi and Kim, 1981). If insulin could be protected from trypsin or chymotrypsin digestion (as in these experiments), it may be a much larger substrate than normally acted upon by the brush-border enzymes. In summary, it appears that luminal and cytosolic peptidases may be largely responsible for insulin degradation in the small intestine.

From these everted gut sac experiments, then, it becomes evident that the apparent permeability of insulin is significantly greater in the latter two thirds of the small intestine than in the duodenum. Compared to distal regions of the small intestine, earlier portions of the intestine have longer villi, and thus more true surface area per unit length of intestine. The duodenal gut sacs, however, showed the poorest absorption of insulin. This may be due, in part, to morphological differences of the cells or the tight junctions. Differences in the depth of the tight junctions and the number of strands involved in the junctions have already been reported between the jejunum and the ileum (Trier and Madara, 1981). On a comparative basis, these experiments tend to suggest that selective delivery of insulin to the latter two thirds of the small intestine may lead to the optimum bioavailability. In situ perfusion experiments in rats will be performed to accurately measure the actual rate of insulin absorption.

Insulin absorption enhancement

With the apparent permeability of insulin in the jejunal and ileal gut sacs being only on the order of $(7–9) \times 10^{-7}$ cm/s, it will be necessary to enhance the absorption of insulin in order to produce reasonable bioavailability (10–20%). Mixed micellar solutions were examined as a means for increasing the fluidity of the mucosal membrane and subsequent enhancement of insulin absorption. The role of bile salt/fatty acid mixed micelles in gastrointestinal absorption is already well established. Dietary fats are emulsified by bile salts in the small intestine. Then, with the aid of pancreatic lipase, monoglycerides and free fatty acids are produced. The monoglycerides, free fatty acids, and bile salts all combine to form mixed micelles from which the monoglycerides and free fatty acids diffuse towards the intestinal membrane where absorption occurs (Vander et al., 1980).

Absorption enhancement of drugs has been shown to occur with mixed micelles in the colon, rectum and small intestine (Muranushi et al., 1980a; Takada et al., 1985; Tomita et al., 1988), and also in the nasal mucosa (Tengamnuay and Mitra, 1990). It is highly unlikely that the hydrophilic insulin molecule would become entrapped in the lipophilic core of the mixed micelles. Rather it is probable that the mechanism of absorption promotion from the mixed micelles requires the fatty acid to become incorporated into the membrane biophase to increase its fluidity (Muranushi et al., 1980a, b). Both Muranishi et al. (1979) and Taniguchi et al. (1980) reported that 40 mM bile

salt in mixed micelles was effective in promoting the absorption of aminoglycosides and heparin in the small intestine. Tengamnuay and Mitra (1990) found that the optimal ratio of bile salt to fatty acid for enhancement of nasal absorption of small peptides was roughly 3 : 1. Therefore, a mixture of 40 mM sodium glycocholate and 15 mM linoleic acid was used in the preparation of mixed micelles. HPLC chromatograms of insulin in mixed micellar solutions did not reveal any instability or change in insulin peak shape or retention.

While repeated, long-term effects of exposure of the gut tissue to mixed micellar solutions were not determined, it has been reported that lipids mixed with bile salts to form mixed micelles prevent the damaging effects that the same concentration of bile salts alone would produce (Feldman et al., 1973; Coleman et al., 1979). Muranishi et al. (1979) and Tengamnuay and Mitra (1990) observed using in situ experiments that the permeability enhancement of mixed micelle solutions was reversible. Experiments with everted gut sacs did not show any significant absorption enhancement for the very water soluble dye, phenol red (Table 4). Thus, aqueous/paracellular pathways may not have been significantly disturbed. Likewise, mixed micellar solutions did not apparently increase the absorption of the large, 20 kDa FITC-dextran molecules. Also, observation of gross morphology with a light microscope did not show any visible differences between gut sacs treated with mixed micelle solutions versus blank modified KRPB buffer solution over the same time periods. From all of these results it may be inferred that during the time course of these experiments the mixed micelles do not appear to cause appreciable damage to the intestinal mucosa.

In mixed micellar solutions insulin permeability through the everted gut sacs was significantly increased in the duodenum ($P < 0.1$) and medial and distal jejunum ($P < 0.05$), but there was insignificant change in the ileum (Fig. 7). Insulin, while a hydrophilic protein, also contains lipophilic regions which are involved in the self-association of insulin to form dimers, tetramers and hexamers. At present, the extent of insulin absorption via transcellular versus paracelluar routes is unknown. Since mixed micellar solutions did not enhance the apparent permeability of the hydrophilic, phenol red or FITC-dextran, the mechanism by which they may promote insulin absorption may be via transcellular transport. In any case, the movement

TABLE 4

Comparison of the apparent permeabilities observed in everted rat small intestine with modified KRPB buffer or mixed micellar solutions

Phenol red	$P_{app} \times 10^5$		Ratio *
	in KRPB ($n = 5$)	with mixed micelles ($n = 5$)	
Duodenum	1.10 ± 0.26	0.87 ± 0.23	0.80 [a]
Medial jejunum	1.22 ± 0.21	1.13 ± 0.14	0.93 [a]
Distal jejunum	1.55 ± 0.32	1.23 ± 0.37	0.79 [a]
Ileum	1.54 ± 0.29	0.71 ± 0.12	0.46 [a]
Insulin	$P_{app} \times 10^7$		Ratio *
	in KRPB ($n = 3$–5)	with mixed micelles ($n = 5$)	
Duodenum	0.78 ± 0.54	6.26 ± 2.62	8.03 [b]
Medial jejunum	4.97 ± 1.51	15.40 ± 4.09	3.10 [c]
Distal jejunum	7.80 ± 1.54	18.40 ± 3.40	2.36 [c]
Ileum	6.82 ± 1.87	6.50 ± 1.76	0.95 [a]

* Ratio of apparent permeability with mixed micelles to apparent permeability in the absence of mixed micelles.

[a] No significant increase in P_{app}.

[b] Significantly greater P_{app} with mixed micelles, $P < 0.1$.

[c] Significantly greater P_{app} with mixed micelles, $P < 0.05$.

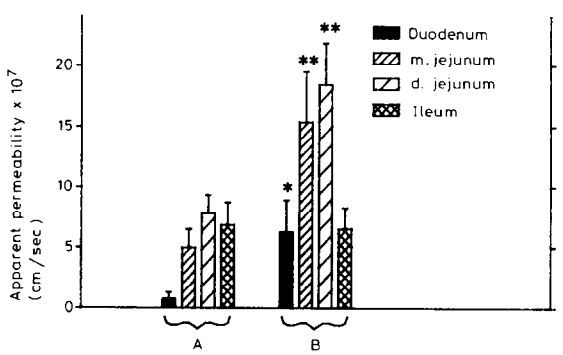

Fig. 7. Apparent permeability (mean ± S.E.) of insulin across everted gut sac segments, with mucosal solutions of 83.3 μM insulin in: A, modified KRPB buffer solution ($n = 5$); or B, modified KRPB buffer solution with 40 mM sodium glycocholate/15 mM linoleic acid mixed micelles ($n = 4$). For A versus B, * $P < 0.1$; ** $P < 0.05$.

of intact insulin across the intestinal membrane was significantly increased (Table 4), and further in situ studies will confirm it.

As shown in Fig. 8, insulin absorption from mixed micellar solutions exhibited a lag time of about 10 to 15 min, after which the insulin steady-state flux was established. This may indicate the necessity for the mixed micellar components to become incorporated into the lipid bilayers prior to the absorption promotion effect. Insulin absorption in four intestinal regions is shown in Fig. 9. The ratio of the apparent permeability of insulin in the presence of mixed micellar solutions to that in the presence of modified KRPB buffer solution was 8.03 for the duodenum, 3.10 for the medial jejunum, 2.36 for the distal jejunum, and 0.95 for the ileum, while the greatest overall apparent permeability for insulin was found in the distal jejunum (Table 4). Even with this increase in insulin absorption, the serosal insulin concentrations at 20–25 min achieved only about 1–2% that of the mucosal insulin concentration. Absorption enhancement experiments using different ratios and/or amounts of fatty acid/bile salt combinations in the mixed micelle solution may further optimize insulin absorption.

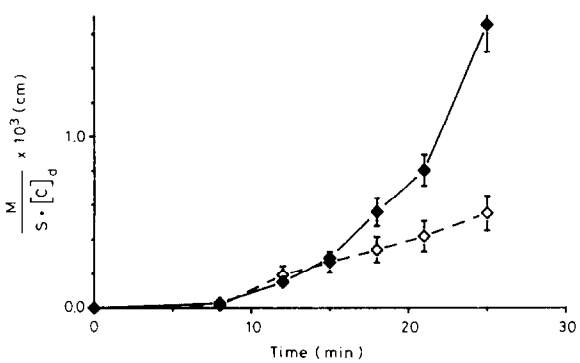

Fig. 8. Effect of mixed micelles on insulin absorption in distal jejunum everted gut sacs, showing mean ± S.E. At start of experiment the mucosal fluid was 83.3 μM insulin in modified KRPB buffer solution, 37 °C, and serosal fluid was blank. *M*, μg of insulin absorbed; *S*, apparent surface area of gut sac; $[C]_d$, donor (mucosal) insulin concentration. ◇------◇ insulin in Ringer ($n = 5$); ◆———◆, insulin in Ringer with mixed micelles ($n = 4$).

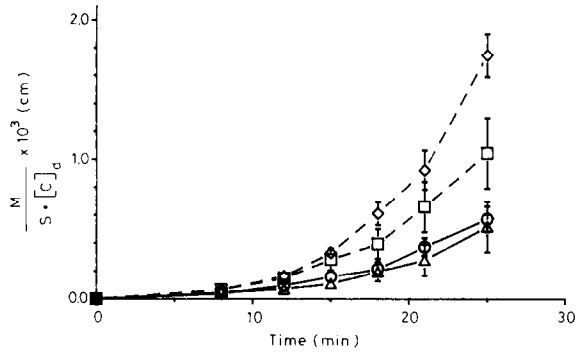

Fig. 9. Comparison of insulin absorption in the presence of mixed micelles into everted gut sacs from four regions of the small intestine from each of five animals (mean ± S.E.). Experiments started with mucosal solutions of 83.3 μM insulin in modified KRPB buffer solution with sodium glycocholate/linoleic acid mixed micelles, and serosal fluid was blank ($n = 5$). ◇------◇, distal jejunum; □-----□, medial jejunum; ○———○, ileum; △———△, duodenum.

The ileum actively transports ionized bile salts, whereas the duodenum and jejunum apparently do not possess such transport systems (Dietschy, 1968). Initially, the absence of any permeability enhancement by mixed micelles in the ileal gut sacs was thought to be the result of this active transport system removing the bile salts from the mixed micellar solutions. Later experiments, however, revealed no direct relationship between the bile salt uptake and the absence of absorption promotion of insulin. Muranushi et al. (1980a) examined the lipid uptake from mixed micellar solutions in the large intestine and found no relationship between the extent of lipid uptake and the magnitude of the permeability enhancement produced by different lipids. Experimenting with a number of various lipids, they concluded that the lipid structure and its interaction with the membrane biophase is responsible for increasing the fluidity of the membrane and subsequent absorption enhancement properties. Therefore, the differences in the interaction of the linoleic acid in the mixed micelle solutions with the morphologically different cells of the duodenum, jejunum and ileum may partially account for the various degrees of absorption enhancement observed in the in vitro gut sac experiments.

Conclusion

Since the oral route of administration is the most convenient and acceptable method of chronic drug administration, studies have been initiated to examine insulin absorption from the small intestine. The initial studies have been directed to identify the optimal site(s) in the small intestine for insulin absorption. There was no evidence of any active transport mechanisms for insulin in any part of the small intestine. Nonfacilitated passive diffusion of intact insulin was noticeable. No significant degradation of insulin by the intact brush border was observed. The apparent permeability of insulin was significantly greater in the jejunum and ileum relative to the duodenum, possibly due to the inherent morphological differences of the various intestinal regions. Insulin absorption studies in human patients lacking pancreatic enzymes have been performed by Crane and Luntz (1968) and by Balsam et al. (1971) where 1% or less of the insulin dose administered was absorbed, yet insulin was delivered to the duodenum or the early jejunum. Had insulin been delivered to more medial or distal regions of the small intestine, absorption may have been greater as would be predicted by the experimental results herein. Selective delivery of insulin to the latter two thirds of the small intestine, which should also decrease the exposure to pancreatic enzymes, may ensure better bioavailability.

Since the apparent intestinal permeability of insulin may be too low to produce any significant blood glucose reduction in live animals, it was necessary to examine methods for absorption enhancement using mixed micellar solutions of linoleic acid and sodium glycocholate. Insulin absorption was significantly enhanced in the duodenum and jejunum, with the highest overall apparent permeability found in the jejunum, where the addition of mixed micelles to the modified KRPB buffer solution increased the P_{app} of insulin from about 7×10^{-7} cm/s to $(1.5\text{–}1.8) \times 10^{-6}$ cm/s. These results are in contrast to the observed lack of permeability enhancement for the small, charged phenol red molecules, and thus the mixed micelles may affect the transcellular transport of insulin rather than the paracellular pathway. However, Tomita et al. (1988) showed that mixed micellular solutions increased the aqueous equivalent pore radius in the colon from 8–9 Å (control) to 14–16 Å, which corresponds closely to the hydrodynamic radius of insulin. These effects must be investigated further using in situ techniques where the true diffusion barrier is the intestinal mucosa alone. The reason for the lack of mixed micellar effect on the permeability enhancement of insulin in the ileum is still unclear.

Summarizing, results from this study tend to suggest that the oral delivery of insulin may be viable. Selective release of insulin in the mid-jejunum would present insulin to the optimal region for absorption, and it would also help to protect insulin from gastric and pancreatic enzymes. To produce adequate insulin bioavailability, the rate of absorption must be greater than the rate of metabolism. Using the comparative results determined in these experiments, in situ perfusions will now be performed in the latter two thirds of the intestine to measure rates of insulin absorption and subsequent glucose lowering in diabetic rats. Mixed micelles enhance the absorption of insulin, but this effect must be optimized by investigating different compositions of the mixed micelles, varying the ratios and amounts of fatty acids and bile salts. The metabolism of insulin by pancreatic enzymes is also currently being examined. Small chemical changes in the insulin molecule might protect susceptible peptide linkages from hydrolysis and might decrease the intestinal metabolism of insulin.

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