Site-Dependent Effect of Aprotinin, Sodium Caprate, Na_2EDTA and Sodium Glycocholate on Intestinal Absorption of Insulin

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In order to determine an advantageous site for intestinal insulin absorption, the hypoglycemic effects of insulin after administration to the duodenum, the jejunum, the ileum and the colon were investigated using an *in situ* loop method. Insulin solution was administered to the various loops of fasted rats with or without aprotinin (AP) as a protease inhibitor, or absorption enhancers such as sodium caprate, Na_2EDTA or sodium glycocholate. An obvious hypoglycemic effect of insulin alone was seen only in the ileum loop washed with phosphate buffered saline. When coadministered with AP, the most remarkably amplified effect was again observed in the ileum. In the ileum, the area under the serum insulin levels *vs.* time curve from 0 to 4 h was linearly related to the logarithm of the AP dose. Both sodium caprate and Na_2EDTA significantly promoted the hypoglycemic effect of insulin at all sites, and their intensity increased towards the distal regions of the intestine. On the other hand, sodium glycocholate improved only colonic insulin efficacy. These results suggest that the ileum seems to be the most useful region in the small intestine for insulin absorption; however, insulin must be protected from proteolysis to enhance its absorption. In addition, the insulin efficacy could be increased by absorption promoters more effectively in the colon than in the small intestine.

Keywords insulin; intestinal absorption; hypoglycemic effect; protease inhibitor; absorption promoter

Introduction

Endogenous insulin is released by the pancreas into tributaries of the hepatic portal vein, resulting in direct delivery of insulin to the liver. The liver is the principal target organ of insulin and also removes half of the insulin presented to it in a single transhepatic circulation.[1] Oral insulin therapy is thought to be close to the physiological state because it may offer a means of improving portal levels of insulin and may also curtail the peripheral hyperinsulinemia. The oral administration of insulin is very useful; however, poor absorbability and low stability of insulin in the gastrointestinal tract make it difficult.

Much evidence that insulin could be absorbed from the intestinal tract has been reported. Insulin can be absorbed from the ileum,[2] the ascending colon[2a] and the descending colon.[3] However, the apparent insulin permeability differs among the various intestinal regions.[4] In addition, the effects of a protease inhibitor and/or an absorption enhancer on the insulin absorption seemed to be different between the ileum and the colon.[2a] The intestinal tract is biologically and physiologically so diversified that it has unique features offering possibilities for selective drug targeting. Thus, an advantageous region for insulin absorption seems to exist in the digestive tract. In order to develop successful strategies for enhancement of the oral bioavailability of insulin, it is very important to investigate insulin absorption from the intestine.

In this study, the insulin absorption from various sites in the rat intestine was compared using the *in situ* ligated loop technique. In a previous paper we reported that the hypoglycemic effect of insulin *via* the oral route could be amplified by the protease inhibitor, aprotinin (AP).[5] Thus, we attempted to determine the effects of AP on the insulin absorption in various intestinal regions. The present studies also examined the insulin absorption promotive effects of sodium caprate, Na_2EDTA and sodium glycocholate in the different regions.

Experimental

Materials Crystalline porcine insulin (Zn-insulin, 26.1 U/mg), sodium glycocholate, Na_2EDTA (ethylenediaminetetraacetic acid, disodium salt, dihydrate), and AP (13.3 TIU/mg) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium caprate was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Pentobarbital sodium was obtained from Pitman-Moore Inc., Mundelein, IL, U.S.A. All other chemicals were of reagent grade.

***In Situ* Absorption Experiments** Male Wistar rats weighing 200—250 g were fasted for 24 h prior to the experiments and were anesthetized by an i.p. injection of 60 mg/kg sodium pentobarbital. The rats were restrained in a supine position on a board which was kept at a surface temperature of 37 °C. A small midline incision was made in the abdomen and 6—7 cm loops of the duodenum, the jejunum, the ileum and the colon were identified and ligated at both ends. The duodenum loop was made at the first portion of the intestine, which was the closest to the stomach. The next portion, 5 cm away from the ligament of Treitz, was utilized as the jejunum loop. The ileum loop was made at the end of the small intestine, just proximal to the *ileo-cecal* junction. The colon loop was made at the ascending colon. In experiments designed to remove luminal enzymes, a washing treatment was performed on each intestinal loop. Both ends of the loop were cannulated with a polyethylene tube, and the loop was washed gently with 10 ml of warmed phosphate buffered saline (PBS: 137 mM NaCl, 2.6 mM KCl, 6.4 mM $Na_2HPO_4 \cdot 12H_2O$ and 1.4 mM KH_2PO_4; pH 7.4; 37 °C). The remaining PBS in the loop was then washed out with air. In the case of the duodenum loop, the bile duct was ligated before the washing treatment. The rats were fixed for 1 h after the operation.

Various test solutions (0.5 ml) prepared with PBS (pH 7.4) were administered directly into the loops. The dose of insulin was fixed at 50 U/kg body weight. In the case of the AP coadministration study, 3 levels of AP dose, 6.7 (L), 13.3 (M) and 26.6 (H) TIU/kg, were used. In order to examine the effect of an absorption enhancer, sodium glycocholate, Na_2EDTA and sodium caprate were each used as a 1% solution. The pH values of the test solutions were measured and adjusted to pH 7.4 by the addition of 0.1 N NaOH or 0.1 N HCl as needed. Approximately 5 min before administration, a 0.2 ml aliquot of blood sample was taken from the jugular vein. Subsequent blood samples were taken at 15, 30, 60, 120, 180 and 240 min after dosing.

In order to calculate the efficacy of insulin intestinal administration relative to i.v., insulin solutions were administered intravenously *via* the jugular vein. In this case, the same operation as in the intestinal administration experiments was performed on the rats. Insulin solutions were prepared by dissolving an appropriate amount of crystalline porcine insulin in PBS. The insulin i.v. doses were 0.5, 1.0 and 3.0 U/kg body weight. A 0.2 ml aliquot of blood sample was collected from the jugular

vein on the opposite side to the injection before and at 5, 15, 30, 60, 120, 180 and 240 min after dosing. Serum was separated by centrifugation at 3000 rpm for 2 min and kept frozen until analysis. The relative efficacy was calculated according to the method described by Morishita *et al.*[5]

Analytical Method The serum insulin levels were measured by enzyme immunoassay (EIA) using an Insulin EIA kit (Dainabot Co., Ltd., Tokyo, Japan). The serum glucose level was determined by the glucose oxidase method using a glucose B-Test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Statistical Analysis All data are given as mean ± S. E. of 4—6 animals. For group comparisons, an analysis of variance (ANOVA) with a one-way layout was applied, followed by the Student's unpaired *t*-test.

Results and Discussion

Changes in Blood Glucose Level after Administration of Insulin Solution to Various Loops Figure 1 shows the changes in serum glucose level following the injection of insulin solution with or without the washing treatment. Administration of insulin alone (50 U/kg) did not decrease the glucose level, as compared with control, at all intestinal regions. When the washing treatment was performed, an obvious decrease in the glucose level was observed only at the ileum. Thus, it was confirmed that the low intrinsic permeability of insulin and/or the enzymatic degradation in the small intestine were major barriers for insulin absortion.

When insulin was coadministered with AP(M), the biological effect of insulin was remarkably amplified, again in the ileum (Fig. 2). Although an amplifying effect of AP(M) was shown in the jejunum with or without the washing treatment, the intensity was much less than that observed in the ileum. No obvious additive effect of AP(M) was seen in the duodenum or the colon in either the presence or absence of luminal enzymes. Similar results concerning the site-dependent action of a protease inhibitor were reported by Kidron *et al.*[2a] They found that insulin coadministered with soybean trypsin inhibitor into the ileum decreased blood glucose concentration, but the effect did not occur in the colon. In addition, it was reported that AP alone had no effect on rectal insulin absorption.[6] Protease inhibitors such as AP and soybean trypsin inhibitor may thus have site-dependent effects on intestinal insulin absorption.

Compared with the distal regions of the small intestine, the proximal regions have longer villi, resulting in a greater surface area for absorption per unit length of intestine. Tight junctions between ileal absorptive cells in primates have greater depth and more strands than those between jejunal absorptive cells.[7] Actually, an effective pore radius has been estimated at 6 Å in the jejunum and 3 Å in the ileum.[8] These morphological differences seemed to be the disadvantageous factors for peptide absorption from the distal small intestine. In this study, however, an obvious hypoglycemic effect of insulin was seen only in the ileum after the washing treatment and when coadministered with AP(M). Recently, it has been proposed that M cells in the Peyer's patches were the major site of pinocytosis for large-sized molecules.[9] Peptides could possibly be absorbed into the lymphatics through the Peyer's patches. The Peyer's patches are located on the antimesenteric wall of the small intestine and are particularly rich in the ileum close to the *ileo-cecal* junction.[9,10] This morphological feature may lead to a much higher absorption of insulin from the ileum than from the jejunum. However, in this study, the extent of insulin absorption *via* the Peyer's patches in the ileum is not clarified. The role of the Peyer's patches on the absorption of insulin should be investigated in the future.

Interestingly, in the ileum region, the serum glucose level after coadministration with AP(M) was not changed until 120 min post-administration by washing treatment (Fig. 2). The biological effect of insulin was, however, elevated relative to the AP doses used in this study (Fig. 3). These results suggest that at the ileum, even when the luminal enzymes are excluded, insulin is still subjected to enzymatic proteolytic degradation. Although enzymatic degradation

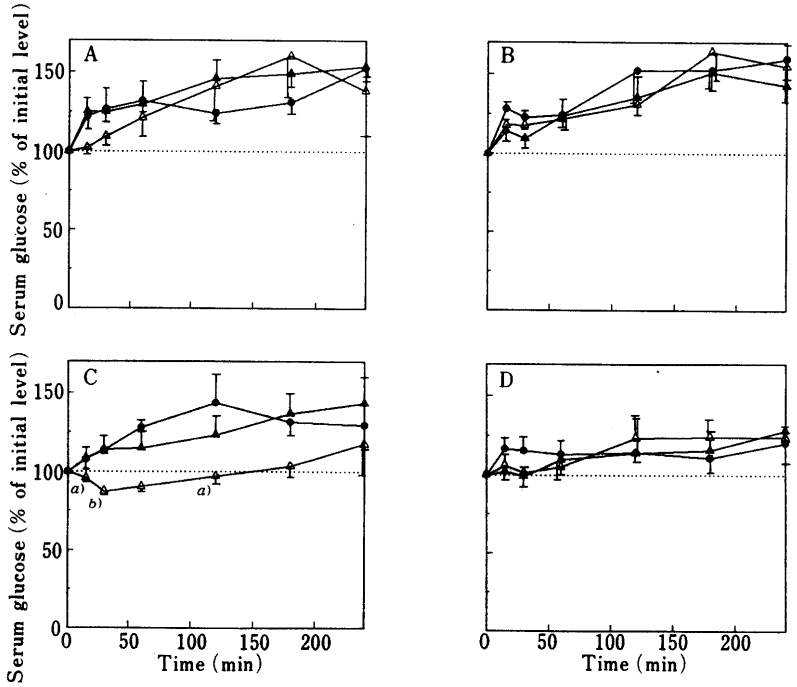

Fig. 1. Effect of Intra-duodenal (A), -jejunal (B), -ileal (C) and -colonic (D) Administration of Insulin on Serum Glucose Levels

●, control (PBS only); ▲, insulin (50 U/kg); △, insulin (50 U/kg) with washing treatment. Comparisons calculated at each period for insulin *vs.* insulin with washing treatment: *a*) $p<0.05$, *b*) $p<0.01$.

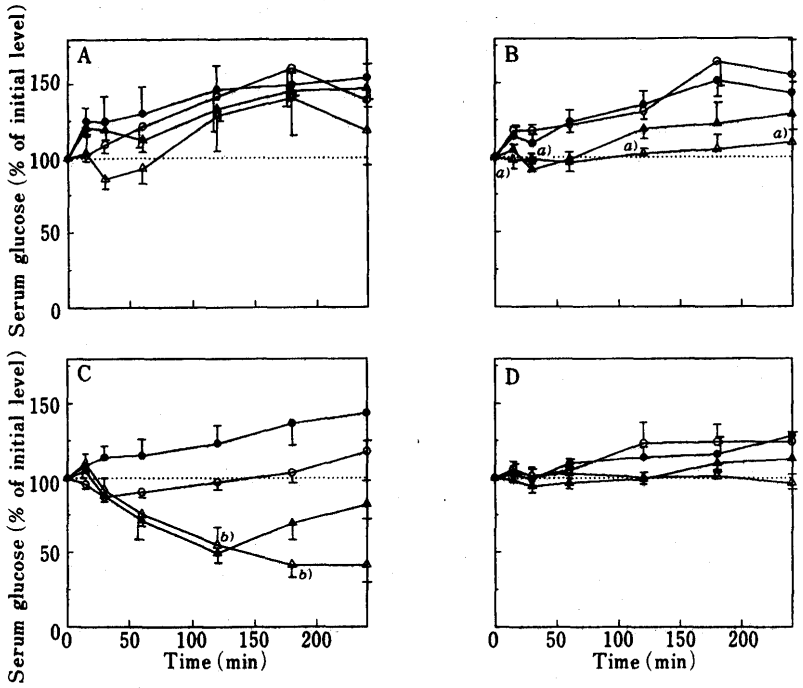

Fig. 2. Effect of Intra-duodenal (A), -jejunal (B), -ileal (C) and -colonic (D) Administration of Insulin with AP (M) on Serum Glucose Levels

●, insulin (50 U/kg); ○, insulin (50 U/kg) with washing treatment; ▲, insulin (50 U/kg) + AP (M: 13.3 TIU/kg); △, insulin (50 U/kg) + AP (M: 13.3 TIU/kg) with washing treatment. Comparisons calculated at each period for insulin with washing treatment *vs.* insulin + AP (M) with washing treatment: *a*) $p<0.05$, *b*) $p<0.01$.

AUC of serum insulin levels was calculated by the trapezoidal rule. In this case, a pre-dose insulin level was previously subtracted from post-dose insulin levels in each rat.

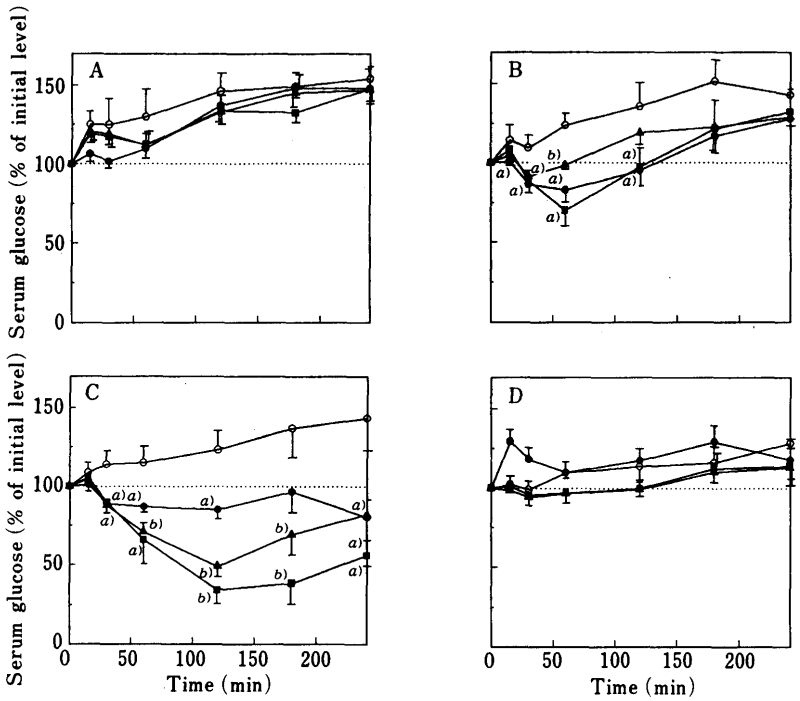

Fig. 3. Effect of Intra-duodenal (A), -jejunal (B), -ileal (C) and -colonic (D) Administration of Insulin with AP (L), AP (M) and AP (H) on Serum Glucose Levels

○, insulin (50 U/kg); ●, insulin (50 U/kg) + AP (L: 6.7 TIU/kg); ▲, insulin (50 U/kg) + AP (M: 13.3 TIU/kg); ■, insulin (50 U/kg) + AP (H: 26.6 TIU/kg). Comparisons calculated at each period for insulin *vs.* insulin + AP (L), AP (M) or AP (H): *a*) $p<0.05$, *b*) $p<0.01$.

in the intestinal lumen is a major barrier for insulin oral delivery, digestive enzymes such as trypsin, chymotrypsin and other pancreatic proteases are also adsorbed within the glycocalyx, which is located external to the microvilli and plays a crucial role in terminal digestion.[11] Further, it was observed that the brush border membrane of the guinea pig did possess endopeptidase activity.[12] In addition, the existence of a chymotrypsin-like protease in the mucosal layer of the small intestine has been reported.[13] Thus, insulin could be degraded even when luminal enzymes were removed.

In this study, there was no evidence of insulin absorption from the duodenum even when insulin was administered with AP(M) after the washing treatment. The duodenum region is rich in pancreatic enzymes and has a much smaller insulin permeability compared to the jejunum and the

ileum.[4] Thus, the duodenum is thought to be unsuitable for selective insulin delivery. Similarly, insulin administered into the colon had no effect on the serum glucose level, regardless of the presence of AP(M) and the washing treatment. Since a smaller amount of peptides is subjected to proteolytic degradation in the large intestine compared to the small intestine,[14] the intrinsic low permeability of insulin seems to be a major barrier in the colon.

The Effect of AP Dose on Insulin Absorption Figure 3 shows the effect of 3 differing levels of AP dose on insulin absorption from the small intestine and the colon. The effect of AP was markedly different for the administered sites. Compared with insulin alone, blood glucose levels observed in the duodenum and the colon did not decrease, even in the presence of AP(H). In contrast, blood glucose levels decreased significantly in the jejunum, and a much greater effect was seen in the ileum. In the ileum, the peak serum insulin level was elevated in an AP dose-related fashion (Fig. 4). The area under the curve (*AUC*) of serum insulin levels or cumulative % change in serum glucose levels *vs.* time profile from 0 to 4 h was linearly related to the logarithm of the AP dose (Fig. 5). From these results, it is expected that in the distal small intestine, insulin would be absorbed according to its ability to protect against enzymatic degradation.

Effect of Absorption Enhancer on the Biological Effect of Insulin Figure 6 shows the changes in serum glucose level following the administration of insulin with sodium caprate, Na_2EDTA or sodium glycocholate. Clearly, the intensity of the promotive effects of the adjuvants varied in relation to the region of administration. Both sodium caprate and Na_2EDTA significantly promoted the hypoglycemic effect of insulin at all sites, and their intensities increased towards the distal regions of the intestine. On the other hand, sodium glycocholate had no significant promoting effect on small intestinal insulin absorption but significantly improved colonic insulin efficacy. The site-dependent insulin ab-

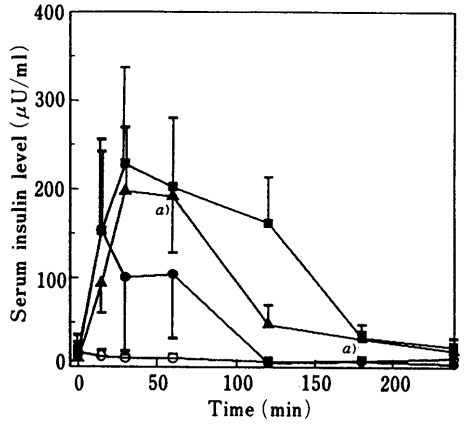

Fig. 4. Effect of AP Dose on Serum Insulin Levels in the Ileum

○, insulin (50 U/kg); ●, insulin (50 U/kg)+AP (L: 6.7 TIU/kg); ▲, insulin (50 U/kg)+AP (M: 13.3 TIU/kg); ■, insulin (50 U/kg)+AP (H: 26.6 TIU/kg). Comparisons calculated at each period for insulin *vs.* insulin+AP (L), AP (M) or AP (H): *a*) $p<0.05$.

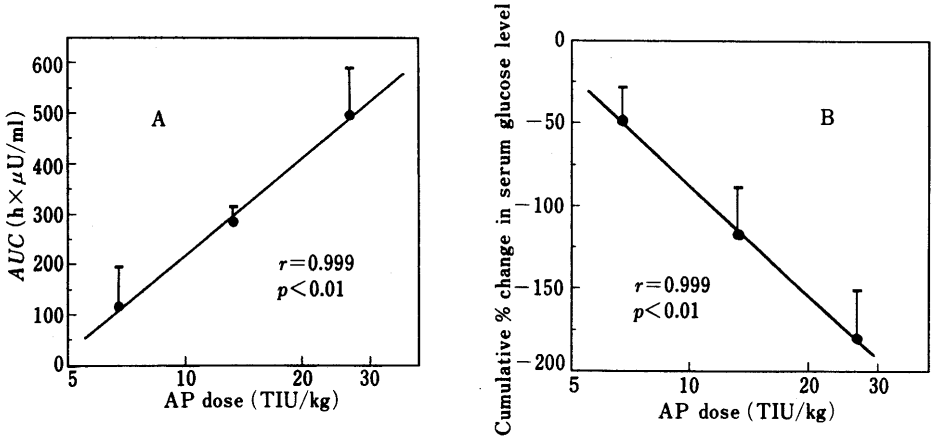

Fig. 5. Relationship between AP Dose and *AUC* of Serum Insulin Levels (A) or Cumulative % Change in Serum Glucose Levels (B)

AUC of serum insulin levels was calculated by the trapezoidal rule. In this case, a pre-dose insulin level was previously subtracted from post-dose insulin levels in each rat.

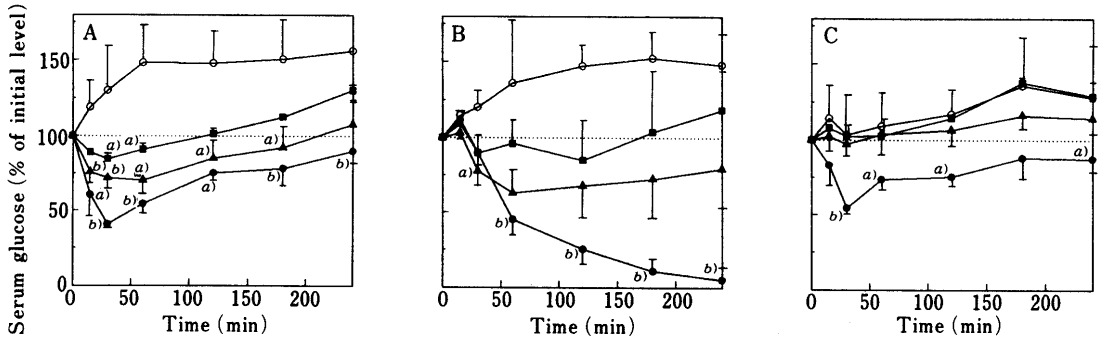

Fig. 6. Effect of Intra-duodenal (○), -jejunal (■), -ileal (▲) and -colonic (●) Administration of Insulin (50 U/kg) with Sodium Caprate, Na_2EDTA or Sodium Glycocholate on Serum Glucose Levels

A, 1.0% sodium caprate; B, 1.0% Na_2EDTA; C, 1.0% sodium glycocholate. Comparisons calculated at each period for insulin *vs.* insulin with an adjuvant: *a*) $p<0.05$, *b*) $p<0.01$.

TABLE I. Comparison of the Percentage Insulin Efficacy Relative to i.v. Observed in Various Intestinal Regions

Treatment	Washing in the loop	Duodenum	Jejunum	Ileum	Colon
50 U/kg	+	0.1	0	0.3	0.1
50 U/kg		0	0	0.1	0.1
with AP (L)		0.1	0.3	0.3	0.3
with AP (M)		0	0.2	1.0	0.2
with AP (M)	+	0.2	0.2	1.8	0.2
with AP (H)		0.1	0.3	2.0	0.2
with sodium caprate		0	0.2	0.5	0.9
with Na_2EDTA		0	0.5	2.8	5.5
with sodium glycocholate		0.1	0.2	0.2	0.7

sorption-promoting actions of Na_2EDTA[6b] and sodium glycocholate[15] among various mucosal sites were previously reported. The effects of absorption promoters seem to vary in different mucosal membranes. However, enhancer activities on insulin absorption in different intestinal regions have not been fully compared. In a recent review by Muranishi,[16] the large intestine, rather than the small intestine, has been shown to be the preferred site for absorption enhancer effectiveness. This is probably due to the higher sensitivity to pore enlargement in the epithelium of the colon than in the small intestine. It is thought that Na_2EDTA acts exclusively at the tight junctions and consequently leads to a loosening of the intercellular spaces. Both sodium caprate and bile salts exert not only surface active action but also enlargement action on the colonic pore sizes.[17] In addition to such a different sensitivity in pore enlargement, the low protease activity may lead to a maximum reduction of blood glucose level in the colon. Further, a chelating agent such as EDTA causes more cellular damage to the mucosa compared with sodium caprate or sodium glycocholate.[16] Thus, Na_2EDTA may exhibit the most remarkable promoting effect both in the small intestine and the colon. It is not clear why the enhancing effect of sodium glycocholate on the small intestinal absorption of insulin occurred to a small extent. However, similar results were found by Kidron *et al.*[2a] who found a much smaller effect of a bile salt in the small intestine than in the large intestine. It is assumed that the upper intestinal mucosa might be uniquely resistant to the attack of exogenous bile acids since it is exposed to a high concentration of bile acids in a physiological state.

Efficacy Relative to i.v. In order to calculate the value of percentage efficacy relative to i.v., a dose/response curve was obtained from the results of i.v. study. That curve gave the following equation.

$$\text{cumulative \% change} = -178.5 \times \log \text{dose} - 188.0; \quad r = 0.960; \quad p < 0.01$$

The relative efficacy was calculated by rearranging this equation so that the i.v. dose gave an equivalent hypoglycemic response to that following intestinal administration. Table I shows the relative efficacies observed in each administered region. It was clearly indicated that when administered together with AP, insulin was absorbed most effectively from the ileum. However, when given with an absorption promoter, the maximum relative hypoglycemic efficacy was obtained from the colonic region. To provide an extensive amount of intact insulin, insulin must be protected from proteolysis in the ileum, but this may not be applicable in the colon. Further, it appears that not only luminal but also membrane proteases might be responsible for insulin degradation in the small intestine.

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