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ABSORPTION OF PROTEIN VIA THE INTESTINAL WALL

A QUANTITATIVE MODEL

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Abstract—Intact, biological active insulin and pancreatic RNase can be absorbed from the intestinal lumen into the blood circulation. The absorption is dependent on the addition of bile acid (sodium cholate) and proteinase inhibitor. The quantitative absorption of insulin and pancreatic RNase has been demonstrated in an *in situ* model. The amount of insulin absorbed after 30 min from the ileum to the mesenteric vein was 0.025% of the initial amount. Sodium cholate (10 mg/ml) and 3000 KIU/ml aprotinin enhanced this absorption by 30 times. The amount of pancreatic RNase which was absorbed from the ileum to the blood was 0.002% of the initial amount during 30 min. Sodium cholate (10 mg/ml) and 3000 KIU/ml aprotinin increased the absorption by a factor of 200. No damage occurred to the intestine during the experimental procedures. The sieving characteristics of the intestinal wall were not altered by the presence of sodium cholate and proteinase inhibitor in the intestinal lumen. These results suggest that sodium cholate and proteinase inhibitors can facilitate the absorption of intact, biologically active proteins across the intestinal wall.

Transport of intact biologically active macromolecules from the intestinal lumen into the blood circulation is a unique phenomenon which differs from the regular process of food digestion and absorption. Intestinal absorption of bioactive peptides and various proteins has been reported during the last decades. The phenomenon has been reviewed in [1]. It is possible to demonstrate that protection against proteolysis is the first step involved in keeping polypeptides intact in the "hostile" intestinal lumen [2, 3]. The second step entails alteration of the mechanisms responsible for the selective absorption of small molecules, to enable absorption of high molecular weight molecules. It has recently been demonstrated that several groups of surfactants can enhance the *in vivo* absorption of biologically active macromolecules such as heparin, insulin and vasopressin [2, 4–10]. The agents used were non-ionic surfactants [6, 9], ionic detergents [4], and bile acids—physiological detergents normally present in the intestinal lumen [2, 7, 8, 10].

This work presents a model for the quantitative measurement of intact insulin and RNase absorbed from the intestine. The model consists of an *in situ* intestinal loop and the amount of insulin and RNase absorbed into the mesenteric vein has been measured using RIA and enzymatic activity, respectively.

MATERIALS AND METHODS

Materials

Insulin–Actrapid 100 i.u./ml was obtained from Novo Industry (Copenhagen, Denmark). Sodium cholate, bovine pancreatic RNase and soybean trypsin inhibitor were purchased from Sigma Chemical Company (St. Louis, MO). Aprotinin (Trasylol®) 10,000 KIU/ml (Kallikrein inactivator units) was obtained from Bayer (Leverkusen, F.R.G.). Glucose oxidase reagent was obtained from Boehringer (Mannheim, F.R.G.). Insulin MAIA RIA kit (using human insulin standards) was obtained from Serono Diagnostic (Coinsins, Switzerland), [^{3}H]uridine (40 Ci/mmole) was purchased from Amersham International (Amersham, U.K.).

Methods

Animal studies. Male rats of the Hebrew University strain weighing 230–280 g were fed with pelleted chow. All the animals were fasted for 16 hr before the beginning of the experiments.

Insulin transport in vivo *to the ileum.* Following surgical operation under ether anesthesia insulin was directly injected into the lumen of the ileum in 0.5 ml of a mixer containing 5 mg sodium cholate and 1000 KIU or 3000 KIU aprotinin in 0.15 M NaCl.

Blood samples used in the determination of glucose concentration were taken from the tail vein 1 hr before administration of the drug, at zero time and following 1, 2 and 4 hr.

Ileal loop. The intestinal–blood drainage–*in situ* preparation was as follows. The rat was anesthetized with sodium barbiturate, 60 mg/kg. A longitudinal incision was made in the abdomen and a loop of the rat ileum, 7 cm long, 6 cm away from the cecum, was identified and clamped at both ends. The main venous trunk in the root of the mesenteric vein was cannulated and allowed to drip. Five samples of blood each of 2 ml, were collected during a period of 30 min, following drug administration. During this period a solution of 0.15 M NaCl was administered through the tail vein to maintain blood volume.

Measurements of the transport of insulin and RNase from the intestinal lumen to the ileal loop to the mesenteric vein. RNase or insulin were injected into the isolated loop, in 0.3 ml of 0.15 M NaCl, and contained 4.5 mg RNase or 12 u insulin, 3000 KIU

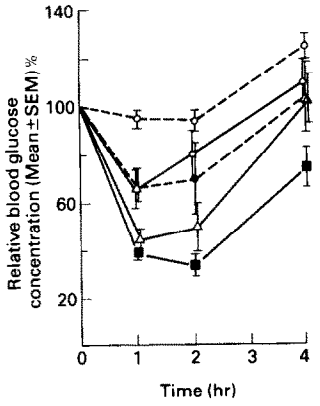

Fig. 1. Changes in blood glucose concentration following injection of 0.5 ml insulin mixed with aprotinin and cholate in 0.15 M NaCl into the ileum. The solutions contained the following: ○---○, 12 u insulin; □——□, 12 u insulin + 10 mg/ml sodium cholate; ●---●, 12 u insulin + 3000 KIU aprotinin; △——△, 12 u insulin + 10 mg/ml sodium cholate + 1000 KIU aprotinin; ■——■, 12 u insulin + 10 mg/ml sodium cholate + 3000 KIU aprotinin. The glucose concentration at zero time served as the base value (100%). Each point represents the mean ± SEM of 6 experiments.

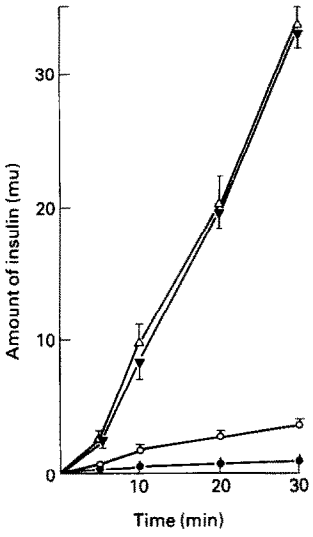

Fig. 2. Cumulative amount of insulin immunoreactivity recovered in mesenteric vein after luminal instillation of insulin with and without cholate and aprotinin into ileal loops *in situ*. The solutions contained the following: ●——●, 12 u insulin; ○——○, 12 u insulin + 10 mg/ml sodium cholate; △——△, 12 u insulin + 3000 KIU aprotinin, ▼——▼, 12 u insulin + 10 mg/ml sodium cholate + 3000 KIU aprotinin. Each point represents the mean ± SEM of 3 experiments.

aprotinin and 3.0 mg sodium cholate.

Insulin was determined in the plasma and in the ileal lumen by radioimmunoassay. The enzymatic activity of RNase was determined, using [^{3}H]-RNA as a substrate. High molecular weight RNA was collected on Whatman 3MM paper, according to the method of Bollum [11]. The amounts of insulin and RNase transported from the intestinal lumen to the blood circulation was calculated from the concentration of the polypeptide in the samples.

Determination of ^{125}I-polypeptide transport through the intestinal wall. The transport of ^{125}I-insulin and its long fragments, which are, cold TCA-precipitable was determined in the *in situ* intestinal blood drainage preparation as described above. 0.3 ml of solution containing 12 u insulin, 10^6 c.p.m. [^{125}I]-insulin, 1 mg soybean trypsin inhibitor and 3.0 mg sodium cholate were injected into the ileal loop segment. Radioactivity was determined in the plasma collected from the mesenteric vein and in the intestinal lumen. [^{125}I]-insulin was precipitated according to the method of Bollum [11]. The ratio between the radioactivity of the long peptides, which are precipitated in cold 5% TCA solution and the total radioactivity was calculated as the percentage of long peptides (insulin and insulin fragments) present in the solution.

[^{125}I]-insulin and peptides were counted in a Kontron Model MR-480 gamma counter.

[^{3}H]-RNA was counted in a Packard Model 300c liquid scintillation counter.

All the results are expressed as the mean ± SEM.

RESULTS

The role of the protease inhibitor, aprotinin, and bile salt, sodium cholate, in the absorption of intact, biologically active insulin was demonstrated in an *in vivo* experiment. Figure 1 shows the changes in blood glucose level following the injection of solutions of insulin with or without aprotinin and sodium cholate into the rat ileum. Injection of insulin alone did not change the blood glucose concentration. The most striking decrease in blood glucose concentration was achieved when insulin was injected together with both compounds. When the amount of aprotinin in this mixture was 3000 KIU the results were as follows. At the time of injection the glucose concentration was 80 ± 8.2 mg/dl (4.4 ± 0.4 mM); following 1, 2 and 4 hr this value had dropped to 32.4 ± 4.6 mg/dl (1.8 ± 0.3 mM), 26 ± 3.3 mg/dl (1.5 ± 0.2 mM), and 59 ± 8.5 mg/dl (3.3 ± 0.5 mM), respectively. Smaller amounts of aprotinin (1000 KIU) were less effective, by 10–15%. When administered together with either aprotinin or cholate, insulin was absorbed less effectively than when given in a mixture containing both these compounds, the changes in blood glucose concentration reflect the qualitative absorption of active insulin.

For quantitative determination of the absorption of macromolecules we developed another method by which the transport of biologically active molecules was measured directly. Figures 2 and 3 demonstrate the results obtained using *in situ* intestinal loop–blood–drainage preparations. The amount of insulin transported from the intestinal lumen to the mesenteric vein followed injection into the loop is shown in Fig. 2. Within 30 min, 1.0 ± 0.3 mu insulin (0.025% of the initial amount) had accumulated in the plasma after being injected without aprotinin and sodium cholate; 3.6 ± 0.4 mu (0.09% of the initial amount) were present when insulin was added together with sodium cholate; 33 ± 2.8 mu (0.82%) when added with aprotinin; and 30 ± 6.0 mu (0.75%) when added with both aprotinin and sodium cholate. The insulin in the samples was measured using a

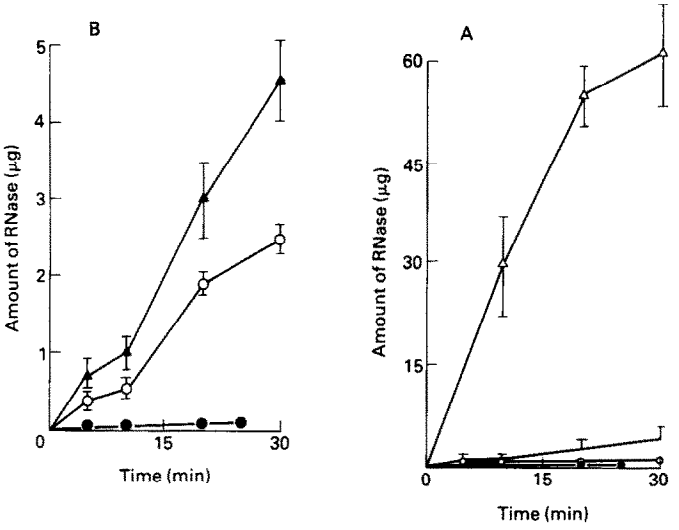

Fig. 3. (A and B). Cumulative amount of RNase recovered in mesenteric vein after luminal instillation of RNase with and without cholate and aprotinin into ileal loops *in situ*. The solutions contained the following: (●——●), 15 mg/ml RNase; ○——○, 15 mg/ml RNase + 10 mg/ml sodium cholate; ▲——▲, 15 mg/ml RNase + 3000 KIU aprotinin; △——△, 15 mg/ml RNase + 10 mg/ml sodium cholate + 3000 KIU aprotinin. Each point represents the mean ± SEM of 3 experiments. (B) is an enlargement part of (A).

radioimmunoassay technique which enables the detection of immunoreactive molecules (intact molecules and immunoreactive fragments).

The best means of determining the intactness of protein molecules is to test their biological activity. For this purpose the pancreatic enzyme RNase, M.W. 13700, was chosen. Figure 3 shows the transport of RNase, following injection of the molecule into the intestinal lumen, to the mesenteric vein. Within 30 min 0.29 ± 0.17 μg RNase (0.002% of the initial amount) had accumulated in the plasma fractions when administered alone, 2.5 ± 0.4 μg (0.05%) when administered with sodium cholate, 4.6 ± 1.6 μg (0.1%) when administered with aprotinin, and 61 ± 7 μg (1.35%) when administered with both compounds.

The polypeptide is absorbed from the intestinal lumen to the blood circulation without damage to the intestinal wall. In order to show this phenomena we measured the sieving properties of the intestinal wall with radiolabeled [^{125}I]-insulin.

In this experiment the transport of TCA precipitable peptides was determined in the *in situ*–blood–drainage preparation. Table 1 summarizes the results of these experiments. The [^{125}I]-insulin reisolated from the intestinal loop at zero time is highly precipitable by cold 5% TCA (90–94%). Thirty minutes after the injection into the intestinal lumen the ratio of TCA precipitable to total counts of the insulin reisolated from the intestinal loop was 13% for insulin alone, 54% for insulin mixed with trypsin inhibitor and for insulin mixed with trypsin inhibitor and cholate it was 80%. The higher the ratio of cold TCA precipitate to total counts the higher the percentage of large peptides of 5 amino acids or more.

The normal effect of the intact intestinal wall is to transfer only the small fragment of the polypeptide (mainly free amino acids)—which are not precipitable in cold TCA. When the sieving properties of the intestine are damaged it is expected that large fragments of [^{125}I]-insulin will be found in the blood circulation in a proportion similar to their concentration in the intestinal lumen.

As can be seen from Table 1, the size of [^{125}I]-fragments in the blood drain is small (the ratio—TCA precipitable [^{125}I]:total counts—18–25%) even when inside the intestinal lumen this ratio is much higher (80%).

The distribution of total radioactivity 30 min after injection is present in Table 2. Insulin alone, without antiproteolytic protection is broken to small fragment, most of them are transported from the intestinal lumen to the intestinal wall and to the blood circulation. Trypsin inhibitor protects insulin against proteolysis. This is reflected in the amount of [^{125}I] fragments which are transported from the intestinal wall to the blood circulation.

DISCUSSION

The main function of the digestive tract is the breakdown and absorption of ingested macromolecules. Generally, the macromolecules lose their biological activity during the initial stages of this process. In the present work we have demonstrated that under certain conditions, intact, biologically active polypeptides can be absorbed from the intestinal lumen into the blood circulation in measurable amounts. For this process two prerequisites have to be fulfilled: first, inhibition of the drastic proteolytic activity along the digestive tract; second, enhancement of the absorption of macromolecules by the intestinal wall.

We have shown that in the intact animal as well as in an *in vivo in situ* model, it is possible to obtain these two requirements by the joint addition of proteinase inhibitor and surfactant to the solution of

Table 1. The relative quantities of [^{125}I]-polypeptides in the intestinal lumen and in the blood circulation

Solution injected into the intestinal lumen	The ratio $\frac{\text{TCA Precipitable } [^{125}\text{I}]}{\text{Total counts}}$		
	In the intestinal lumen		In the blood
	Zero time	30 min	30 min
[^{125}I]-Insulin	0.90	0.13 ± 0.06	0.18 ± 0.01
[^{125}I]-Insulin + trypsin inhibitor	0.90	0.54 ± 0.2	0.26 ± 0.04
[^{125}I]-Insulin + cholate +trypsin inhibitor	0.94	0.80 ± 0.17	0.25 ± 0.07

Each value represents the mean ± SEM of 3 experiments.

Table 2. The relative distribution of [^{125}I] 30 min after the injection of [^{125}I]-polypeptide into the intestinal lumen

Solution injected into the intestinal lumen	Percent of [^{125}I] counts		
	In the intestinal lumen	In the intestinal wall	In the blood
[^{125}I]-Insulin	39 ± 10	19 ± 5	42 ± 17
[^{125}I]-Insulin + trypsin inhibitor	76 ± 2	14 ± 4	10 ± 2
[^{125}I]-Insulin + cholate +trypsin inhibitor	84 ± 3	8 ± 3	8 ± 6

Each value represents the mean ± SEM of 3 experiments.

polypeptide to be absorbed. In earlier studies it was found [1] that small amounts of peptides and proteins, as well as some inert particles can, and do cross *in vivo* the normal small intestine in an intact form. In neonate mammalians [12–14] this absorption was estimated to be in the range of 10^{-6} to 10^{-5} of the total amount administered. In our *in situ* adult rat intestine model it was possible to enhance the absorption of enzymatically active RNase during an experimental period of 30 min to a total of more than 1.3×10^{-2} (1.3%) of the amount added. The molecule was protected against proteolytic activity by aprotinin and its absorption was promoted by sodium cholate. In the absence of these two compounds, enzymatically active RNase was absorbed 200-fold less efficiently than in their presence.

There is a dramatic difference in results depending on whether measurements are made using radioimmunoassay (insulin) or enzymatic activity (RNase). This is quite evident when Figs 1, 2 and 3 are compared. Unlike the results obtained using different admixtures of RNase (Fig. 3) or the *in vivo* experiment with insulin (Fig. 1), there was no apparent difference in the quantity of insulin absorbed when the molecule was administered jointly with aprotinin or with both aprotinin and sodium cholate (Fig. 2). This is because even when aprotinin alone was added to the insulin, the large fragments of insulin absorbed were of sufficient size to react with the added antibody. The results of the insulin *in vivo* (Fig. 1) and the RNase *in situ* experiments (Fig. 3) are more precise since they are related to the biological activity of the polypeptides.

Skogh [15] has shown that the TCA precipitable radioactivity in the blood can be drastically reduced when mice were given non-radioactive NaI before intragastric administration of ^{125}I-polypeptide. According to Skogh's work, a large part of the TCA precipitable ^{125}I-lable in the blood is due to free Na ^{125}I bound to circulating plasma proteins.

The measurement of TCA precipitable radioactivity in the blood after oral administration of ^{125}I-lable polypeptide cannot serve as a quantitative model. We have, therefore, used ^{125}I-insulin only to determine whether sodium cholate and proteinase inhibitor do not change the sieving characteristics of the intestinal wall (Tables 1 and 2). Most of the ^{125}I-insulin in the intestinal lumen is TCA precipitable even 30 min after its administration together with sodium cholate and soybean-trypsine inhibitor (80% ± 17%). At the same time most of the ^{125}I-lable in the blood is not TCA precipitable (26% ± 4%).

The results summarized in Tables 1 and 2 prove that the intestinal wall is still selective in its transport properties under the experimental conditions. It allows mainly the transport of small molecules, while the larger molecules (which are TCA precipitable) remain inside the intestinal lumen. We have made microscopic studies (light and electron microscopy) of intestinal slices after the addition of the various insulin–aprotinin–sodium cholate solutions, and following the injection of saline (control). There were no detectable differences in the intestinal macro or micro structures in the different systems.

There are several hypotheses to explain the mechanism(s) by which macromolecules may be transported through the intestinal wall. Murakami *et al.* [16] suggested that there is a correlation between the hydrophobicity and the ability of the bile salt to

bind calcium ions and their ability to promote rectal absorption of sodium ampicillin. Gordon *et al.* [17] found a correlation between the hydrophobicity of the bile salts steroid nucleus and the adjuvant potency for nasal insulin absorption. In both cases the effect of sodium cholate on the rectal absorption of sodium ampicillin or on the nasal absorption of insulin was almost negligible. In this report we have demonstrated that the absorption of insulin from the ileum to the blood circulation using sodium cholate is almost equal to the ileal absorption of insulin mixed with sodium deoxycholate [2]. Similar results were found by Hirai *et al.* [18] on the nasal absorption of insulin in rats. The change in the intestinal permeability to insulin occurs when the concentration of the bile acids are higher than their critical micellar concentration (CMC). It was suggested by Feldman and Gibaldi [19] that this change is a result of the interaction of bile salts micell with the phospholipids of the enterocytes membrane. The exact mechanism is still to be determined.

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