Enteral Absorption of Insulin in Rats from Mucoadhesive Chitosan-Coated Liposomes

Hirofumi Takeuchi,[1,2] Hiromitsu Yamamoto,[1] Toshiyuki Niwa,[1] Tomoaki Hino,[1] and Yoshiaki Kawashima[1]

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Purpose. The mucoadhesiveness of polymer-coated liposomes was evaluated to develop a novel drug carrier system for oral administration of poorly absorbed drugs such as peptide drugs.

Methods. Multilamellar liposomes consisting of dipalmitoylphosphatidylcholine (DPPC) and dicetyl phosphate (DCP) (DPPC:DCP = 8:2 in molar ratio) were coated with chitosan (CS), polyvinyl alcohol having a long alkyl chain (PVA-R) and poly (acrylic acid) bearing a cholesteryl group. The adhesiveness of the resultant polymer-coated liposomes to the rat intestine was measured in vitro by a particle counting method with a Coulter counter. The CS-coated liposomes containing insulin were administered to normal rats and the blood glucose level was monitored.

Results. The existence of polymer layers on the surface of liposomes was confirmed by measuring the zeta potential of liposomes. The CS-coated liposomes showed the highest mucoadhesiveness and the degree of adhesion was dependent on the amount of CS on the surface of the liposomes. The blood glucose level of rats was found to be significantly decreased after administration of the CS-coated liposomes containing insulin. The lowered glucose level was maintained for more than 12h after administration of the liposomal insulin, which suggested mucoadhesion of the CS-coated liposomes in the intestinal tract of the rats.

KEY WORDS: liposome; mucoadhesion; chitosan; oral administration.

INTRODUCTION

Liposomes have received much attention as potential drug carriers for improving enteral absorption of poorly absorbed drugs including peptide drugs such as insulin. There have been several attempts to demonstrate the effectiveness of orally administered insulin liposomes (1). However, the results of these studies indicate that the influences of the liposomal formulations on drug absorption are not predictable or reproducible, while some of them have indicated the occurrence of a marked hypoglycemic response.

Among the attractive means of improving the bioavailability of drugs are mucoadhesive dosage forms (2). Since mucoadhesion can prolong the residence time of drug carriers at the absorption sites, improved drug absorption is expected with a combination of mucoadhesiveness and controlled drug release from the devices. One of the most extensively studied mucoadhesive dosage forms is a tablet for oral or buccal administration (3–6). The devices are prepared by formulating mucoadhesive polymers such as hydroxypropylmethylcellulose and Carbopol, which is a high molecular weight poly(acrylic acid) copolymer, loosely cross-linked with divinyl glycol. The mucoadhesive properties of these polymers and devices have been evaluated using various methods (6,7). A multi unit bioadhesive system was reported to have been prepared by coating microspheres of poly-hydroxyethyl-methacrylate with mucoadhesive polymers using laboratory-scale equipment (8,9). Recently, Pimienta et al: (10) investigated the bioadhesion of hydroxypropylmethacrylate nanoparticles or isohexylcyanoacrylate nanocapsules coated with poloxamers and poloxamine on rat ileal segments in vitro using a labeled compound.

The aim of the present study was to develop mucoadhesive liposomal dosage forms to facilitate the enteral absorption of poorly absorbed drugs. The liposomes were coated with several polymers including chitosan (11), which are expected to be mucoadhesive. The mucoadhesion of the resultant liposomes was evaluated in vitro using isolated rat intestine. Finally, in vivo drug absorption tests were carried out in rats by intragastric administration of the polymer-coated liposomes containing insulin.

MATERIALS AND METHODS

Materials

L-α-dipalmitoylphosphatidylcholine (DPPC, Nippon Oil and Fats Co.), dicetyl phosphate (DCP, Sigma), insulin from bovine pancreas (Sigma), pyrene (Nacalai Tesque) and poly(acrylic acid) (PAA, Sigma) were used as received. All other reagents were of analytical grade. Chitosan (CS) and polyvinyl alcohol having a long alkyl chain at the end of the molecule (PVA-R) were gifts from Katakurachikkarin Co. (Japan) and Kuraray Co. (Japan), respectively. Chitosan (CS) is a deacetylated chitin (poly(N-deacetylglucosamine) and industrially prepared by hydrolyzing the aminoacetyl groups of chitin in aqueous alkaline solution. CSs deacetylated to various degrees and of various molecular weights are available. Poly(acrylic acid) bearing cholesteryl group (PAA-Chol) was prepared by the method reported by Arnold et al. (12). The structure and properties of polymers used in this study are shown in Table I.

Preparation of Liposomes and Polymer-coated Liposomes

Multilamellar liposomes were prepared by the hydration method. In a typical procedure, 24mg (32.7 μ mol) of DPPC and 4.47mg (8.17 μ mol) of DCP were dissolved in a small amount of chloroform, and the solution was rotary evaporated at 50°C to finally obtain a thin lipid film. The thin lipid film was dried in a vacuum oven over night to ensure complete removal of the solvent, and hydration was carried out with 0.5ml of phosphate buffer solution (pH 7.4) by vortexing, followed by incubation at 10°C for 30 min.

For polymer coating of liposomes, an appropriate amount of PVA-R and PAA-Chol were dissolved in phosphate buffer solution (pH 7.4); in the case of CS solution acetate buffer solution (pH 4.4) was used. An aliquot of liposome suspension was mixed with the same volume of polymer solution of various concentrations (up to 1.5%). Thus, the final concentrations of

[1] Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502, Japan.
[2] To whom correspondence should be addressed.

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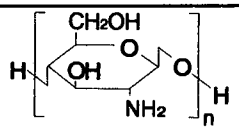
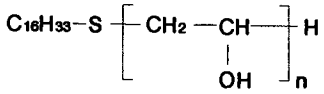
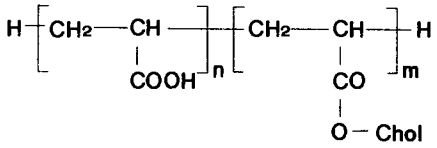
Table I. Properties of Polymers Used for Liposome Coating

Polymer	Structure	Note
Chitosan		Degree of deacetylation = 85% Molecular weight = aprox. 150,000
PVA-R		Degree of polymerization = 480
PAA-Chol		Molecular weight of PAA = 250,000

liposomes and the polymer were 28.5 mg/ml and ≦ 0.75 %, respectively. The concentration of noncoated liposomes was adjusted to that of the coated ones by diluting with the corresponding buffer solutions.

Pyrene-loaded liposomes were also prepared by the hydration method, in which a thin lipid film of DPPC (32.7 μ m) and DCP (8.17 μ m) containing 10 μ m of pyrene was hydrated with 1ml of phosphate buffer solution (pH 7.4).

In preparing insulin-loaded liposomes, a thin lipid film of DPPC (32.7 μ m) and DCP(8.17 μ m) was hydrated with 0.5 ml of insulin solution (conc. of insulin = 2 mg/ml), which was prepared by mixing 0.01N HCl insulin solution with the same volume of acetate buffer (pH 4.4). Polymer coating was carried out in the same manner as described above.

Properties of Liposomes

The mean diameter of liposomes were measured with Cis-1 (Galai Inc.), which is a laser-based time of transition analysis system combined with an image analysis system. The change in surface properties of liposomes was evaluated by measuring the zeta potential of the particles with a zeta meter (Laser Zee Meter 501, Penkem, Inc.). In measuring the zeta potential of polymer-coated liposomes, the liposome samples were diluted with a large amount of buffer solution having an appropriate pH.

Mucoadhesion Test

The mucoadhesion test was carried out using intestines (15cm long) isolated from male Wistar rats. After washing the intestine with saline solution, it was filled with a liposome suspension diluted 100-fold with phosphate buffer solution (pH 7.4 or 6.5), acetate buffer solution (pH 5.6), disintegration solution No.1 (pH 1.2) specified in JP XII or saline solution (pH6.0), and then sealed with closers. The intestines were incubated in saline solution at 37°C for 1 h and the number of liposome particles was measured with a Coulter counter (Coulter Multisizer II, Coulter) both before and after incubation. The number of liposome particles was adjusted to the range of 500–5000 by diluting the liposome samples with an appropriate amount of buffer solution.

Pyrene-loaded liposomes adhering to the luminal surface of the rat intestine were observed with a fluorescence microscope (AXIOPHOT, Karl Zeiss).

Administration of Insulin-loaded Liposomes to Rats

The absorption test was carried out using male Wistar rats (12–13 weeks old, 220–250g) fasted for the 24h before administration of the liposomes. An insulin-loaded liposome suspension (24IU/rat) was administered intragastrically. As a reference, an equivalent amount of insulin solution was administered. The liposome suspensions were administered as prepared, because little amount of insulin was detected in the dispersing medium after loading of insulin. Although CS coating caused a leakage of some amount of insulin entrapped, the CS-coated liposomes were also used as prepared. A 200 μl blood sample was obtained from the carotid vein at an appropriate interval to determine the glucose level. The plasma glucose levels were measured using a commercially available kit (Glucose CII-test WAKO, Wako Pure Chemical Ind. Co., Japan).

RESULTS AND DISCUSSION

Polymer Coating of Liposomes

It has been reported that the physicochemical properties of liposomes such as stability were improved by surface modification with polymers (13–14). In the present study, surface modification of liposomes was conducted with three different polymers (PAA-Chol, PVA-R and CS) expected to confer on the resultant liposomes mucoadhesive properties. In coating of liposomes, the cholesteryl group of PAA-Chol and hexadecyl group (-R) of PVA-R were expected to function as anchors which could penetrate into the phospholipid bilayer. The CS-coated liposomes were considered to be formed via ionic interaction between the positively charged CS and negatively charged DCP on the surface of the liposomes.

To confirm the surface modification of liposomes with the polymers, the zeta potential of the liposomes was measured. The zeta potentials of the polymer-treated liposomes were found

to vary with the concentration of polymer solution used for coating (Fig. 1).

The changes in zeta potential of the PAA-Chol-coated liposomes were attributed to changes in the electric charge of the polymers fixed on the surface of the liposomes. The decrease in zeta potential of CS-coated liposomes suggested the consumption of anionic charge on the surface of the liposomes by ion-complex formation between DCP on the surface of liposomes and CS. In the case of the noncharged polymer, PVA-R, the formation of a polymer layer on the surface of the liposomes might have led to moving of the sheared plane to the solution side, which could then be responsible for the change in zeta potential. These results confirmed the existence of fixed polymer layers on the surface of the liposomes.

The mean diameters (number-volume) of liposomes measured with Cis-1 before and after CS coating were 5.15 μm (sd: 2.53 μm) and 4.96 μm (sd: 2.37 μm), respectively, which suggested that little aggregation of liposome particles occurred during the polymer coating process. Similar results were observed in the case of PVA-R or PAA-Chol coating.

Mucoadhesion of Polymer-coated Liposomes to the Rat Intestine

A variety of studies on the mucoadhesive properties of tablets or granules have been performed and the methods for evaluating the mucoadhesiveness of a polymer itself have also been extensively investigated (6,7). However, the number of reports dealing with the mucoadhesion of particulate systems is small. The method we devised for evaluating the mucoadhesive properties of the polymer-coated liposomes was a particle counting method, in which the number of liposome particles was measured with a Coulter counter after incubating the liposome suspension in the isolated rat intestine. Since the size distribution of the liposomes was observed to be unchanged after incubation, the adhesive % was calculated using the following equation.

$$\text{Adhesive } \% = (\text{No} - \text{N}_S)/\text{No} \times 100$$

where No and Ns are the number of liposomes before and after incubation, respectively. The particle counting method was adopted after confirming the existence of a linear relationship between the lipid concentration (μ mol lipid/ml) and the measured number of liposomes (Fig. 2).

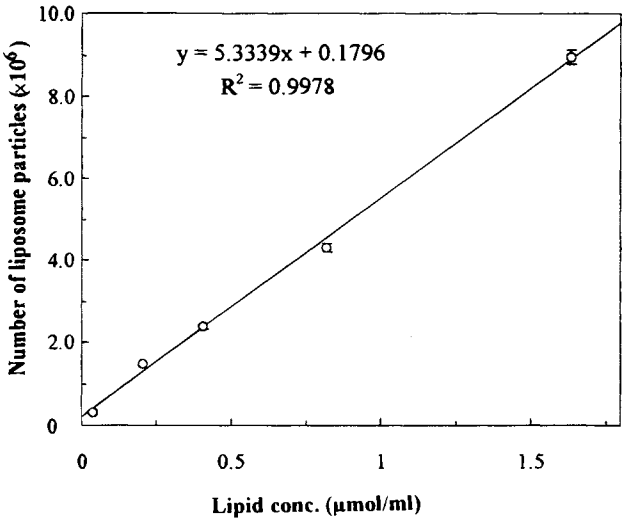


Fig. 2. Relationship between the number of liposome particles and lipids concentration. Symbols represent mean ± standard deviation (sd) of three experiments.

As shown in Fig. 3, the adhesive % of polymer-coated liposomes was significantly higher than that of noncoated liposomes, which suggested that the mucoadhesive properties of the polymer-coated liposomes were conferred by the polymer layer fixed on the surface of the liposomes. When the concentration of CS used for coating was decreased, the adhesive % of CS-coated liposomes decreased. This result also indicated the importance of the polymer layer in mucoadhesion. CS-coated liposomes showed the highest adhesive % among the three different polymer-coated liposomes. Recently, Lehr et al. (11) found that a cationic polymer, chitosan, is strongly mucoadhesive, as are anionic polymers such as Carbopol, by measuring the force of detachment of swollen polymer films from pig

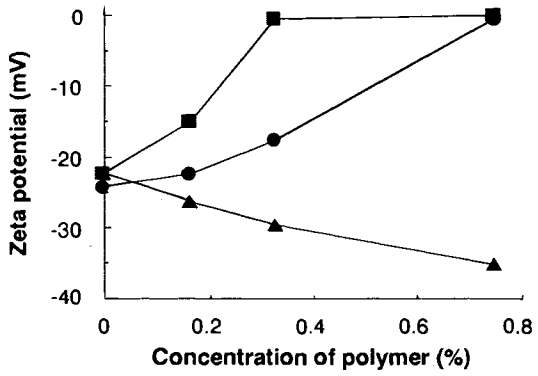


Fig. 1. Zeta potential of liposomes coated with various polymers in phosphate buffer solution (pH 7.4). ●: Chitosan, ■: PVA-R, ▲: PAA-R. Lipid composition: DPPC:DCP = 8:2.

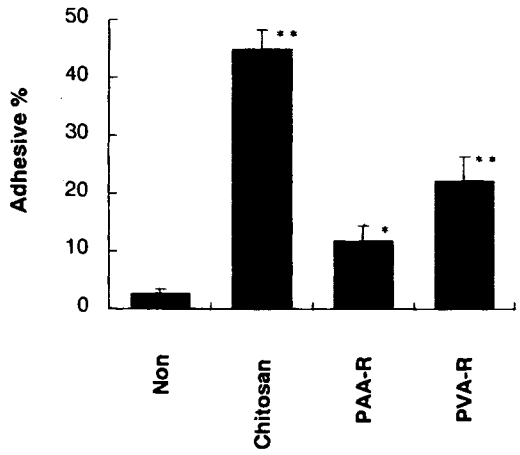


Fig. 3. Adhesive % of liposomes coated with various polymers to the rat intestine. Lipid composition: DPPC:DCP = 8:2. Polymer concentration: 0.75%. Dispersion medium: phosphate buffer solution (pH 7.4). The results are expressed as mean ± sd of three experiments. Significantly different from the value for noncoated liposomes at $p < 0.01$ (**) and $p < 0.05$ (*).

intestinal mucosa. The stronger mucoadhesiveness of the CS-coated liposomes in the present study was consistent with their results. However, the adhesion mechanism of CS-coated liposomes to the rat intestine could not be explained by only the ionic interaction between CS and the mucosa because the zeta potential of the CS-coated liposomes was almost zero when they were dispersed in pH 7.4 phosphate buffer solution (Fig. 1).

The adhesion tests of CS-coated liposomes dispersed in saline solution were carried out using various parts of the small intestine, which were cut to a length of 15cm. These parts of the small intestines were designated S.I.(15), S.I.(30), S.I.(45) and S.I.(60), where the number in parentheses denotes the distance from the lower end of the duodenum. They are corresponding to the upper jejunum, lower jejunum, upper ileum, and lower ileum. A considerable adhesive % of CS-coated liposomes was observed for each part, while a little adhesive % was observed in the case of noncoated liposomes (Fig. 4(A)). Considering the pH change in the intestinal tract, similar adhesion tests were conducted with changing of the pH of the medium in which the liposomes were dispersed. A difference in the adhesion % between CS-coated and noncoated liposomes was again observed as shown in Fig.4(B).

Next, the adhesion test was conducted with the CS-coated or noncoated liposomes containing pyren to confirm the mucoadhesion of CS-coated liposomes fluorescence microscopically. After the adhesion test, the intestine was washed with a large volume of saline solution and the luminal surface of the intestine was observed with a fluorescence microscope. A large number of liposomes were observed in the micrograph when the CS-coated liposomes were used in the adhesion test, while no liposome particles were found in the case of noncoated liposomes.

Oral Administration of Insulin-loaded Liposomes to Rats

To confirm the usefulness of the mucoadhesive liposomes in improving the bioavailability of drugs, an in vivo test was carried out with insulin-loaded liposomes coated with CS. Although conflicting data regarding the usefulness of insulin-loaded liposomes for oral administration, it seems possible that liposomes promote the enteral absorption of insulin (15). The mucoadhesive properties of CS-coated liposomes were expected to increase the probability of insulin absorption in the intestinal tract by increasing the residence time and possibly the concentration of insulin in the mucosa. A longer residence time of the liposomes might lead to prolongation of the observed therapeutic effect, i.e., hypoglycemic responses.

The CS-coated or noncoated liposomes containing insulin and an insulin solution as a reference were administered to normal rats orally through an intragastric tube, and the blood glucose level was monitored. A different pattern of change in basal blood glucose level as a function of time was observed in each case as shown in Fig. 5(A). In the case of insulin solution, the basal glucose level showed a minimum at 1h after administration, and immediately recovered to the initial level. Administration of noncoated insulin-loaded liposomes caused a significant reduction in basal glucose level for 3h from 1h after administration, after which the level gradually increased to the initial level. A marked reduction in basal blood glucose level was observed 30 min after administration of the CS-coated insulin-loaded liposomes, although little reduction was detected in the case of the noncoated insulin-loaded liposomes or insulin solution at that time. The most striking result was that the reduced basal blood glucose level was maintained up to at least 12h after administration of CS-coated insulin-loaded liposomes. This prolonging effect of CS-coated liposomal systems could be attributed to their mucoadhe-

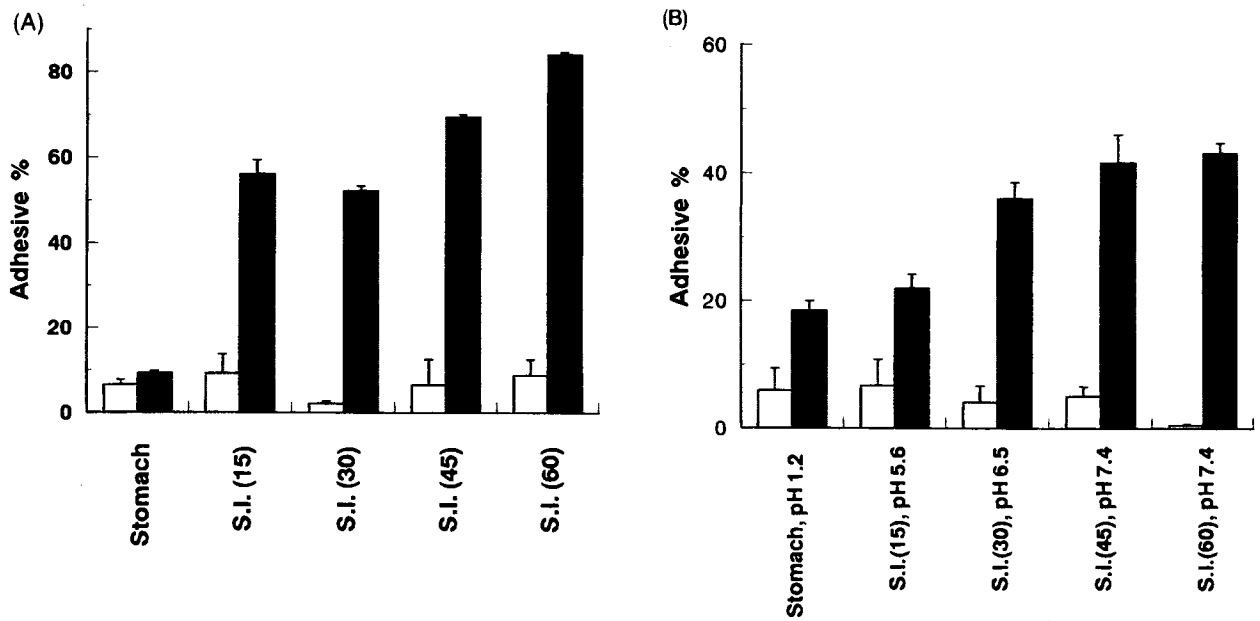


Fig. 4. Adhesive % of CS-coated liposomes dispersed in (A) saline solution or (B) buffer solutions of various pHs at varius parts of the rat intestine. □: Noncoated liposome, ■: CS-coated liposome. Lipid composition: DPPC:DCP = 8:2. Polymer concentration: 0.75%. Dispersion medium: saline solution (pH 6.0), phosphate buffer solution (pH 7.4 or 6.5), acetate buffer solution (pH 5.6) or disintegration solution No. 1 (pH 1.2) specified in JP XII. The results are expressed as mean ± sd of two experiments.

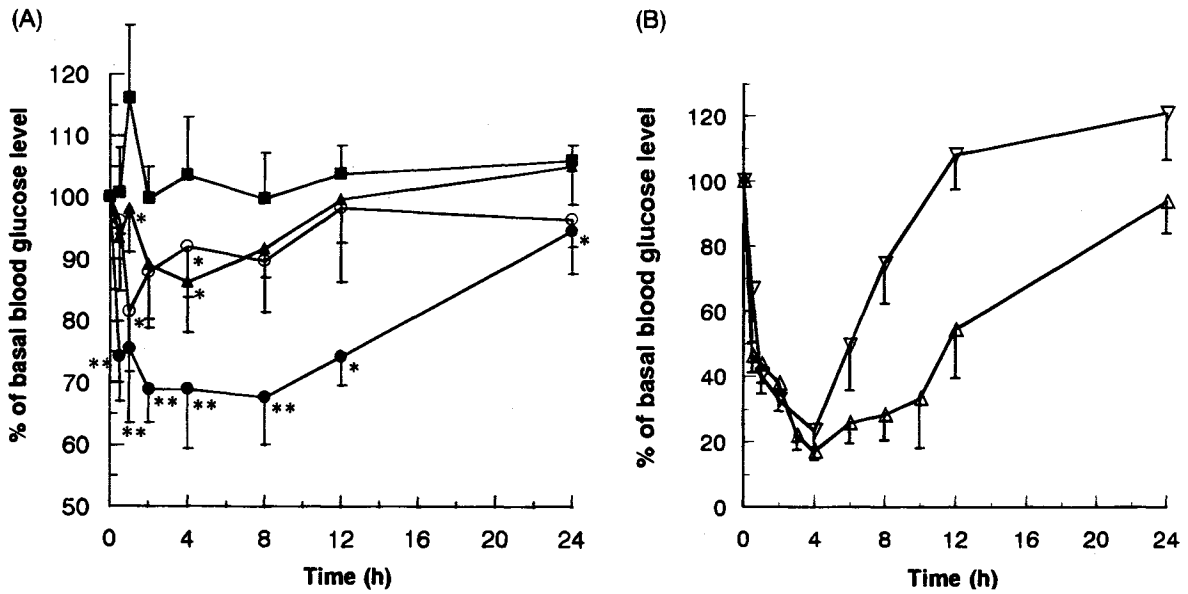


Fig. 5. Change in basal blood glucose level (%) after (A) oral administration of an insulin solution or insulin encapsulated in liposomes and (B) subcutaneous injection of insulin solution. ○: noncoated liposome, ●: CS-coated liposome; ■: control, ▲: insulin solution. △: subcutaneous injection of insulin solution (5IU/rat); ▽: subcutaneous injection of insulin solution (1IU/rat). The dose of insulin for oral administration was 24IU/rat. Symbols represent mean ± sd of six experiments for oral administration of noncoated and CS-coated liposomes, five experiments for control and oral administration of insulin solution, and three experiments for subcutaneous injection of insulin solution. Statistically significant difference from control: $p < 0.01$, **; $p < 0.05$, *.

sive properties. It is presumed that an insulin molecule released from the liposomes in the mucous layer can be absorbed without being enzymatically degraded.

To investigate the relative effectiveness of insulin-loaded liposomes orally administered, two different doses of insulin solution were injected subcutaneously in rats as a reference. The basal glucose level changes after the subcutaneous injection are shown in Fig. 5(B). The area of reduced basal blood glucose level up to 24h after oral administration of insulin-loaded liposomes was compared with that in the case of subcutaneous injection. The relative effectiveness of insulin-loaded CS-coated liposomes orally administered was calculated by comparing these values per unit dose. The calculated value was 10% in comparing with the case of 5 IU injection. When comparing with the case of 1 IU injection, the value was calculated to be 5%. The values for noncoated liposomal insulin and insulin solution were less than one-fifth that for CS-coated liposomal insulin.

Although more detailed studies are required to clarify the mechanisms of drug absorption from polymer-coated liposomes, the results presented here confirm the usefulness of polymer-coated liposomes in enhancing the enteral absorption of poorly absorbed drugs such as insulin.

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