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Effects of Oral Administration of Positively Charged Insulin Liposomes on Alloxan Diabetic Rats: Preliminary Study

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Synopsis

Insulin encapsulated in liposomes of various lipid compositions were prepared. The amount of insulin trapped in these liposomes increased in the order, negatively charged liposomes <neutral liposomes <positively charged liposomes. In positively charged liposomes, the amount of insulin trapped increased with increase in the amount of amphiphile stearylamine. Under the conditions tested, the highest insulin content (about 50%) was obtained with liposomes composed of phosphatidyl choline / cholesterol / stearylamine in a molar ratio of 7 / 2 / 2.25. These liposomes were stable on incubation for 3 hr at 37°C in solutions of pepsin, trypsin, and pancreatin, and after these incubations, a considerable amount of insulin was still associated with the liposomes. However, the liposomes released almost all the insulin into the medium on treatment with bile.

When the liposomes were administered orally to rats in the 3rd phase of acute alloxan diabetes, reduction of the blood glucose level was observed in 7 of 11 animals, the reduction persisted for several hours and was ranging from 30 to 75%.

In alloxan diabetic rats showing hyperglycemia for 3 to 6 months, the liposomes also increased the glucose tolerance in half the animals tested.

Oral administration of insulin without loss of its biological activity has been attempted in several laboratories: oral administration of negatively charged insulin liposomes was effective in streptozotocin-induced diabetic rats, but not in normal animals (Patel and Ryman, 1976, 1977); intragastric administration of neutral insulin liposomes significantly decreased the blood glucose in normal and diabetic rats (Gregoriadis *et al.*, 1976; Dapergolas and Gregoriadis, 1976, 1977); oral administration of water-in-oil-in-water type emulsions of insulin was also examined (Shichiri *et al.*, 1974, 1976). We have been using liposomes as a model of thyroid hormone secretion (Kawada *et al.*, 1974, 1975, 1976). The present paper reports the trapping of insulin in various kinds of liposomes. Finding that insulin was trapped most efficiently in positively charged liposomes and that this type of liposomes was considerably stable, we then studied the effects of oral administration of positively charged insulin liposomes on alloxan-induced diabetic rats.

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Materials and Methods

Preparation of insulin liposomes

Egg yolk lecithin was obtained by the method of Pangborn (1951). Charged amphiphile used were

dicetylphosphate (Sigma Chem. Co., St. Louis), and stearylamine (Wako Pure Chem. Indust., Tokyo).

For negatively charged liposomes, lecithin, dicetylphosphate, and cholesterol (Wako) were dissolved in 1.5 m*l* chloroform in the various molar ratios indicated. For positively charged liposomes, stearylamine was used instead of dicetylphosphate. To prepare neutral liposomes the charged amphiphile was omitted. Approximately 30 mg of total lipids were used. All experiments were carried out at room temperature unless otherwise noted, and air in the vessels used was replaced with nitrogen when necessary.

A thin lipid film was prepared on the inside wall of a small flask by evaporating the lipid solution in a rotary evaporator. The film was then dried in a desiccator *in vacuo* for 3 hr. Two mg of insulin (25.3 IU/mg, Sigma) was dissolved in 1 m*l* of 0.01 N HCl and the solution was neutralized by adding an equal volume of 0.1 M phosphate buffer (pH 7.5). The insulin solution and 2 m*l* of 0.1 M phosphate buffer (pH 7.5) were placed in the flask containing the lipid film and a homogeneous emulsion was made by swirling the flask for 10 min. The emulsion was kept to stand for 60 min in a water bath at 25°C, and then sonicated for 1 min at 4°C using a 12 mm titanium probe at 150 W in a Branson Sonifier, Model W-185. The resulting liposomes were then precipitated by centrifugation at 100,000×*g* for 60 min, and the precipitate was carefully resuspended in an appropriate volume of 0.1 M phosphate buffer (pH 7.5).

For determination of the amount of trapped insulin in liposomes, ^{131}I- or ^{125}I-labeled insulin was used as a marker. The radioactive insulin was prepared according to the method of Hunter and Greenwood (1962). Good separation of iodinated insulin fraction from inorganic iodide and iodinated bovine serum albumin was attained by Sephadex G-25 column chromatography. Two m*l* of the non-labeled insulin solution (1 mg/m*l*), 1 m*l* of the ^{131}I- or ^{125}I-labeled insulin solution (approx. 100,000 cpm), and 1 m*l* of 0.1 M phosphate buffer (pH 7.5) were placed in the flask containing the lipid film. Then liposomes were prepared as described above.

Examination of the stability of insulin liposomes in vitro under various conditions

The enzymes used in this experiment were pepsin (from hog stomach mucosa, 3,035 U/mg protein, trypsin (from bovine pancreas, 12,700 U/mg protein) (Sigma) and pancreatin (N.F., Difco Lab., Detroit).

^{125}I-labeled insulin liposomes prepared as above were used to determine the stability of liposomes under various conditions. One m*l* of suspension of the labeled liposomes in 0.1 M phosphate buffer (pH 7.5) was counted (A cpm), and it was mixed with each 1 m*l* of the following solutions; solution I (JP) (isotonic HCl solution, pH 1.2*), solution II (JP) (isotonic phosphate solution, pH 7.5), pepsin solution (weight ratio of trapped insulin to enzyme, 200 : 1; 0.1 M glycine buffer, pH 1.2), trypsin solution (weight ratio of trapped insulin to enzyme, 10 : 1; isotonic 0.1 M Tris-HCl buffer, pH 8.0), pancreatin solution (0.2 mg/m*l*, 0.15 M phosphate buffer, pH 7.5), and bile diluted 2-, 4-, and 16-fold with 0.1 M phosphate buffer, pH 7.5. The bile had been collected by cannulation from rats starved overnight. As controls, the liposomal suspension was mixed with phosphate buffer (pH 7.5) or Triton X-100 solution (final concentration of 0.5% with 0.1 M phosphate buffer, pH 7.5). The mixtures were incubated for 3 hr at 37°C, cooled in an ice-water bath and centrifuged at 100,000×*g* for 60 min. The radioactivity of the precipitates was counted (B cpm) and the stability of liposomes was expressed as the percent released of radioactivity, i.e. 100 (A−B)/A.

Animal experiments

Male Wistar strain rats weighing 200–300 g were fed for 3 hr each day between 14.00 and 17.00 h for ten days. At 10.00 h on day 11, alloxan (4 mg/100 g body wt.) was administered to each animal intravenously, and food was given from 18.00 to 21.00 hr. On the assumption that liposomes should pass from the stomach to the intestine more easily when the stomach is empty, the animals were starved as described above before liposome administration. The initial blood sample was taken at 10.00 h on day 12, immediately followed by oral administration of 2 m*l* of insulin liposome suspension in 0.1 M phosphate buffer (pH 7.5) or 2 m*l* buffer only by a stomach tube. Thereafter, blood was taken from the tail vein at the times indicated and the concentration of blood sugar was measured by the conventional method with o-toluidine-borate reagent or the enzymic method using ATP (Boehringer Mannheim, GmbH), NADP (Kyowa Hakko Kogyo, Tokyo), hexokinase and glucose-6-phosphate dehydrogenase (Oriental Yeast Co., Tokyo).

Six alloxan diabetic rats showing hyperglycemia for 3 to 6 months were repeatedly used for the glucose tolerance test. For the control experiment, the animals were starved for 24 hr. Each rat was orally given 3 g/kg body wt. of glucose as a 20% solution. Blood was taken from the tail vein over a period of 8 hr at the time indicated and blood glucose was determined enzymically. On day 2 and 7 after the control experiment, the same rats were treated again in the same way and liposomal insulin (approx. 20 IU/rat) was administered intragastrically 30 min after glucose loading.

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* Isotonicity was attained by adding NaCl. The pH values shown here indicated final pH after the liposome suspension and each test solution were mixed.

Results

Trapping of insulin liposomes

As shown in Table 1, the amount of insulin trapped in liposomes was greatly influenced by the lipid composition: the trapping was highest in positively charged liposomes and lowest in negatively charged ones. The increase in the relative content of cholesterol tended to decrease the insulin content in positively charged liposomes, increase the content in neutral liposomes, and produced no effect on the content in negatively charged liposomes.

In positively charged liposomes, the molar proportion of amphiphile stearylamine influenced the content: doubling insulin the molar percentage of stearylamine resulted in trapping of insulin twice with all compositions of lipids tested. The greatest amount was trapped in liposomes with a molar ratio of phosphatidyl choline/cholesterol/stearylamine of 7/2/2.25 under our experimental conditions. Therefore, liposomes of this composition were used in the subsequent experiments.

Stability of insulin liposomes in vitro

As seen in Fig. 1, about 25% of the initial radioactivity of insulin was recovered in the 100,000×g supernatant at 0-time, probably due to the mechanical destruction of liposomes or the presence of extremely small unsedimentable liposomes, or both. This value was regarded as a back ground count, and subtracted from the values obtained after various treatments. Incubation with trypsin or pancreatin for 3 hr at 37°C caused no significant increase in the release of radioactivity compared with the control. Incubation with pepsin resulted in a slightly higher release, but the value was not significantly different from that of the incubation in the acidic medium, solution I. The small increase in the release of radioactivity ob-

Table 1. Trapping of insulin in liposomes.

Lipid comp.	μmoles of PC	Total lipids (mg)	Insulin (%)
PC/Ch/SA			
7/2/1	35	30.4	25.7
7/2/2.25	33	30.3	48.1
6/3/1	32	30.7	20.7
6/3/2.25	30	30.4	43.1
5/4/1	28	30.3	18.7
5/4/2.25	26.5	30.5	32.7
PC/Ch/DCP			
7/2/1	33.5	30.4	2.5
6/3/1	30	30.1	3.0
5/4/1	26.5	30.2	2.5
PC/Ch			
7/2	36.5	30.3	3.6
6/3	33	30.1	4.5
5/4	29.5	30.4	5.3

PC: Lecithin, Ch: Cholesterol, SA: Stearylamine, DCP: Dicetylphosphate.

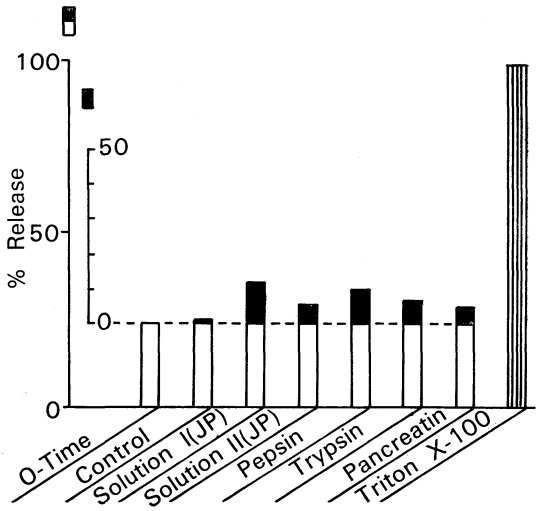

Fig. 1. Stability of positively charged insulin liposomes under various conditions. Released % of ^{125}I-insulin into each medium during 3 hr incubation at 37°C is shown as a mean of triplicates. Standard deviations are not indicated, since they are negligibly small. Released % of the labeled insulin from liposomes in the control without incubation is shown as "0-Time". Closed bars indicate the values subtracting (broken line) the value of "0-Time".

served in the acidic medium is discussed in the next section. Thus under these *in vitro* conditions a considerable amount of insulin remained trapped in the liposomes after incubation for 3 hr under proteolytic conditions. Triton X-100 caused almost the complete release of insulin from the liposomes. Moreover, the liposomes were highly sensitive to bile (Fig. 2), 2-fold, and 4-fold dilutions of bile resulting in almost 100% release of labeled insulin from the liposomes, although 16-fold dilution of bile resulted in only 24% release.

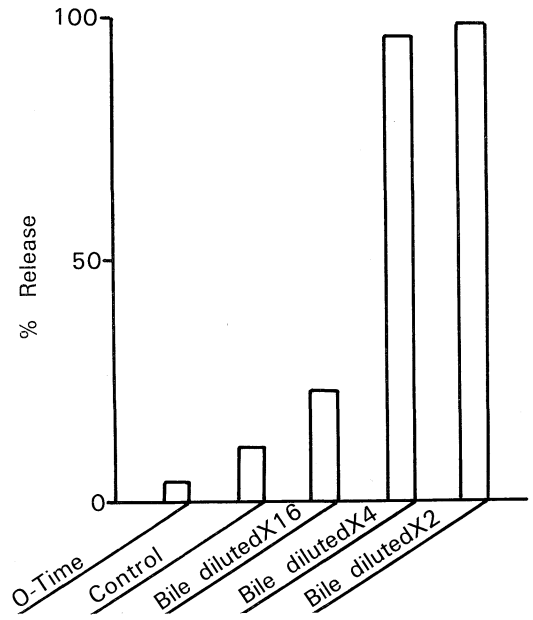

Fig. 2. Stability of positively charged insulin liposomes in bile diluted 2-, 4-, and 16-fold with 0.1 M phosphate buffer, pH 7.5. The other explanation is given in the legend of Fig. 1.

Effect of oral administration of insulin liposomes

1) *Effect of liposomal insulin on rats with acute diabetes*

Insulin liposomes were given to rats after alloxan injection. At this time the rats had been starved for 17 hr. As shown in Fig. 3, the blood sugar level of individual rats varied at 28 hr after alloxan administration. In 4 rats the level increased

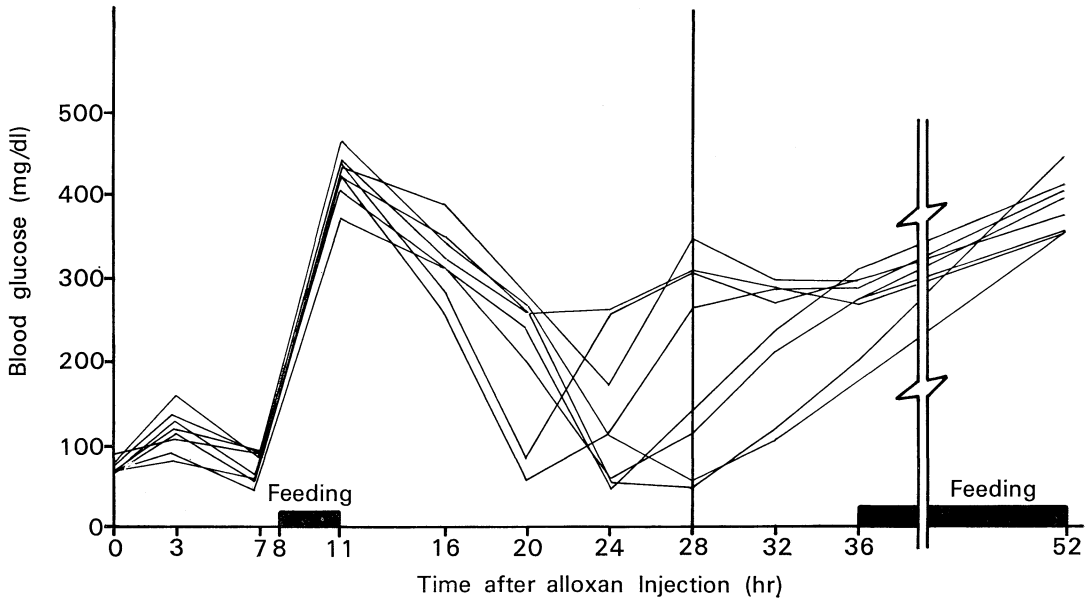

Fig. 3. Change in blood sugar levels of individual rat 28 hr after alloxan administration (40 mg/kg body wt.). Blood glucose was determined by the enzymic method.

thereafter for 8 hr at least. In the same 8 hr period, the remaining 4 rats showed almost constant level of blood glucose or slight decrease (less than 10%). Similar patterns of change in blood sugar were obtained in another experiment using 10 rats. Therefore, according to our experimental conditions, from 28 hr to 36 hr at least after alloxan administration no significant reduction of blood glucose was observed on starved rats.

On the basis of these control experiments, the change in blood glucose levels was examined after oral administration of insulin liposome or 0.1 M phosphate buffer. The results are shown in Fig. 4 (effective cases) and Fig. 5 (non-effective cases). In these Figures, blood glucose concentrations are shown as percentage of values at 0-time, i.e. the concentrations just before administration of insulin liposomes. Liposomal insulin markedly decreased the blood

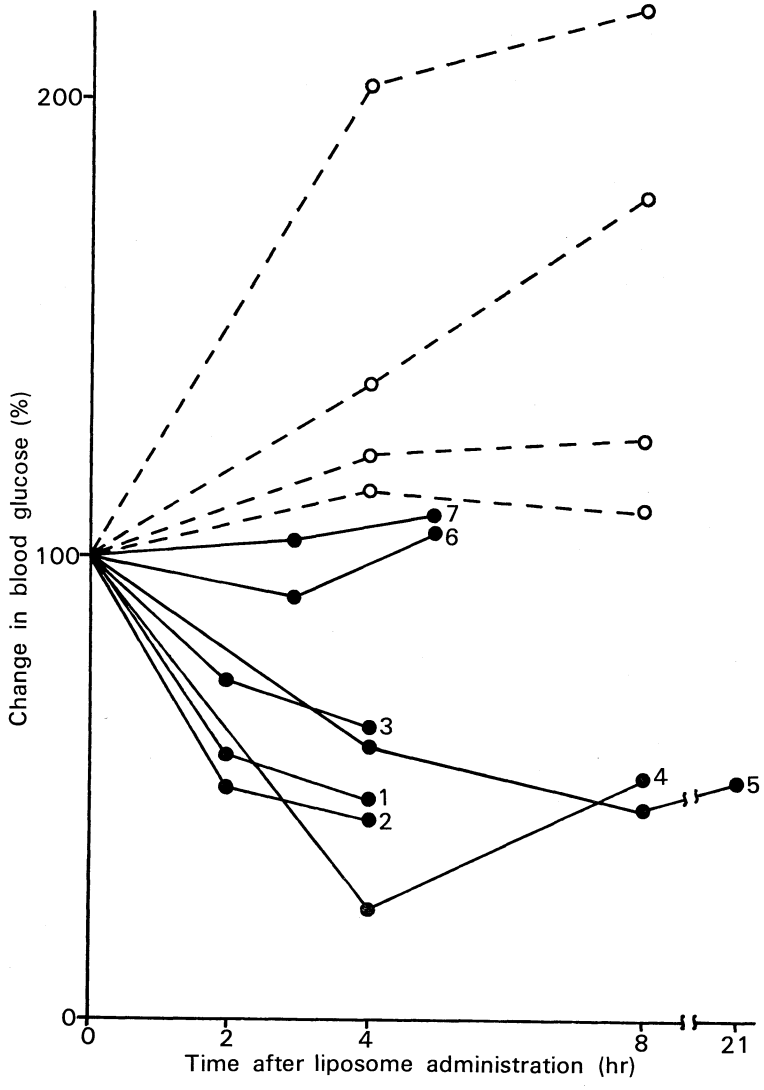

Fig. 4. Effective cases of oral administration of liposomal insulin (approx. 20 IU/rat) on rats with acute alloxan-induced diabetes (solid lines). Blood glucose concentrations are shown as percentages of values at 0-time, i.e. concentrations just before administration of insulin liposomes. Broken lines indicate the levels of blood glucose of control rats given 0.1 M phosphate buffer by oral administration. Blood glucose was determined by the method using o-toluidine-borate reagent. The blood glucose level at 0-time of each rat was as follows; rat No. 1. 266 mg/d*l*, No. 2. 164 mg/d*l*, No. 3. 230 mg/d*l*, No. 4. 320 mg/d*l*, No. 5. 322 mg/d*l*, No. 6. 235 mg/d*l*, and No. 7. 215 mg/d*l*.

glucose level (in cases Nos. 1, 2, 3, 4 and 5) 30 to 75% below the initial level within 4 hr. In two cases (No. 4 and 5), the observation was continued for more than 4 hr. The blood sugar reducing effect persisted for 8 to 21 hr. Liposomal insulin also seemed to be effective in cases No. 6 and 7, as explained later. The cases in which liposomes were not effective (4 of 11 rats) are shown in Fig. 5.

2) *Effect of insulin liposomes on glucose tolerance in rats with chronic alloxan diabetes*

The effect of liposomal insulin was also examined by the glucose tolerance test on alloxan diabetic rats that had shown hyperglycemia for 3 to 6 months. The results are shown in Fig. 6. In 6 of 12 cases (i and ii in a and b, ii in c, and ii in d) increased glucose tolerance was observed after oral administration of insulin liposomes. One rat (ii in b) died owing to the extremely low level of blood glucose.

Discussion

In this work we found that the association of insulin with liposomes was greatly influenced by the molar percentage of stearylamine in the liposomes. The net charge of insulin in the solution used for preparation of insulin liposomes is negative, since the isoelectric point of insulin is about pH 5.3. Therefore, the increased association of insulin with liposomes on raising the molar percentage of stearylamine is presumably due to increase in the electrostatic and hydrophobic interaction of insulin with positively charged liposome membranes.

The increased release of labeled insulin from liposomes observed in acidic medium could be explained by a change in protein-

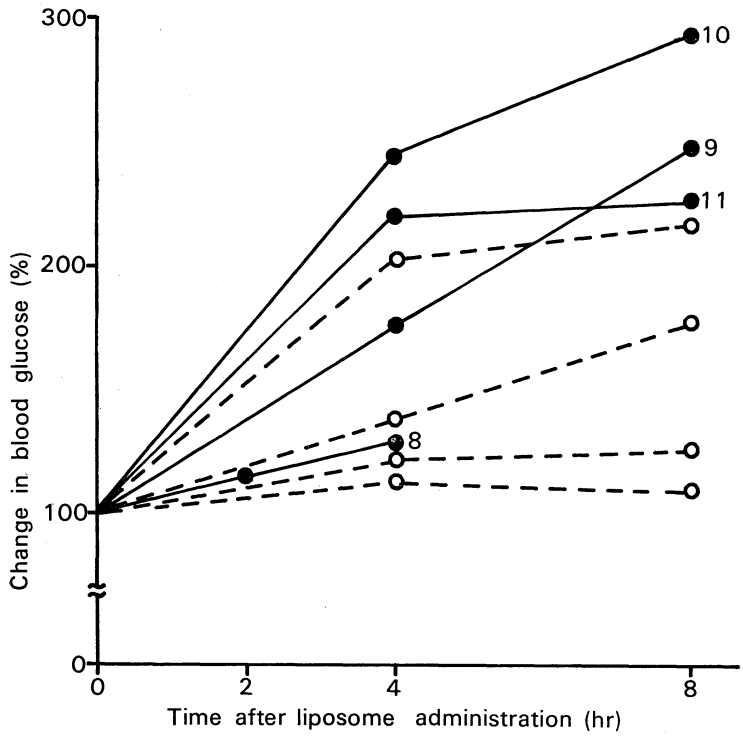

Fig. 5. Non-effective cases of oral administration of liposomal insulin on rats with acute diabetes. Details are given in the legend of Fig. 4. The blood glucose level at 0-time of each rat was as follows; rat No. 8. 251 mg/d*l*, No. 9. 132 mg/d*l*, No. 10. 128 mg/d*l*, and No. 11. 192 mg/d*l*.

lipid interactions with insulin. Insulin bound to the outermost positively charged lipid layer of the liposomes would be released at pH value below the isoelectric point. Thus at pH 1.2 in solution I or in pepsin solution, the net negative charge of insulin should be reversed, resulting in release of insulin.

In this work we found that the oral administration of insulin liposomes to rats

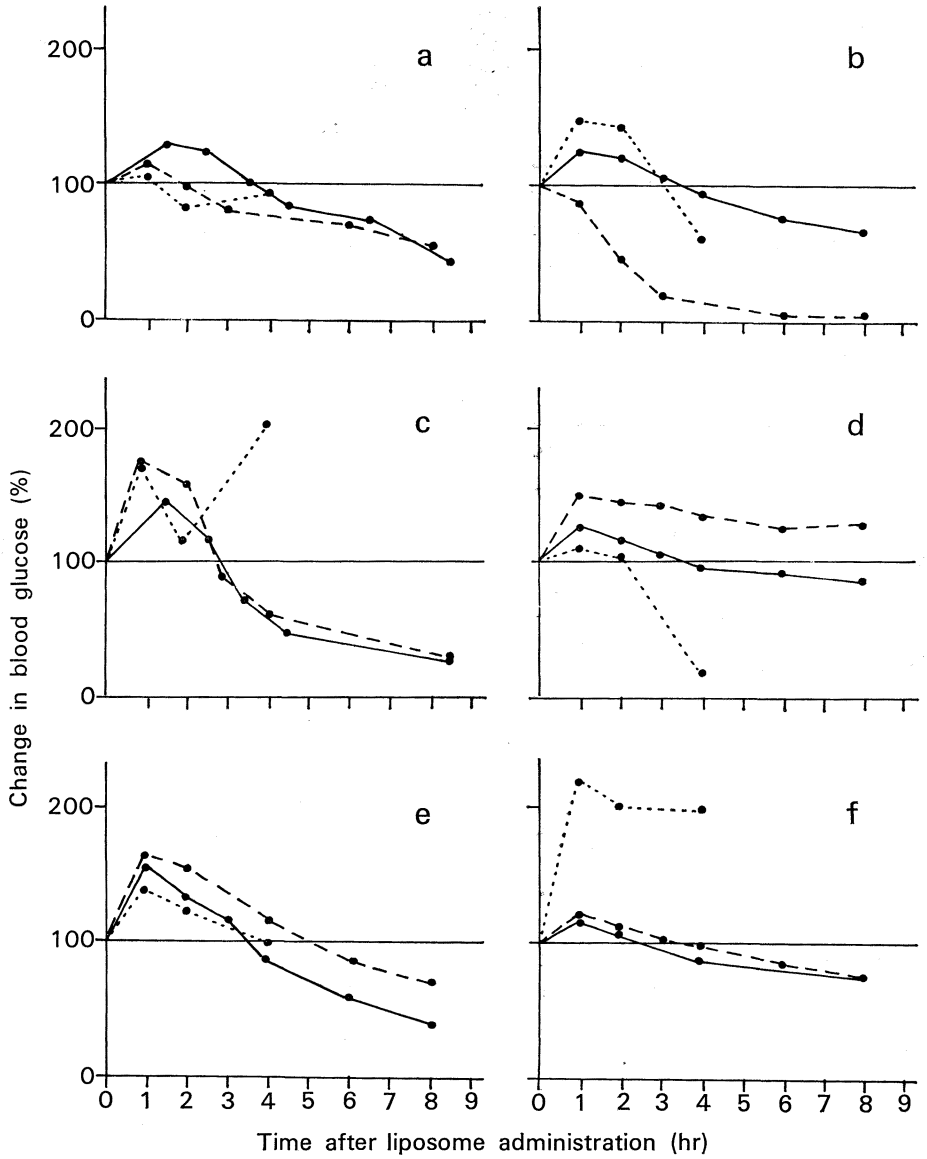

Fig. 6. Effect of oral administration of liposomal insulin (approx. 20 IU/rat) on glucose tolerance in rats with alloxan diabetes showing hyperglycemia for 3 to 6 months. Blood glucose concentrations are shown as percentages of values at 0-time, i.e. concentrations just before administration of insulin liposomes. Blood glucose was determined by the enzymic method. Solid line indicates control. Dotted line (i, trial on day 2) and broken line (ii, trial on day 7) indicate liposomal insulin. Details are given in the text.

with acute diabetes clearly reduced the blood sugar levels in 5 of 11 rats. Although the liposomes did not reduce the blood sugar level in two cases (No. 6 and 7 in Fig. 4), they seemed to be effective for the following reasons. As shown in Fig. 3, individual blood glucose levels 28 hr after alloxan administration were variable. However, in the animals showing the low blood glucose level at this time, the level tended to increase subsequently. Considering this, the initial blood glucose levels of rats No. 6 and 7 were rather low (235 and 215 mg/d*l*, respectively). If insulin had not been given to these animals, the levels of glucose would probably have increased in the next few hours, but in fact they remained almost constant for 6 hr after administration of liposomal insulin. The liposomal insulin probably prevented an increase in blood glucose in these animals.

It is reported that introduction of insulin into isolated jejunal loops decreases the blood sugar and that its effect is increased by addition of bile salts (Davenport, 1962). On the basis of these findings and our finding that liposomes were stable in the pepsin solution, it seems probable that in our experiments, the insulin liposomes passed into the intestinal lumen without destruction by gastric proteolytic enzymes and that in the intestine the insulin was released by the surfactant activity of bile salts, as observed in our *in vitro* experiments (Fig. 2).

The presence of intact insulin liposomes in the serum after their oral administration has been suggested by Dapergolas and Gregoriadis (1976). However, as Davenport (1962) and Shichiri *et al.* (1974) described, the size of liposome should be small enough to come in sufficiently close contact with the intestinal mucosal surface to be absorbed. Electron microscopy showed that our liposomes prepared by the method of Dapergolas and Gregoriadis (1976) ranged from 0.1 to 1 μm in diameter and thus they were probably too big to be absorbed unchanged from the intestine. Recently, Patel and Ryman (1977) gave no evidence to support the fact that liposomes appeared in the serum after their oral administration.

The optimal feeding condition for administration of insulin liposomes requires examination. In the experiment reported by Dapergolas and Gregoriadis (1979), liposomes were administered to fed rats. In our experiment starved animals were used. As shown in Fig. 3, the blood sugar level was variable at the time liposomes were given, probably owing to this starvation.

Acknowledgement

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