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Application of Liposomes to the Pharmaceutical Modification of the Distribution Characteristics of Drugs in the Rat[1,2]

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In order to examine the possibility of utilizing liposomes as drug carriers, the fate of ^{14}C-inulin and ^{131}I-insulin entrapped in negatively or positively charged liposomes after intravenous injection was investigated in rats.

The clearance rate from plasma of ^{14}C-inulin entrapped in liposomes was faster than that of free ^{14}C-inulin, and decreased with the increase of the liposomal concentration. Further, liposome-entrapped drugs were recovered mainly in the liver, especially in Kupffer cells, as well as the spleen, and such hepatic accumulation was reduced by pretreatment with methyl palmitate to a great extent.

From these results, it appears that the large proportion of drugs entrapped in liposomes is delivered into the RES intact and their distribution pattern depended on the liposomal surface properties, but not on the kind of drugs entrapped.

The use of liposomes as drug carriers has been noted recently. In the previous paper[1b] from our laboratory, it was reported that the duration of the intramuscular absorption of some drugs can be achieved by entrapping them into phosphatidylcholine liposomes. Attempts to deliver amyloglucosidase,[4] chelating agents,[5] and actinomycine D[6] into the liver and the spleen have been reported. However, there appears to be much less known about the detailed mechanism of such unique liposomal effects.

The present studies were undertaken to investigate the fate of drugs entrapped in liposomes after intravenous administration in an attempt to examine the possibility of utilizing liposomes for modifying the drug distribution characteristics.

Experimental

Materials——^{14}C-Inulin and ^{131}I-insulin were purchased from Japan Radioisotope Association (Tokyo, Japan). Cholesterol, dicetyl phosphate, stearylamine, triolein, and choline chloride were of analytical grade and obtained commercially. Phosphatidylcholine was purified using a modification of the method of Rhodes and Lea.[7] After extraction from egg yolks with chloroform–methanol (1:1), the extract was chromatographed on alumina (Merck, for chromatograph) and eluted with chloroform–methanol (1:1). The solvent of the eluate was evaporated, and the residue was dissolved in chloroform. The precipitate produced by the addition of acetone to the chloroform solution was collected and redissolved in chloroform and passed through a silicic acid (Mallinckrodt) column. After removal of contaminating lipids with chloroform–methanol (9:1), pure phosphatidylcholine was eluted with chloroform-methanol (7:3). The purity was checked by thin-layer chromatography (TLC) on a silica gel G plate with chloroform–methanol–water (65:25:4) system. Purified phosphatidylcholine was dissolved in chloroform and stored under nitrogen at −5°.

1) *a*) This paper constitutes the 10th report in a series of "Biopharmaceutical Studies on the Parenteral Preparations." *b*) Preceding paper, Part IX: E. Arakawa, Y. Imai, H. Kobayashi, K. Okumura, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), **23**, 2218 (1975).
2) Part of this work was presented at 94th Annual Meeting of Pharmaceutical Society of Japan, Sendai, April, 1974.
3) Location: *Yoshidashimoadachi-cho, Sakyo-ku, Kyoto, Japan.*
4) G. Gregoriadis and B.E. Ryman, *Europ. J. Biochem.*, **24**, 485 (1972).
5) Y.E. Rahman, M.W. Rosenthal, and E.A. Cerny, *Science*, **180**, 300 (1973); Y.E. Rahman, M.W. Rosenthal, E.A. Cerny, and E.S. Moretti, *J. Lab. Clin. Med.*, **83**, 640 (1974).
6) G. Gregoriadis, *FEBS Letters*, **36**, 292 (1973).
7) D.N. Rhodes and C.H. Lea, *Biochem. J.*, **65**, 526 (1957).

Preparation of Liposomes——Liposome suspension containing ^{14}C-inulin (10 μCi/ml) or ^{131}I-insulin (200 μCi/ml) were prepared by the same method described in the previous paper.[1]

Procedure of Animal Experiment——Male Wistar albino rats weighing 180—230 g were used in all experiments.

After anesthesia with pentobarbital and injection of heparin sodium (100 unit/100 g body weight) into the femoral vein, a polyethylene tubing (0.50 mm i.d., 0.80 mm o.d.) was inserted into the carotid artery.

Controlling the body temperature at 36±1°, drugs were administered from the opposite femoral vein for about 1 min. At regular time intervals, blood samples were collected into heparinized tubes through the polyethylene tubing inserted previously into the carotid artery. After centrifugation at 3000 rpm for 5 min, plasma was assayed for ^{14}C or ^{131}I activity.

Animals were killed by complete bleeding 30 or 60 min later, and various tissues were excised, soaked in saline, blotted on filter papers, weighed, and analyzed.

Preparation of Injections——Liposomes prepared with 10 μmoles phosphatidylcholine, 30 μmoles cholesterol, and either 3.3 μmoles dicetyl phosphate or stearylamine were passed through a Sephadex G-75 column (16 mm i.d., 170 mm length) and eluted with buffered saline (NaH_2PO_4–Na_2HPO_4 pH 7.0 buffer and 0.9% NaCl solution, 1/10 and 9/10 by volume, respectively). The turbid liposomal elution, 5.5 ml, was collected and diluted to 22.0 ml with buffered saline.

To prepare a suspension of high liposomal concentration, drug-containing liposomes were diluted four times with a suspension of drug-free liposomes.

As aqueous injectable solutions, ^{14}C-inulin and ^{131}I-insulin were dissolved in buffered saline to 0.23 μCi/ml and 200 μCi/ml, respectively.

These preparations were given to rats at a dosage of 1 ml/100 g body weight.

Pretreatment with Methyl Palmitate——Rats were pretreated with methyl palmitate according to Wooles, *et al.*[8] Methyl palmitate was suspended in 5% dextrose solution with 0.1% Tween 20 at the concentration of 100 mg/ml and sonicated at about 50° (100 W, 100 min) to make emulsions. This emulsion was prepared immediately prior to injection. Rats, received two daily intravenous injection of methyl palmitate in the amount of 120 mg/100 g body weight, were used in distribution experiments 24 hr after the last injection.

Pretreatment with Choline and Triolein——The distribution of ^{14}C-inulin entrapped in liposomes was determined in rats pretreated with choline chloride using the method of Heller[9] or with triolein emulsions in the amount of 25 mg/100 g body weight by the method of Altura, *et al.*[10]

Isolation of Kupffer Cells——Isolation of Kupffer cells from the rat liver was performed essentially according to Peake, *et al.*[11] Rats were bled 15 min after injection and remaining blood was cleared by perfusion through the *thoracic aorta* with about 70 ml of saline. The liver was then removed, washed quickly in saline, and sliced into 3—5 mm fragments. These fragments were incubated in 25 ml of Pronase (Kaken Chem. Co. Ltd., 4500 p.u.k./g) solution in Gey's balanced salt solution[12] by vigorous stirring on a magnetic stirrer at 37°. After 60 min, the mixture was centrifuged at 300 ***g*** for 3 min and washed two times with Gey's solution. The sediment containing rich Kupffer cells, swollen overnight by the addition of 6 ml of 0.5N NaOH, was dissolved by heating to 90—95° for 30 min. To the supernatant 1 ml of 3N NaOH was added. After cooling, each fraction was made up to 50 ml. Samples of 5 ml were withdrawn and protein was precipitated by the addition of 3 ml of 10% $ZnSO_4$. After centrifugation, radioactivity of 1 ml of the supernatant was determined.

Analytical Methods——a) ^{14}C-Inulin: The whole tissues were weighed and radioactivity of these small pieces was determined by the same method reported in the previous paper.[13] In the case of plasma sample, 15 ml of NT-scintillation medium and 0.5 ml of 1 N HCl were added to 0.2 ml of plasma, shaked vigorously, and the radioactivity was determined.

b) ^{131}I-Insulin: ^{131}I activity of tissues was determined according to the previous paper.[1] The radioactivity of 1 ml of plasma in a polyethylene tube was determined in the well-type scintillation counter (TEN model Ats-121).

Results

(1) Clearance of ^{14}C-Inulin from Plasma

Figure 1 shows the clearance from plasma of ^{14}C-inulin in buffer solution and entrapped in negatively charged liposomes composed of phosphatidylcholine, cholesterol, and dicetyl

8) N.R. Di Luzio and W.R. Wooles, *Am. J. Physiol.*, **206**, 939 (1964).
9) J.H. Heller, *Science*, **118**, 353 (1953).
10) B.M. Altura and S.G. Hershey, *J. Pharmacol. Exptl. Therap.*, **175**, 555 (1970).
11) I.R. Peake, *Internal. J. Vit. Nutr. Res.*, **43**, 291 (1973).
12) G.O. Gey and M.D. Gey, *Am. J. Cancer*, **27**, 45 (1936).
13) H. Kobayashi, T. Nishimura, K. Okumura, S. Muranishi, and H. Sezaki, *J. Pharm. Sci.*, **63**, 580 (1974).

phosphate in a molar ratio of 3:9:1 following intravenous injection. Plasma level at 1 min after injection was used as the 100% value to minimize the inter-subjects difference. Free ^{14}C-inulin disappeared from the circulation very slowly after distribution equilibrium was reached, while ^{14}C-inulin entrapped in liposomes was removed much more rapidly. The clearance was delayed with the increase of liposomal concentration ranging from 1 to 6.5 mg lipid/ml.

(2) Tissue Distribution of ^{14}C-Inulin

Figures 2 and 3 show the distribution of ^{14}C-inulin to various organs 60 min after injection. In the figures, the distribution is represented as % of dose per gram tissue or that in whole tissue. Although there was no significant accumulation of free ^{14}C-inulin in tissues except in the kidney, the marked recovery of ^{14}C-inulin-entrapped liposomes per gram liver and spleen

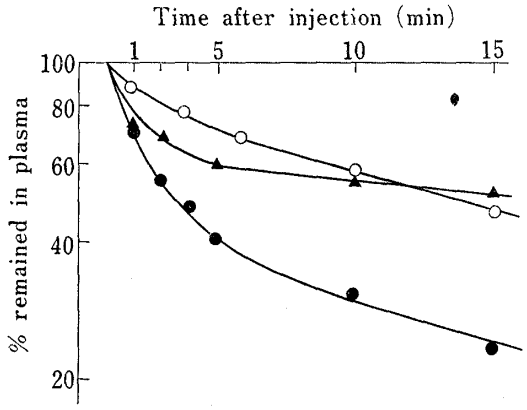

Fig. 1. Clearance of ^{14}C-Inulin from Plasma after Intravenous Injection

—▲—: buffer solution
—●—: liposome suspension (PC: CH:DP=3:9:1) 1 mg lipid/ml
—○—: liposome suspension (PC: CH: DP=3:9:1) 6.5 mg lipid/ml
PC: phosphatidylcholine
CH: cholesterol
DP: dicetyl phosphate
Each point represents the mean value of at least five experiments

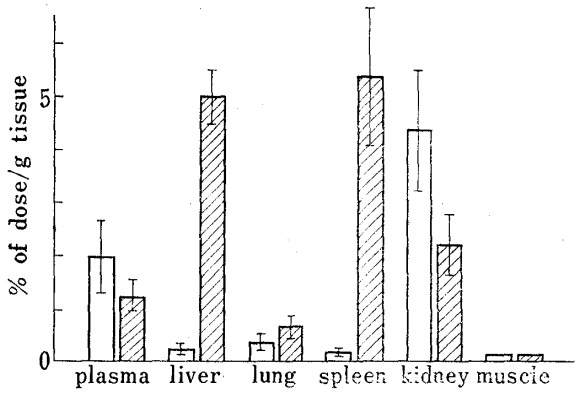

Fig. 2. Tissue Distribution of ^{14}C-Inulin 60 min after Intravenous Injection

□: buffer solution
▨: liposome suspension (PC: CH: DP=3: 9 1, 1 mg lipid/ml)
Each column represents the mean value of at least five experiments. Vertical bars indicate ± S.D.

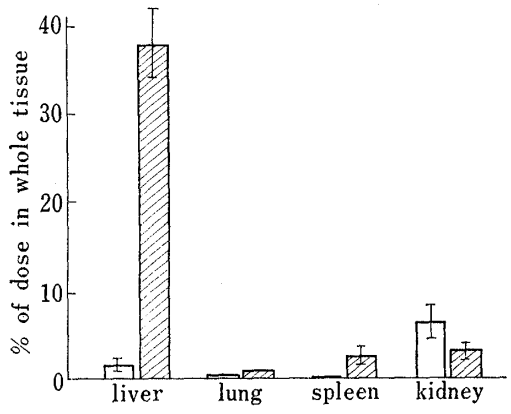

Fig. 3. Tissue Distribution of ^{14}C-Inulin 60 min after Intravenous Injection

Each column represents the mean value of at least five experiments. Vertical bars indicate ± S.D. Key: Refer to Fig. 2 for the column in this graph.

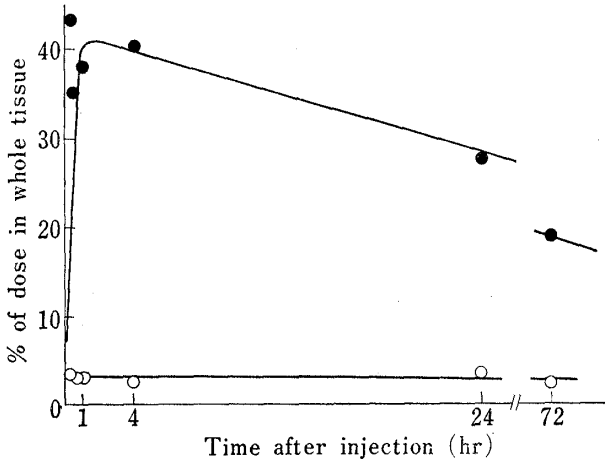

Fig. 4. Clearance of ^{14}C-Inulin Entrapped in Liposomes from the Liver and the Spleen

—●—: liver —○—: spleen
iposome suspension: (PC: CH: DP=3: 9: 1, 1 mg lipid/ml)
Each point represents the mean value of at least five experiments.

was observed. However, the total recovery of ^{14}C-inulin entrapped in liposomes in the liver was 38.0±3.8% and that in the spleen was only 2.70±0.56%.

(3) Disappearance of ^{14}C-Inulin from the Liver and the Spleen

The time-courses of the disappearance of ^{14}C-inulin entrapped in liposomes and accumulated in the liver and the spleen is shown in Fig. 4. As is evident from the figure, the considerable amount of ^{14}C-inulin was remained in these tissues even 72 hr after administration.

(4) Distribution of ^{14}C-Inulin between Kupffer and Parenchymal Cells in the Liver

The uptake of ^{14}C-inulin in the liver was examined on a cellular level as shown in Table I.

Table I. Distribution of ^{14}C-Inulin between Kupffer and Parenchymal Cells in the Liver 15 min after Intravenous Injection

	% in Kupffer cell rich fraction	% in parenchymal cell debris
Buffer solution	20.9± 8.2	79.1± 8.2
Liposome suspension[a)]	69.6±11.0	30.4±11.0

a) PC: CH: DP=3: 9: 1, 1 mg lipid/ml
PC: phosphatidylcholine, CH: cholesterol, DP: dicetyl phosphate
Each value represents the mean±S.D. of at least five experiments.

In contrast to free ^{14}C-inulin which was found largely in the parenchymal cell fraction, ^{14}C-inulin entrapped in liposomes was recovered in the Kupffer cell rich fraction up to about 70%.

(5) Effect of Methyl Palmitate on the Tissue Distribution of ^{14}C-Inulin Entrapped in Liposomes

Figure 5 shows the effect of pretreatment with methyl palmitate on the distribution of ^{14}C-inulin entrapped in liposomes to various organs. From this figure, it becomes clear that the accumulation in the liver was greatly reduced by the prior injection of methyl palmitate.

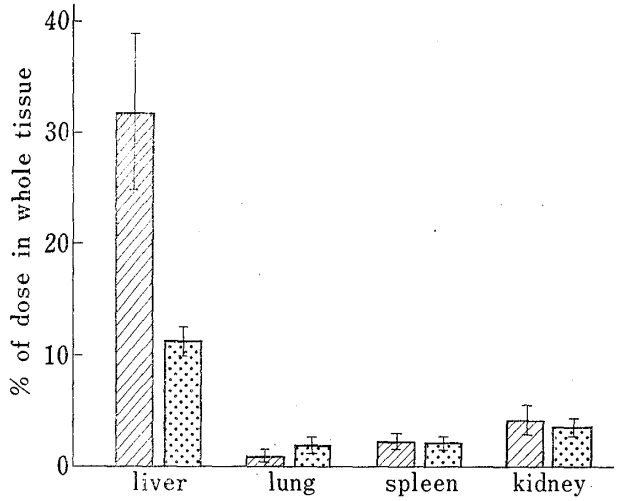

Fig. 5. Effect of Methyl Palmitate on the Tissue Distribution of ^{14}C-Inulin Entrapped in Liposomes 30 min after Intravenous Injection

liposome suspension: (PC: CH: DP=3: 9: 1, 1 mg lipid/ml)
▨: control
▩: pretreated with methyl palmitate
Each column represents the mean value of at least five experiments. Vertical bars indicate±S.D.

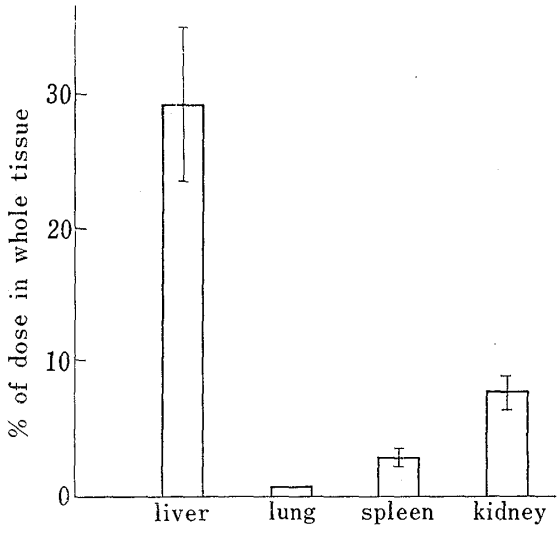

Fig. 6. Tissue Distribution of ^{14}C-Inulin Entrapped in Positively Charged Liposomes 60 min after Intravenous Injection

liposome suspension: (PC: CH: SA=3: 9: 1, 1 mg lipid/ml)
PC: phosphatidylcholine
CH: cholesterol
SA: stearylamine
Each column represents the mean value of at least five experiments. Vertical bars indicate±S.D.

(6) Effect of Charge of Liposomes on the Tissue Distribution of ^{14}C-Inulin Entrapped in Liposomes

Figure 6 reveals the distribution of ^{14}C-inulin entrapped in positively charged liposomes composed of phosphatidylcholine, cholesterol, and stearylamine in a molar ratio of 3:9:1 60 min after intravenous injection. Compared with negatively charged liposomes, less amount of the drug was recovered in the liver and *vice versa* in the kidney.

(7) Tissue Distribution of ^{131}I-Insulin

Figure 7 shows the distribution of ^{131}I-insulin in buffer solution and entrapped in liposomes composed of phosphatidylcholine, cholesterol, and dicetyl phosphate in a molar ratio of 3:9:1 60 min after intravenous injection. Contrary to large accumulation in the kidney of free ^{131}I-insulin, ^{131}I-insulin entrapped in liposomes was recovered in the liver as well as the spleen at high concentration.

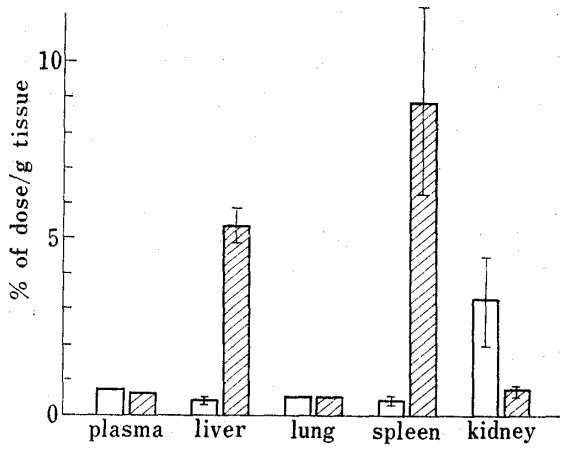

Fig. 7. Tissue Distribution of ^{131}I-Insulin 60 min after Intravenous Injection

Each column represents the mean value of at least five experiments. Vertical bars indicate±S.D. Key: Refer to Fig. 2 for the column in this graph.

Discussion

Since the tissue distribution of drugs after vascular administration depends mainly on the properties of drugs themselves, side effects caused by their distribution into non-intended organs have been inevitable. Recently, "drug delivery system" continues to receive attention and new pharmaceutical dosage forms as well as manners of administration which offer distinct advantage over conventional methods are being extensively investigated.

Liposomes are spherules of 1—50 mμ consisting of concentric lipid bilayers and persumed to be phagocytosed by the reticuloendothelial cells similarly to colloidal carbon and metals.[14] Therefore, it appears possible to deliver drugs primarily to the reticuloendothelial system (RES) by entrapping them into liposomes.

Since the purification of phosphatidylcholine by alumina chromatography described in the previous paper[1] could not remove sphingomyelin and lyso-phosphatidylcholine completely, the modified method of Rhodes and Lea[7] was used in this experiment. By this method phosphatidylcholine was almost completely purified.

In order to clarify the fate of drugs entrapped in negatively charged liposomes, their clearance from plasma and the distribution were compared with those of non-entrapped drugs. The clearance rate from plasma of ^{14}C-inulin entrapped in liposomes was faster than that of free ^{14}-C-inulin, and declined with the increase of the liposomal concentration. Such decline of the clearance rate is one of the main characteristics of the phagocytosis by the RES, which was reported in the case of colloidal carbon[15] and lipid emulsions.[16]

Soon after administration of ^{14}C-inulin entrapped in liposomes, radioactivity was recovered in the liver and the spleen, the reticuloendothelial organs, at high concentration, and slowly disappeared. However, the role of the spleen in the total recovery was much less important. The liver, the most important organ for the distribution of ^{14}C-inulin entrapped in liposomes, contains two major type of endocytosing cells, namely, parenchymal and Kupffer cells. Kupf-

14) Y.E. Rahman and B.J. Wright, *J. Cell. Biol.*, **59**, 276a (1973).
15) B.M. Halpern, *J. Pharm. Pharmacol.*, **11**, 321 (1959).
16) R.P. Cornell and T.M. Saba, *Am. J. Physiol.*, **225**, 1300 (1973).

fer cells belong to the RES and occupy about 30% of the liver.[17] Whereas most of free ^{14}C-inulin was found in the parenchymal cell fraction, 70% of ^{14}C-inulin entrapped in liposomes was found in the Kupffer cell rich fraction. From this result, the amount of ^{14}C-inulin entrapped in liposomes per gram of Kupffer cells is estimated to be more than five times as much as that per gram of parenchymal cells.

From the pattern of the clearance from plasma and that of the tissue and cell distribution, the large proportion of ^{14}C-inulin entrapped in liposomes appears to be incorporated into the reticuloendothelial cells intact. From this viewpoint, the effects of enhancement and inhibition of reticuloendothelial activity on the fate of ^{14}C-inulin entrapped in liposomes were studied. Although no effect was observed by stimulating pretreatment with choline and triolein emulsions, extensive reduction of hepatic uptake was observed with methyl palmitate.

As mentioned above, it has been made clear that drugs can be delivered mainly to the RES by entrapping into liposomes. It might be possible, therefore, to alter the distribution characteristics of drugs by specific manipulation of liposomal surface properties. In the present investigation, some difference in the tissue distribution of ^{14}C-inulin entrapped in liposomes negatively charged by dicetyl phosphate and positively charged by stearylamine was observed. Such differences *in vivo* and *in vitro* were also reported by Gregoriadis[6] and Magee, *et al.*[18] respectively. This effect of the liposomal charge could be attributed to the following possibilities: the difference in the affinity of tissues or the change of the physicochemical properties of liposomes themselves. As noted above, the distribution pattern of drugs entrapped in liposomes depended on the liposomal surface properties, but not on the kind of drugs because the tissue distribution of ^{131}I-insulin entrapped in negatively charged liposomes was similar to that of ^{14}C-inulin.

Thus, this finding raises the possibility that we might alter distribution characteristics of drugs in the clinical field by means of intravenous administration of a suspension of drug-containing liposomes.

17) K. Akazaki and M. Kojima, "Seirigaku-taikei," Vol 2, Igaku-shoin Co., Tokyo, 1968, p. 357.
18) W.E. Magee and O.V. Miller, *Nature*, **235**, 339 (1972).