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Effects of Dose and Vesicle Size on the Pharmacokinetics of Liposomes

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The blood concentration and urinary excretion after intravenous injection of liposomes containing ^{3}H-inulin at three dose levels were examined in rats. A pharmacokinetic model for the dose dependency is postulated based on the results. The effects of vesicle size were also examined and the biological disposition was simulated. Factors affecting the liposomal disposition are discussed.

Dose dependency of the disposition was observed at three dose levels (1.8, 14.9 and 70.3 μmol total lipids/rat) of liposomes having a mean diameter of 0.22 μm. The results suggest the existence of saturable processes in the uptake of liposomes by the reticuloendothelial system (RES) and leakage of inulin from liposomes, and a first-order rate process in the release of inulin from the cell after degradation of liposomes taken up by the RES. However, simulation of the data obtained after high-dose administration was unsuccessful with this model, the blood clearance of liposomes being faster than calculated. It is suggested that first-order processes may operate in parallel with the saturable processes of uptake and leakage.

The effects of the size of liposomes (mean diameter: 0.15, 0.22 and 0.43 μm) on the disposition were also examined. The patterns of the blood levels and urinary excretion of small liposomes and large liposomes were very similar to those of the high-dose and low-dose experiments, respectively, in spite of the very similar dose levels of total lipids administered.

Simulation suggested that the dose expressed in terms of the number of vesicles was suitable for evaluation of the liposomal disposition. The results indicate that the size and number of liposomes are important variables affecting the disposition of liposomes and they should be controlled strictly in liposomes for *in vivo* use.

Keywords——liposome; pharmacokinetics; dose dependency; simulation; vesicle number; blood clearance; urinary excretion; reticuloendothelial system

Introduction

The use of liposomes as effective drug carriers is currently of great interest[2] and many investigators have reported on the tissue distribution[3-12] or biological fate[13-18] of intravenously injected liposomes. From these studies it has become apparent that a substantial fraction of intravenously injected liposomes are rapidly taken up by the reticuloendothelial system (RES) in the liver and spleen, while some liposomes release their contents in the circulation owing to interactions with blood components. The disposition of liposomes is known to be affected by the liposomal size, dose, surface charge, and lipid composition. Smaller liposomes (small unilamellar vesicles (SUV)) are cleared from circulation more slowly than the larger ones (multilamellar vesicles (MLV) or reverse-phase evaporation vesicles (REV)).[19,20] This is considered to be due to differences of uptake by the liver, since larger liposomes are taken up by the Kupffer cells and SUV are taken up by both Kupffer cells and parenchymal cells.[21,22]

It was reported that positively charged liposomes showed a longer half-life in the circulation than negatively charged or neutral ones.[19] The lipid composition of the liposomes

also affected their stability in the blood. Liposomes containing cholesterol or sphingomyelin are stabilized in the blood[13,23,24] and those composed of phospholipids having higher gel-liquid transition temperatures are more stable.[25] It is well known that the disposition of liposomes is dose-dependent; at high dose the uptake by the RES is saturated and a high blood concentration per dose is obtained.[6] This is also observed after the predosing of empty liposomes or other colloids.[13,26-28] Thus, the disposition of liposomes in the body is affected by many factors.

For the evaluation of liposomes as drug carriers *in vivo*, it is necessary to analyze their disposition with an appropriate pharmacokinetic model.[29] Juliano and Stamp analyzed the pharmacokinetics of liposomes containing antitumor drugs in terms of a two-compartment model,[30] and Hwang *et al.* studied the kinetics of hepatic uptake and degradation of unilamellar liposomes.[31] Freise *et al.* and Abraham *et al.* reported on the pharmacokinetics of liposomes in rats[32] and rabbits,[33] respectively. However, these studies were not aimed at evaluation of the disposition of liposomes themselves but at pharmacokinetic analysis of the drug content. Recently, the effects of liposomal properties (*e.g.* size and drug-release properties[34] and surface charge[35]) on the pharmacokinetics of liposomes have been studied.

In this study, we investigated the pharmacokinetics of liposomes by using inulin as an aqueous space marker, and following the blood concentration and urinary excretion. The pharmacokinetic model postulated in the present study involves two major processes, *i.e.* uptake by the RES and degradation in the blood. Factors affecting the liposomal disposition are discussed.

Experimental

Materials——Egg L-α-phosphatidylcholine (PC), L-α-dipalmitoylphosphatidic acid (PA) sodium salt and D-α-tocopherol (α-T) were purchased from Sigma Chem. Co. (St. Louis, MO). PA was extracted from acidic aqueous solution with chloroform and methanol (9 : 1) prior to use. Cholesterol (CH) and inulin were from Kanto Chem. Co. (Tokyo). ^{3}H-Inulin was from New England Nuclear (Boston, MA).

Preparation of Liposomes——The lipid composition of the liposomes used in this experiment was PC, PA, CH and α-T at a molar ratio of 4/1/3/0.1. Reverse-phase evaporation vesicles (REV) were prepared as described in the previous paper,[36] and sized by extrusion and dialysis as described in the previous paper.[37] The lipid concentration of the liposome preparation was measured by the method of Bartlett after extraction with chloroform and methanol (9 : 1).[38] The size of the liposomes was measured with a photon correlation spectrometer (model N_4, Coulter Electronics, Hialeah, FL).

Animal Experiment——Male albino Wistar rats (body weight; 200 ± 10 g) anesthetized with ether were cannulated in the femoral vein, femoral artery and bladder. Two cannulas were inserted into the bladder, one for sampling and one for washing. Each operated rat was placed in a Bollman cage. After recovering from the anesthesia, it was injected with liposome suspension through the femoral vein cannula. Blood and urine samples were collected from the cannulas inserted into the artery and bladder, respectively.

Determination of Radioactivity——Radioactivity in blood and urine samples after intravenous injection of liposomes encapsulating ^{3}H-inulin as an aqueous marker was determined as follows. A 0.5 ml aliquot of 30% H_2O_2 and 0.5 ml of 2 N KOH solution in isopropanol were added to 0.2 ml of blood sample in a liquid scintillation vial, and the mixture was stirred gently but sufficiently, then left at room temperature overnight. Next, 10 ml of liquid scintillation cocktail (Scintisol EX-H, Wako Pure Chem. Co., Osaka) was added, and after mixing, 1 ml of 10% acetic acid was added for neutralization. The mixture was allowed to stand at room temperature for at least 3 h to dissipate chemiluminescence, and the radioactivity was counted with a liquid scintillation counter (Aloka LSC-673, Tokyo). The urine sample was diluted with distilled water to 10 ml. A 0.2 ml aliquot of H_2O_2 and 0.2 ml of 2 N KOH solution were added to 1 ml of the diluted sample. After bleaching and digestion overnight as described above, 10 ml of the cocktail and 0.5 ml of 10% acetic acid were added to the sample, and the mixture was treated as described for the blood sample.

Computation——Computations were carried out on a digital computer system (HITAC M-280/S-810) at the computer center of the University of Tokyo. The program for simulation was written by the authors in Fortran,[37] and the library program SALS[39] at the center was used for the nonlinear least-squares analysis.

Results and Discussion

Disposition of Free and Liposome-Encapsulated Inulin

Liposomes (mean diameter: 0.22 μm, 16.2 μmol of total lipid) and free inulin were injected intravenously into rats. As shown in Fig. 1, free inulin was eliminated very rapidly from the blood and excreted into the urine. Over 90% of injected radioactivity was excreted within 2 h after injection. Therefore, with inulin as a liposomal aqueous marker, it is considered that the radioactivity found in the blood represents liposomes in the blood. The activity in the urine represents inulin that has leaked from liposomes, and the excretion rate reflects the rate of the leakage.

After injection of liposomes containing inulin, the cumulative excretion curve showed a biphasic increase, *i.e.* an initial rapid phase followed by a linear and slow phase. It is suspected that the former represents the rapid leakage of inulin from liposomes in the circulation and the latter may reflect the gradual degradation of liposomes in the RES cells.

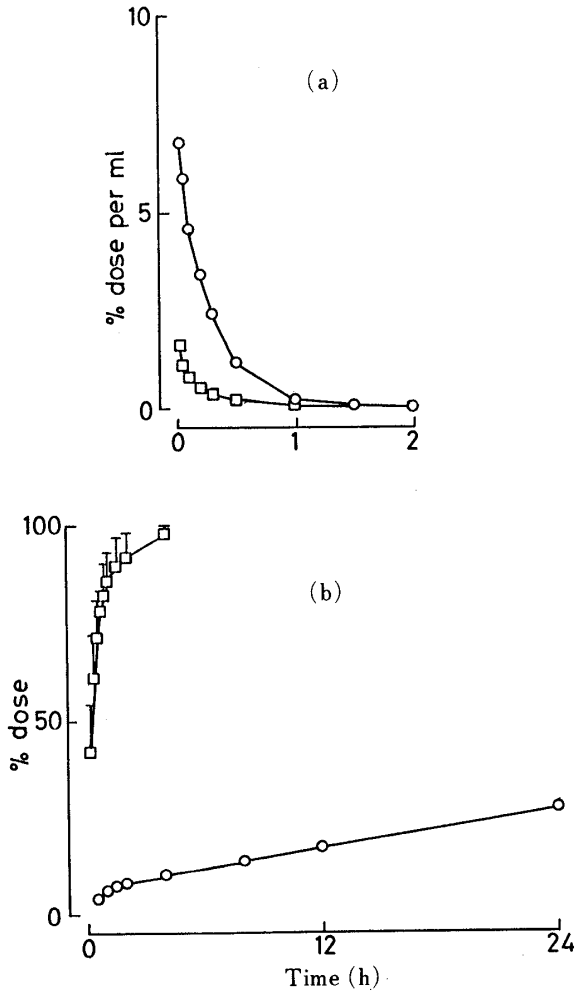

Fig. 1. Time Courses of Blood Levels (a) and Cumulative Urinary Excretions (b) after Intravenous Injection of Free (□) and Liposome (○)-Encapsulated ^{3}H-Inulin

Liposomes were composed of PC/PA/CH/α-T in the molar ratio of 4 : 1 : 3 : 0.1, and the mean diameter was 0.22 μm. The liposomal lipid dose was 16.2 μmol. Values are means ± S.D. of four animals.

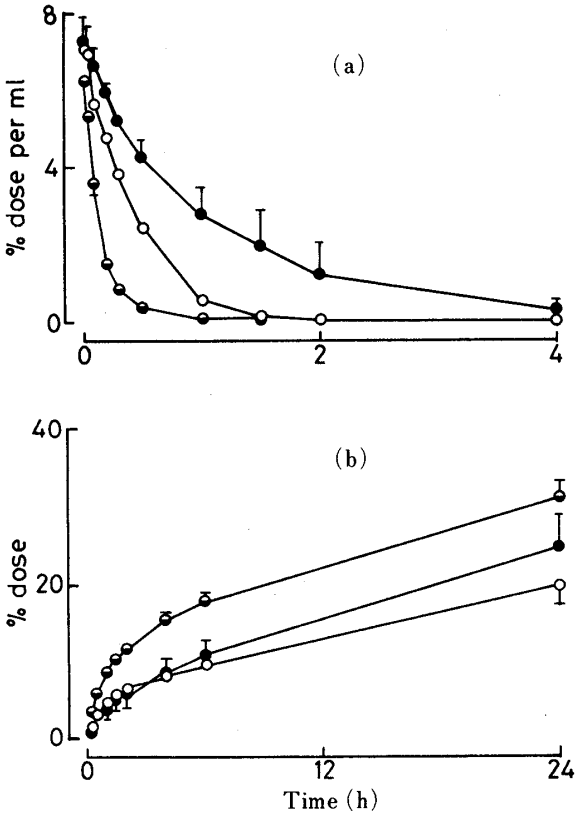

Fig. 2. Effect of Liposomal Dose of on Blood Clearance (a) and Cumulative Urinary Excretion (b)

Liposomes had the same composition and mean diameter as those in Fig. 1. Liposomal doses were 1.8 μmol (low dose, ◒), 14.9 μmol (medium dose, ○) and 70.3 μmol (high dose, ●). Values are means ± S.D. of three animals.

Effect of Dose

Liposomes with a mean diameter of $0.22 \pm 0.03\,\mu m$ were injected intravenously into rats at a dose of $1.8\,\mu mol$ (low dose), $14.9\,\mu mol$ (medium dose) or $70.3\,\mu mol$ (high dose) in total lipid. As shown in Fig. 2, in spite of the same size and lipid composition of the liposomes, the patterns of disposition were different at the three dose levels. The blood levels were prolonged and excretion in the urine was decreased in terms of percent per dose as the dose was increased. Cumulative urinary excretion curves showed a linearly increasing phase from 6 to 24 h after the administration (although no data were obtained between 6 and 24 h in this experiment, linearity of the excretion was confirmed by sampling at 8 and 12 h after administration in the preliminary experiments shown in Fig. 1).

As shown in Fig. 2, the patterns of blood clearance of liposomes at the three dose levels are different. The half-lives of the liposomes obtained from the initial phase were 35.5, 15.8 and 5.4 min at high dose, medium dose and low dose, respectively. The urinary excretions were also different, especially at the initial phase, being higher at low dose and lower at high dose. The subsequent phase did not show any significant dose dependence.

These observations suggest that the clearance of the liposomes from the blood involves both a saturable uptake process and a saturable excretion process in the urine. However, it seems unlikely that all excretion in the urine results from elimination in the blood, and a substantial contribution from liposomes taken up by the RES is likely. The slow and linear excretion at the later phase after the disappearance of the radioactivity in the blood seems to be due to the slow degradation of liposomes in the RES, and this seems not to be saturable.

Modeling

Pharmacokinetic modeling of the *in vivo* disposition of liposomes was attempted in order to elucidate the contributions of the leakage and RES uptake to the blood clearance and urinary excretion, and to simulate the *in vivo* disposition of liposomes having various properties. The following assumptions were used.

1) The disposition of liposomes *in vivo* is governed by the RES uptake and by destruction in the blood.

2) The uptake and destruction processes are saturable.

3) The liposomes taken up by the RES are disintegrated in the cells and their contents are released into the blood in an apparent first-order process.

Regarding the RES uptake of liposomes, limited capacity on the cell surface was assumed because it is considered that the process might be initiated by binding of the liposomes. This might be the rate-limiting step of the process, which can be expressed as follows;

$$L_B + B_F \underset{k_{-B}}{\overset{k_B}{\rightleftharpoons}} L_S \xrightarrow{k_U} L_I + B_F$$

where L_B, L_S and L_I are liposomes in the blood, bound to the cell surface and in the cell, respectively, k_B is the second-order rate constant for production of L_S, and k_{-B} and k_U are first-order rate constants of reduction of L_S to L_B and B_F, and L_I and B_F, respectively. B_F is the number of free binding sites. Actually the binding site means the site at which internalization of liposomes occurs, and it seems to be reasonable that the number of such sites (B_T) is limited and k_{-B} is very small compared with k_B, as follows;

$$B_T = L_S + B_F$$

$$k_B \gg k_{-B} \fallingdotseq 0$$

Based on these assumptions, a pharmacokinetic model of liposomal disposition *in vivo* was postulated (model I in Fig. 3). The liposomes in the blood release their contents with Michaelis–Menten kinetics (V_m and K_m), and are taken up by the RES through the saturable

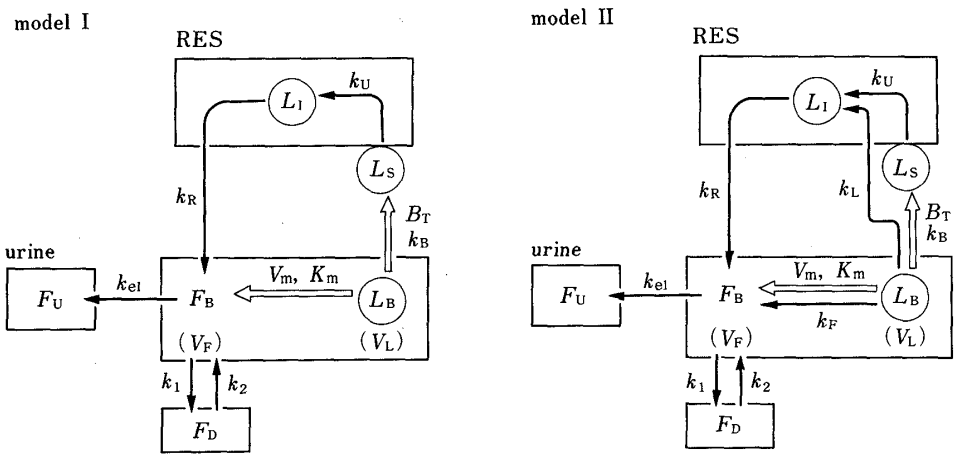

Fig. 3. Pharmacokinetic Models for *in Vivo* Fate of Liposomes after Intravenous Injection

Compartments:
L_B, liposomes in the blood;
L_S, liposomes on the cell surface of the RES;
L_I, liposomes in the cells of the RES;
F_B, free inulin in the blood;
F_D, distribution of free inulin;
F_U, free inulin excreted into urine.
Parameters:
V_L, apparent distribution volume of liposomes;
V_F, apparent distribution volume of free inulin;
k_1, k_2, first-order rate constant of inulin distribution;
k_{el}, first-order rate constant of inulin excretion;
V_m, maximum velocity of inulin leakage from liposomes in the blood;
K_m, Michaelis–Menten constant of inulin leakage from liposomes in the blood;
B_T, number of binding sites on the cell surface;
k_B, second-order rate constant of liposome uptake;
k_U, first-order rate constant of liposome transfer into the cells;
k_R, first-order rate constant of inulin leakage from liposomes in the cells;
k_L first-order rate constant of liposome uptake by the RES;
k_F, first-order rate constant of inulin leakage from liposomes in the blood.

process described above. The internalized liposomes disintegrate in the cells and release their contents into the blood with first-order kinetics (k_R). Inulin in the blood (F_B) obeys a two-compartment (F_D, k_1 and k_2) model and is excreted (k_{el}) in the urine (F_U).

Parameter values used for solving the differential equations numerically by the Runge–Kutta method are listed in Table I. Simulations of the blood levels and urinary excretion were carried out with these parameter values. The initial values of these parameters, from which the values listed in Table I were obtained, were as follows. Distribution volume of liposomes (V_L) was obtained by extrapolation of the semilogarithmic plots of the initial phase of blood clearance to time 0. Parameter values of inulin (V_F, k_1, k_2 and k_{el}) were obtained from the results of leasts-squares fitting analysis by the SALS program on the data after intravenous injection of free inulin (Fig. 2). Parameter values of the saturable processes (V_m, K_m, B_T, k_B and k_U) were estimated from Lineweaver–Burk plots of mean urinary excretion rate and mean blood concentration, and by calculation from the values obtained from plots of amount in the RES (total minus amounts in the blood and urine) and mean blood concentration up to 15 min after administration of the medium dose. The value of release from the cells (k_R) was estimated from the slope in the later phase of urinary excretion. Based on these initial values, curve fitting was attempted to the data at the three dose levels by simulation with gradual changes of the parameter values. The computation was carried out after normalizing the dose to 100 for the modium dose, and fitting to the data on blood concentration (% dose/ml) and cumulative amount excreted into the urine (% dose).

TABLE I. Parameter Values Used for Simulation with Model I

Name	Unit	Value
Dose	—	100.0
V_L	ml	13.2
V_F	ml	54.8
k_1	h^{-1}	4.74
k_2	h^{-1}	4.82
k_{el}	h^{-1}	4.39
V_m	Dose·h^{-1}	4.0
K_m	Dose	2.0
B_T	Dose	50.0
k_B	Dose^{-1} h^{-1}	0.1
k_U	h^{-1}	2.0
k_R	h^{-1}	0.01

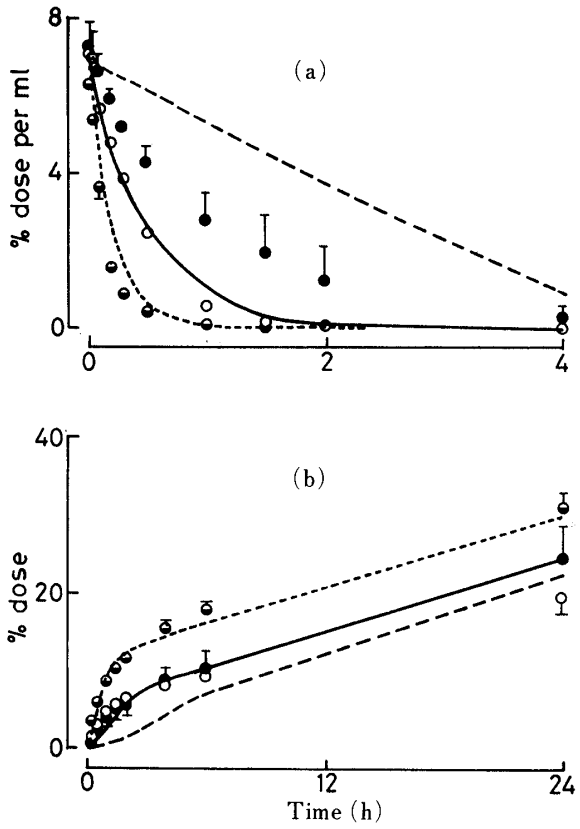

Fig. 4. Calculated Time Courses of Blood Concentration (a) and Cumulative Urinary Excretion (b) Using Model I with the Parameter Values Listed in Table I at Low Dose (---◒---), Medium Dose (—○—) and High Dose (--●--)

The experimental data are taken from Fig. 2.

A good fit to the observed data at low dose and medium dose, but not at high dose, was obtained, as shown in Fig. 4. Beaumier *et al.*[40] proposed a kinetic model having a first-order rate process for the uptake of liposomes by the liver in parallel with a saturable process, and at higher dose the contribution of the first-order rate process would be higher. It is considered that non-saturable processes such as agitation by the blood stream and/or a detergent effect of the blood components may contribute to the degradation process in blood. Thus, first-order rate processes were added to the uptake process (k_L) and degradation process (k_F) in parallel with the saturable processes of model I shown in Fig. 3. The new model is shown in Fig. 3 as model II.

The parameter values of k_L and k_F were estimated as 0.3 and 0.05 h^{-1} by simulation, respectively. Simulations were carried out in the new model with the parameter values shown in Table I and these two new parameter values. The results (Fig. 5) showed good agreement with the experimental data. This indicates that the model and parameter values are appropriate for pharmacokinetic evaluation of the *in vivo* disposition of liposomes, at least of this size class.

Effect of Size

It is well known that the uptake by the liver and the physico-chemical properties of REV and SUV are different,[4] but the differences of disposition of liposomes having rather similar sizes are not clear. This is important, because liposomes prepared by general methods show

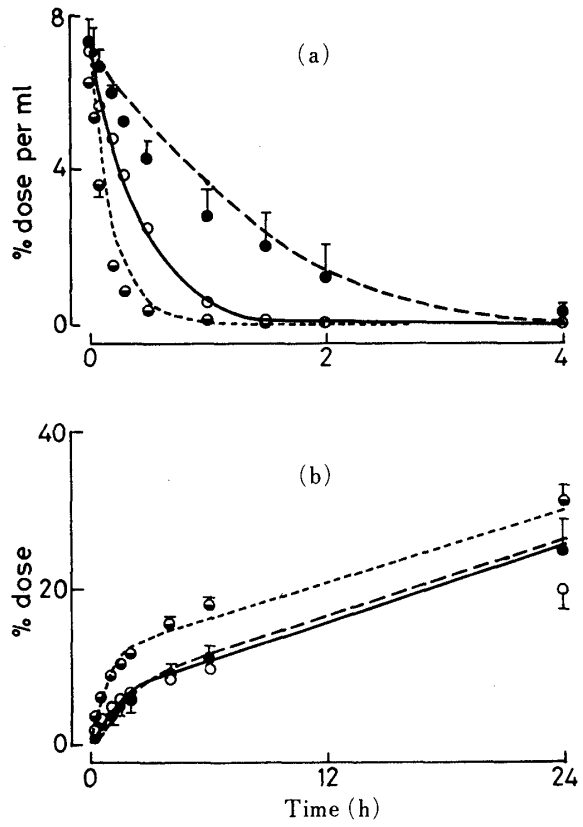

Fig. 5. Simulated Time Courses of Blood Concentration (a) and Cumulative Urinary Excretion (b) Based on Model II at Various Dose Levels

Details are the same as in Fig. 4.

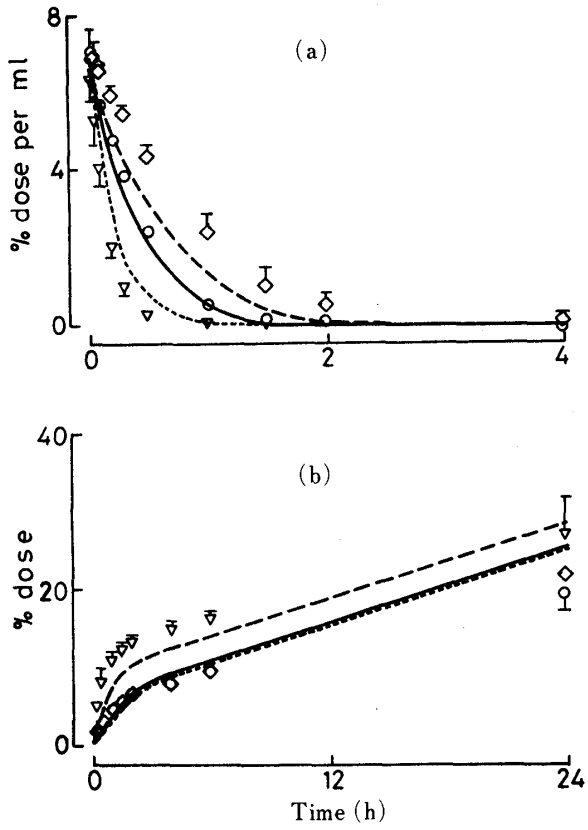

Fig. 6. Effects of Liposomal Size on Blood Concentration (a) and Cumulative Urinary Excretion (b)

Values are the mean±S.D. of three experiments and curves show the results of simulation with model II based on the number of vesicles as a measure of the dose after injection of small liposomes (◇, ----), medium liposomes (○, ——) and large liposomes (▽, ------).

some variation in size. The disposition of liposomes having slightly different sizes (mean diameters of 0.15 ± 0.03, 0.22 ± 0.03 and $0.43\pm0.10\,\mu$m for small, medium and large liposomes, respectively) were examined. The results were analyzed in terms of model II shown in Fig. 3.

In spite of the very similar doses as total lipids (12.4, 14.9 and 16.1 μmol for small, medium and large liposomes, respectively) remarkable differences were observed in their disposition, as shown in Fig. 6. This observation suggests that even small differences of liposomal size affect the disposition. Therefore, the liposomes for biological use should be strictly sized.

Simulation was carried out with the same parameter values as in Table II. Besides the total lipids, the number of the vesicles and the total surface area (reflecting the amount of the lipid molecules on the surface of the vesicles) were taken as measures of the dose.

The number of the vesicles (N) and the total surface area (TSA) were calculated as follows[41];

$$N=\frac{TVL+TVW}{\frac{4}{3}\pi R^3}$$

$$TSA = N \cdot 4\pi R^2 = \frac{3(TVL + TVW)}{R}$$

where TVL is the total volume of lipids (volume of lipids used in the liposome preparation; the specific volume is taken as 1.0). TVW is the total volume of water encapsulated, and is calculated from the encapsulation efficiency of the aqueous marker (^{3}H-inulin) in the liposome preparation. R is the radius of the vesicles. These values are listed in Table II.

TABLE II. Dose Parameters for Liposomes of Various Sizes

Size	Mean diameter (μm)	Lipid amount (μmol)	Number (10^{12})	Surface area (m^2)
Small	0.15	12.4	14.9	1.1
Medium	0.22	14.9	9.4	1.5
Large	0.43	16.1	1.8	1.1

The blood clearance and urinary excretion after the administration of the liposomes of three different sizes were simulated using these measures of the dose. The best fit was obtained when number of the vesicles was used (Fig. 6). Therefore it is likely that the mechanism governing liposome disposition *in vivo* is a function of the number of vesicles.

Abra and Hunt reported the dispositions of differently sized liposomes in mice and found that surface area was important as well as number.[4] They used SUV as small liposomes. However, the mechanisms of liposomal uptake by RES[22] and the stability in the blood[20] may be different for SUV and larger liposomes, so a direct comparison cannot be made. We could not simulate the disposition of SUV with the model and parameter values presented here, and it seemed likely that the fate of SUV is controlled by different factors from those that affect larger liposomes. Therefore, the conclusions presented in this paper may be restricted to liposomes in the size range of 0.1—0.4 μm.

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