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Fate of Lipid and Encapsulated Drug after Intramuscular Administration of Liposomes Prepared by the Freeze-Thawing Method in Rats

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In order to assess the utility of liposomes prepared by the freeze-thawing (FT) method as a drug carrier, the fate of the liposomes themselves and of encapsulated drug following intramuscular administration was investigated in rats. Liposomes were prepared from yolk phospholipid, and ^{3}H-labeled dipalmitoyl-phosphatidylcholine (^{3}H-DPPC) and ^{14}C-labeled inulin were employed as markers of the lipid and the drug, respectively. Liposomes prepared by the ordinary hydration (HY) method were also tested for comparison. The inulin-encapsulating efficiencies (*EN*%) of liposomes prepared by the FT, HY with buffer, and HY with distilled water methods were 60%, 4%, and 45%, respectively.

At 24 h after intramuscular injection of liposomes, about half of the injected ^{3}H-DPPC still remained in the injection site regardless of the preparation method. A considerable amount of ^{3}H-DPPC was detected in the regional lymph nodes, suggesting a significant contribution of the lymphatic route in the absorption of liposomes. On the other hand, the disappearance of ^{14}C-inulin from the muscle varied with *EN*%, and liposomes prepared by the FT method showed higher retention than those prepared by the HY method. The difference in absorption was well reflected in the plasma concentration and urinary recovery of ^{14}C-inulin. Accumulation of ^{14}C-inulin in the lymph nodes was also observed. The concentration ratios of ^{14}C-inulin to ^{3}H-DPPC in the muscle and lymph nodes increased with time, indicating that the lipid was absorbed faster than ^{14}C-inulin. Liposomes prepared by the FT method were concluded to be effective in retarding the absorption and enhancing the lymphatic delivery of the drug, giving high *EN*% by a simple procedure.

Keywords——liposome; freeze-thawing method; encapsulation efficiency; ^{3}H-dipalmitoyl-phosphatidylcholine; ^{14}C-inulin; intramuscular injection; slow absorption; sustained release; lymph node accumulation

Liposomes are well known as a potential drug delivery system,[1] and localized delivery by liposomes has been demonstrated for many drugs given *via* various injection routes, such as inulin (*i.m.*),[2] melphalan (*s.c.*),[3] steroid (intraarticular),[4] cytosine arabinoside (*i.p.*)[5] and albumin (intratesticular).[6] The delivery efficiency of liposomes appears to depend on pharmaceutical characteristics such as sustained release and site-specific distribution potentials, as well as more basic physicochemical properties such as drug encapsulation efficiency, size,[2,7,8] surface charge, or fluidity.[9]

In ordinary procedures for preparing liposomes, the encapsulated drug must be separated from unencapsulated (free) drug and concentrated, because the encapsulation efficiency is relatively low. In previous investigations, we presented two novel methods for preparing liposome formulations which are applicable to therapeutic use; *i.e.*, the freeze-thawing (FT) method[10] and the freeze-drying method.[11] From the viewpoint of pharmaceutical manufacture, these methods have the advantages that they can produce liposomes without the use of any organic solvents or detergents and they are compatible with sterilization and industrial scale up requirements; further, high encapsulation efficiency can be obtained by

simple manipulation. Liposomal suspension prepared by the FT method contains free drug as well as encapsulated drug, but its drug-encapsulation efficiency ($EN\%$) can be increased to as much as 80% by choosing suitable preparation conditions.[12] Therefore, FT liposomes with high $EN\%$ might be used as an effective drug carrier without any pretreatment, such as gel filtration, ultrafiltration, or sedimentation.

In the present investigation, the biopharmaceutical utility of the liposome formulation prepared by the FT method was examined by comparing the muscular absorption and lymphatic delivery characteristics of the lipid component and encapsulated inulin. Inulin was employed as a model drug since it is rapidly excreted by glomerular filtration, and is neither metabolized nor distributed to intracellular space.[13]

Experimental

Meterials——[Methoxy-^{14}C] inulin (^{14}C-inulin) and [2-palmitoyl-9,10-^{3}H] L-α-dipalmitoylphosphatidylcholine (^{3}H-DPPC) were purchased (New England Nuclear, Boston, Mass., U.S.A.) and their specific activities were 41.9 mCi/g and 60 Ci/mmol, respectively. The manufacturer's estimates of radiochemical purity were 99% for ^{14}C-inulin and 98% for ^{3}H-DPPC (determined by gel permeation chromatography (GPC) and thin layer chromatography (TLC)). Yolk phospholipid (YPL) was prepared from egg yolk as described previously.[10] Cold inulin was purchased from Nakarai Chemicals Co., Kyoto, Japan. All other chemicals used were of the highest grade commercially available.

Preparation of Liposomes——Five kinds of liposomes with different encapsulation efficiencies were prepared by the FT method[10] or the thin film hydration (HY) method as described below.[14] In these preparations, the concentrations of cold and radioactive inulin in the aqueous phase were adjusted to 2 mg/ml and 2 μCi/ml, respectively. As a marker for the phospholipid phase, 100 μCi of ^{3}H-DPPC was added to 100 mg of YPL.

a) FT Method: The dried YPL[12] was dispersed in distilled water by sonication under nitrogen on an ice bath and the resulting YPL suspension (YPL 10 w/v%) was mixed with an equal volume of distilled water containing inulin (2 mg/ml). The mixture was frozen at −20 °C for 24 h, thawed at room temperature and shaken for 20 min using a Vortex mixer (FT-W). The obtained FT-W liposomes were subjected to gel filtration on a Sepharose 4B column and free inulin was removed (FT-G). The radioactivities of ^{14}C-inulin and ^{3}H-DPPC in the collected liposomal suspension were determined and then adjusted to an appropriate concentration.

b) HY Method: A solution of YPL in chloroform in a round bottomed flask was evaporated at 30 °C. The dried YPL on the flask was dispersed into buffered saline solution (0.05 M Tris–HCl, pH 7.4 and 0.9% NaCl) containing inulin by shaking with a Vortex mixer for 20 min at room temperature (HY-B). Liposomes were also prepared by dispersing YPL thin film into concentrated inulin distilled water solution, followed by dilution with distilled water (HY-W). The amount of YPL and the concentration of inulin solution were adjusted to be the same as in the case of FT liposomes. HY-W liposomes were also subjected to gel filtration, and liposomal suspension without free inulin was obtained (HY-G).

The osmotic pressure of these liposomes was adjusted to be isotonic with NaCl for the *in vivo* experiment.

Encapsulation Efficiency Determination——Encapsulation percent ($EN\%$) of inulin in each liposome formulation was determined by gel filtration on a Sepharose 4B column (1.6 cm × 22 cm).[10] Liposomes were eluted with 0.05 M Tris–HCl buffer, pH 7.4.

Drug Release Experiment——The release of inulin from liposome formulations was determined by a modification of the dynamic dialysis system of Meyer and Gutman[15] with the apparatus shown in Fig. 1. Six milliliters of a liposome formulation containing inulin was added to Visking dialysis tubing as the inner solution, and a beaker

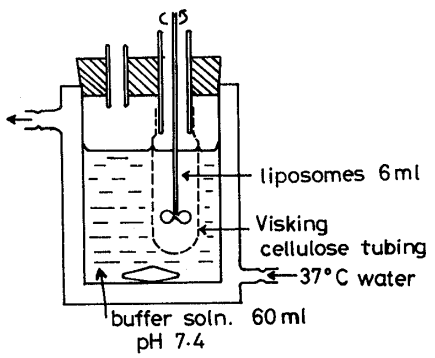

Fig. 1. Apparatus Used to Study the Release of Inulin from Liposome Formulations

containing 60 ml of 0.05 M Tris–HCl buffer (pH 7.4) as the outer solution was maintained at 37 °C by circulating water. The inner and outer solutions were stirred at the rates of 150 and 500 rpm, respectively. At fixed time intervals, 1 ml of the outer solution was withdrawn for assay.

Animal Experiment——Male Wistar albino rats weighing between 180 and 200 g were used for all animal experiments. The *i.m.* absorption experiments were performed according to the method of Kakemi *et al.*[16] Liposome formulation (100 μl) was injected with a microliter syringe into the left thigh muscle (musculus rectus femoris) of rats under light ether anesthesia. At 0.5, 2, 8, 16 and 24 h after *i.m.* administration of liposomes, the rats were anesthetized with ether. Blood samples were obtained from the descending aorta, and the left thigh muscle and ipsilateral iliac lymph nodes were removed, weighed and analyzed for total radioactivity. For determining urinary excretion of radioactivities, rats were housed in metabolic cages after *i.m.* injection, and urine samples were collected for 24 h.

Radioactivity Analysis——Sample solution (1 ml) in the *in vitro* experiment was mixed well with 10 ml of scintillation medium (Univer-Gel II, Nakarai Chemicals Co., Kyoto, Japan) and radioactivity was determined in a liquid scintillation system (Packard, Tri-Carb 460).

The radioactivity of muscle, lymph node, plasma and urine was determined by the modified method reported by Mahin and Loftberg.[17] The muscle was digested with 4 ml of 2 N NaOH at 50 °C for 2 h, and diluted to 7 ml with distilled water. Then, 0.2 ml of sample solution was put into a counting vial and 0.2 ml of perchloric acid (60%) and 0.2 ml of hydrogen peroxide (35%) were added. The mixture was heated at 70 °C for 1.5 h with occasional agitation. After cooling to room temperature, 10 ml of the scintillation medium was added and samples were analyzed for ^{3}H and ^{14}C contents.

A measured weight of excised lymph node, 0.2 ml of the plasma or 0.2 ml of urine was put into the vial, and the radioactivity was determined as described above.

Results

Characteristics of Prepared Liposomes

In Table I, the characteristics of five kinds of liposome formulations tested in the present study are summarized. The concentrations of YPL, ^{3}H-DPPC and inulin were adjusted to be the same in FT-W, HY-W and HY-B. FT-G and HY-G have low inulin contents, because free inulin was removed by gel filtration. The YPL content of HY-G was also lower than those of the others. The range of particle size was estimated to be 0.1 to 1 μm in all the formulations, as reported previously.[10]

As shown in the gel filtration pattern presented in Fig. 2, FT-W liposomes showed relatively high $EN\%$ (60%), while only 4% of inulin was encapsulated in the liposomal fraction in HY-B liposomes prepared by the ordinary method. FT-G and HY-G were estimated to have $EN\%$ values of about 100%.

In Vitro Release of Inulin from Various Liposomal Formulations

The time courses of remaining percentage of ^{14}C-inulin (X_t) in the Visking tubing during release experiments are shown in Fig. 3 for the various kinds of liposome formulations. The log (X_t) values decreased almost linearly with time at the initial stage in all liposome formulations, as represented by solid lines. FT-G and HY-G liposomes showed very slow disappearance of ^{14}C-inulin from the inner phase, suggesting that the release rate of ^{14}C-inulin

TABLE I. Characteristics of Liposomes Used in This Experiment

Formulation	YPL mg/ml	^{3}H-DPPC μCi/ml	Inulin μg/ml	^{14}C-Inulin μCi/ml	$EN\%^{a)}$	$EN_R\%^{b)}$
FT-W	2.5	2.5	50	0.5	60	58
FT-G	2.5	2.5	30	0.3	(100)	95
HY-B	2.5	2.5	50	0.5	4	5
HY-W	2.5	2.5	50	0.5	45	47
HY-G	2.0	2.0	20	0.2	(100)	90
Solution	—	—	50	0.5	0	0

a) Determined by the gel filtration method. *b*) Calculated from the results of the release experiment.

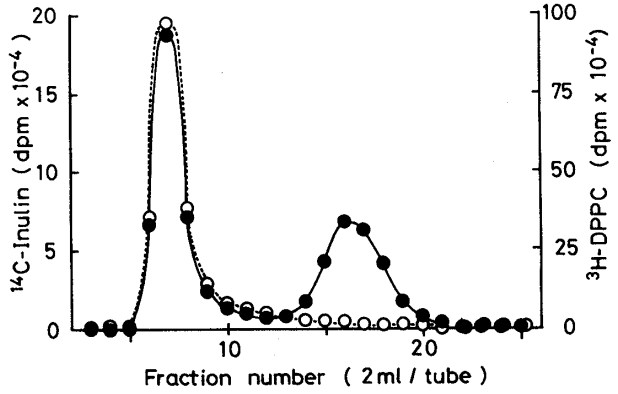

Fig. 2. Gel Filtration of Inulin/YPL Liposomes Prepared by the FT Method (FT-W)

○, ^{3}H-DPPC; ●, ^{14}C-inulin.

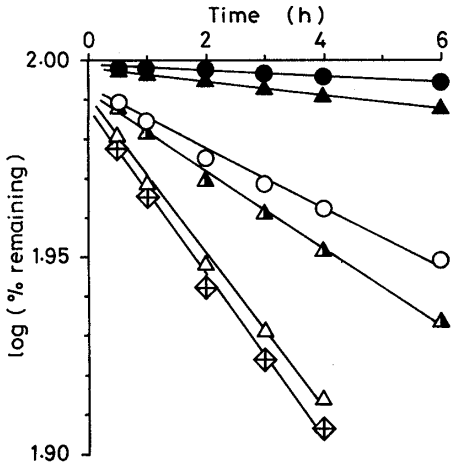

Fig. 3. Release of ^{14}C-Inulin from Liposome Formulations

●, FT-G; ○, FT-W; ▲, HY-G; ▲, HY-W; △, HY-B; ⊕, solution.

from liposomal particles was small.

The amount of ^{14}C-inulin transferring through the Visking membrane depends on the concentration gradient of its free form between the inner and the outer phases. Since the volumes on both sides of the membrane were constant and the concentration in the outer solution was negligible at the initial stage, the amount of free inulin inside the tube should be the determinant of the initial transfer rate. Accordingly, the following equation can be obtained;

$$dX_t/dt = -k \cdot X_f \quad (1)$$

where X_f is the percent amount of free ^{14}C-inulin in the inner space of the tube and k is the transfer rate constant for free ^{14}C-inulin (min^{-1}). Since k is constant regardless of formulation type, the ratios of dX_t/dt values of liposome formulations to that of ^{14}C-inulin solution ($X_f = 100\%$) represent the free fractions of the drug. Thus, the encapsulation efficiencies of these formulations were calculated from *in vitro* release data, and are also listed in Table I as $EN_R\%$. The calculated values were in good agreement with those obtained in the gel filtration experiment.

Disappearance of ^{3}H-DPPC and ^{14}C-Inulin from the Injection Site after *i.m.* Administration of Liposomes

The time courses of disappearance of ^{3}H-DPPC and ^{14}C-inulin from the injection site (the left thigh muscle) after *i.m.* administration of five liposome formulations are shown in Figs. 4 and 5, respectively.

All liposome formulations showed almost the same pattern of ^{3}H-DPPC disappearance; about 10% of injected ^{3}H-DPPC was lost during the initial 30 min, and thereafter it disappeared more slowly following first-order kinetics. About 50% of the dose still remained after 24 h. These results indicate that the difference in the preparation method (FT or HY) did not affect the absorption behavior of ^{3}H-DPPC in liposomes.

The disappearance of ^{14}C-inulin also showed a biphasic pattern with rapid disappearance in the initial 2 h and a successive slow disappearance following apparent first-order kinetics.

The kinetic parameters which characterize the disappearance behavior of ^{3}H-DPPC and ^{14}C-inulin were determined by the back-projection method using Eqs. 2 and 3, and the results are summarized in Table II.

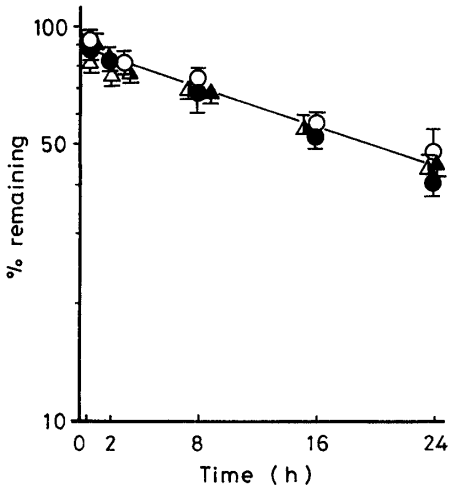

Fig. 4. Disappearance of ^{3}H-DPPC from the Thigh Muscle after Injection of Liposome Formulations

Symbols are the same as in Fig. 3. Results are expressed as the mean ± S.D. of at least four rats.

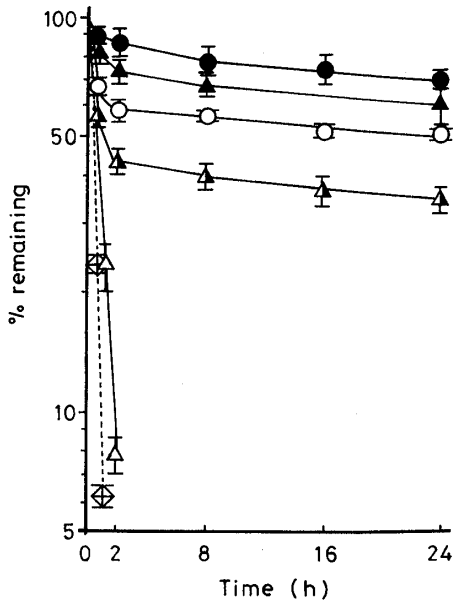

Fig. 5. Disappearance of ^{14}C-Inulin from the Thigh Muscle after Injection of Liposome Formulations

Symbols are the same as in Fig. 3. Results are expressed as the mean ± S.D. of at least four rats.

TABLE II. Kinetic Parameters for Disappearance of ^{14}C-Inulin and ^{3}H-DPPC from the Thigh Muscle

Formulation	First phase	Second phase			
	α (h^{-1})	B_0 (%)		β ($\times 10^{-2}$, h^{-1})	
	^{14}C-Inulin	^{3}H-DPPC	^{14}C-Inulin	^{3}H-DPPC	^{14}C-Inulin
FT-W	3.1	86	60	2.3	0.61
FT-G	1.9	88	88	3.3	0.87
HY-B	3.4	—	—	—	—
HY-W	2.8	75	43	1.8	0.66
HY-G	2.9	87	74	2.7	0.75
Solution	3.6	—	—	—	—

$$\ln(\text{remaining \% of dose}) = \ln(100 - B_0) - \alpha \cdot t \quad \text{(for the first phase)} \tag{2}$$

$$\ln(\text{remaining \% of dose}) = \ln(B_0) - \beta \cdot t \quad \text{(for the second phase)} \tag{3}$$

In these equations, α and β are the disappearance rate constants for the first and the second phases, and B_0 represents the percent amount of drug whose disappearance behavior can be described by Eq. 3. Because enough data could not be obtained for calculating α values, only B_0 and β were calculated for ^{3}H-DPPC.

The first phase disappearance rate constants (α) of ^{14}C-inulin are nearly equal to each other, suggesting that this phase represents the absorption of unencapsulated inulin. The β values did not differ much among preparation methods in the cases of both ^{3}H-DPPC and ^{14}C-inulin. However, the β values of ^{3}H-DPPC were several times larger than those of ^{14}C-inulin.

Lymphatic Transport of ^{3}H-DPPC and ^{14}C-Inulin

The radioactivities of ^{3}H-DPPC and ^{14}C-inulin in the left iliac lymph node, which is on the lymphatic absorption pathway from the injection site,[9] were determined and the results are shown in Figs. 6a and 6b, respectively.

The concentration of ^{3}H-DPPC in the lymph node became high in the early phase after injection and then gradually decreased, but remained at a rather high level after 24 h in all three liposome formulations (FT-G, HY-G, FT-W). The radioactivities of ^{14}C-inulin for FT-G, HY-G and FT-W remained at high levels up to 24 h. However, the solution and HY-B gave very low levels.

Plasma Level and Urinary Excretion of ^{3}H-DPPC and ^{14}C-Inulin

Figure 7 shows the plasma concentrations of ^{3}H-DPPC and ^{14}C-inulin following injection of six formulations. With all the liposome formulations, ^{3}H-DPPC was detected at a very low level. The plasma level of ^{14}C-inulin was also very low for FT-G and HY-G. FT-W, HY-W and HY-B showed higher levels at 30 min after injection, but these rapidly decreased to the same low level as in the case of ^{14}C-inulin solution.

Table III summarizes the amounts of ^{14}C-inulin and ^{3}H-DPPC recovered in urine during

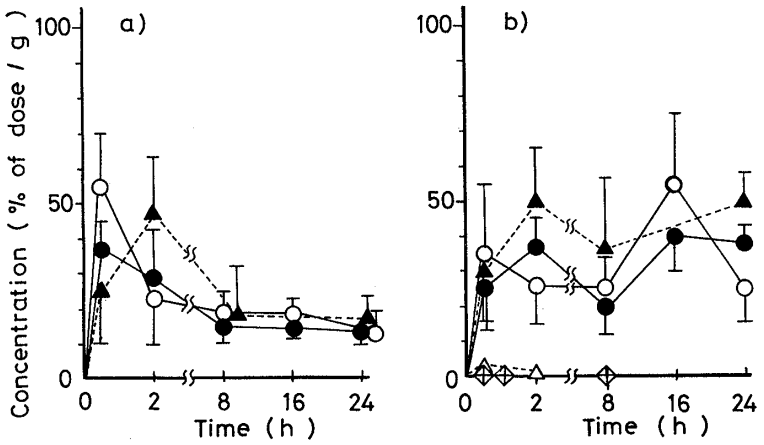

Fig. 6. Concentrations of ^{3}H-DPPC (a) and ^{14}C-Inulin (b) in the Left Iliac Lymph Nodes after *i.m.* Injection of Liposome Formulations

●, FT-G; ○, FT-W; ▲, HY-G; △, HY-B; ⊕, solution.

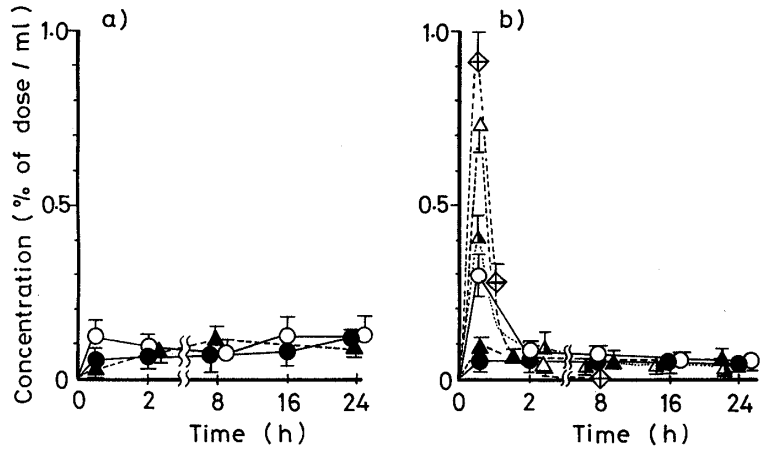

Fig. 7. Plasma Concentrations of ^{3}H-DPPC (a) and ^{14}C-Inulin (b) after Intramuscular Administration of Liposome Formulations

Symbols are the same as in Fig. 3. Results are expressed as the mean ± S.D. of at least four rats.

TABLE III. Recovery of Radioactivity in Urine within 24 h

Formulation	^{3}H (% of dose)	^{14}C (% of dose)
FT-W	8.01±1.28	39.82±2.59
FT-G	9.86±1.79	10.01±2.53
HY-B	9.93±3.32	89.84±2.57
HY-W	10.01±1.36	48.59±4.11
HY-G	8.99±2.12	18.57±2.39
Solution	—	90.68±2.78

Each value is the mean±S.D. of at least four rats.

TABLE IV. Distribution of Radioactivity in Tissues and Urine at 24 h after *i.m.* Injection of FT-W Preparation into Rats

Tissue	^{3}H		^{14}C	
	Amount (% dose)	Concn. (% dose/g)	Amount (% dose)	Concn. (% dose/g)
Thigh muscle	51.4		51.9	
Lymph node	—	12.6	—	25.0
Liver	4.1	0.55	0.8	0.11
Spleen	0.11	—	0.27	—
Plasma	2.6	0.17	0.8	0.05
Urine	8.0	—	39.8	—

24 h. The results show that the excreted amounts of ^{14}C-inulin depended strongly on the preparation method, while those of ^{3}H-DPPC did not and were at a very low level compared with ^{14}C-inulin.

Tissue Distribution of ^{3}H-DPPC and ^{14}C-Inulin at 24 h after *i.m.* Administration of FT-W Liposome

The radioactivities in various tissues at 24 h after *i.m.* injection of FT-W preparation are shown in Table IV. The concentrations of both ^{3}H-DPPC and ^{14}C-inulin in regional lymph nodes were very much higher than those in other organs.

Discussion

In the present investigation, the fate of liposome formulations after *i.m.* injection was examined by tracing the movement of phospholipid (^{3}H-DPPC) and the model drug (^{14}C-inulin) among the muscle, regional lymph nodes, plasma, urine, and other organs.

After *i.m.* injection of all liposome formulations, about 10% of injected ^{3}H-DPPC disappeared from the injection site, but the remainder was retained in the muscle for a considerable period (Fig. 4). This rapid disappearance is presumed to correspond to the absorption of particularly small liposomes or molecular phospholipid. Direct intrusion of liposomes into the lymph or blood vessels during the injection procedure may not be negligible. At the same time, injected ^{3}H-DPPC was mostly transferred to the regional lymph nodes, suggesting a significant contribution of the lymphatic pathway in the muscular absorption of liposomes (Fig. 6). On the other hand, only a small amount of ^{3}H-DPPC was detected in plasma, indicating that only a small part of injected liposomal particles was transported into the blood circulation (Fig. 7). However, further information on the systemic

disposition characteristics of phospholipid is necessary for the quantitative analysis of the absorption behavior of liposomes. In these experiments, the preparation method of liposomes had no significant effect, and it is considered that all liposome formulations tested in this study have rather similar properties as regards size, structure, *etc.*

On the other hand, the transfer of ^{14}C-inulin after *i.m.* injection did vary with the preparation method of liposomes, or more specifically, with the encapsulation efficiency. In Fig. 8, the B_0 values (Table II), plasma concentrations (Fig. 7) and excreted amount in urine (Table III) of ^{14}C-inulin after injection of various liposome formulations are plotted against the encapsulation efficiency (EN_R%, Table I) of each formulation. As shown in Fig. 8, the B_0 value linearly increased and the plasma level and urinary recovery decreased with an increase of EN_R%. A plot of these data against EN% (Table I) gave almost the same results (not shown). The marked dependency of these values on EN_R% suggests that the free fraction of inulin in each formulation is rapidly absorbed by blood capillaries and then rapidly excreted in urine. In Fig. 8, the amount of ^{3}H-DPPC excreted in urine is also plotted for each formulation, but the values were very low and did not depend on EN_R%. Based on these results, it is suggested that a prolonged supply of drug at the injection site can be efficiently achieved by employing a liposome formulation with high EN_R%.

To elucidate the absorption behavior of liposomes more clearly, the concentration ratio of ^{14}C-inulin to ^{3}H-DPPC at each experimental period after injection of various liposome formulations was calculated and results were compared by standardizing the initial values to be one.

As shown in Fig. 9, the ^{14}C/^{3}H ratio in the muscle increased with time in the case of FT-G liposomes, and this is consistent with the fact that the β values of ^{14}C-inulin were smaller than those of ^{3}H-DPPC (Table II). In the case of FT-W, the ^{14}C/^{3}H ratio first dropped, and thereafter increased, as in the case of FT-G. The drop is considered to correspond to the rapid absorption of the unencapsulated free inulin.

The time courses of the ^{14}C/^{3}H ratio in the lymph nodes for the three liposome formulations are shown in Fig. 10. The ratio in all cases clearly increased with time, and the extent of the increase was higher in the lymph nodes than in the muscle.

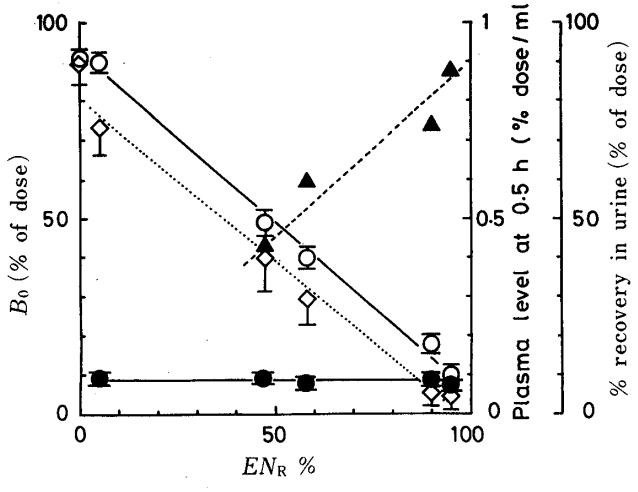

Fig. 8. Relationship between *in Vivo* Behavior of ^{14}C-Inulin and ^{3}H-DPPC after *i.m.* Injection and EN_R% Obtained from the Release Experiment

▲, B_0; ◇, plasma concentration of ^{14}C-inulin at 0.5 h; ○, % recovery of ^{14}C-inulin in urine until 24 h; ●, % recovery of ^{3}H-DPPC in urine until 24 h.

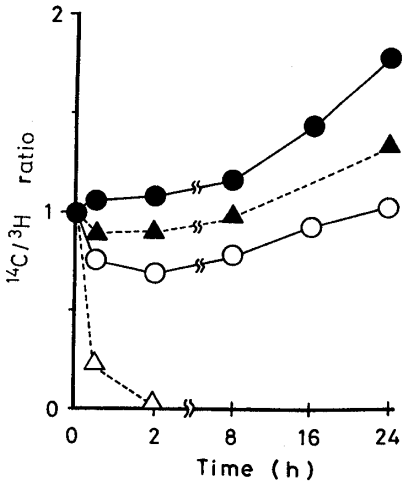

Fig. 9. ^{14}C/^{3}H Ratio in the Muscle Injection Site

●, FT-G; ○, FT-W; ▲, HY-G; △, HY-B.

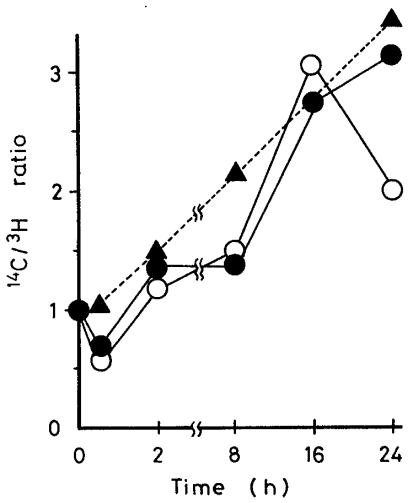

Fig. 10. $^{14}C/^{3}H$ Ratio in the Left Iliac Lymph Nodes

●, FT-G; ○, FT-W; ▲, HY-G.

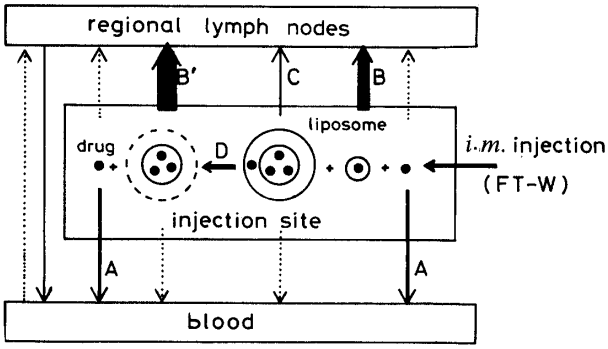

Fig. 11. Schematic Representation of the Drug Transfer Following Intramuscular Injection of FT Liposomes

A, transfer of free drug; B, B′, transfer of small liposomes included in the liposomal preparation from the beginning and those generated from larger liposomes by ablation at the injection site, respectively; C, transfer of larger liposomes; D, ablation of liposomes.

If ^{14}C-inulin is dispersed uniformly in all liposomes and is absorbed in an encapsulated form, the β values of ^{3}H-DPPC and ^{14}C-inulin should be the same and consequently the $^{14}C/^{3}H$ ratio should be constant. The several-fold difference of β values between them indicates that the distribution of inulin in liposomes is not uniform. Considering this irregular distribution, the following two mechanisms may be considered for these phenomena, based on the facts that free inulin was absorbed rapidly, but encapsulated inulin scarcely leaked out from liposomes, and that oligolamellar structures were observed in FT liposomes:[12]

1) Liposomes have a large aqueous space in the center, and thus the concentration of ^{14}C-inulin per unit lipid is higher in the center part of liposome than in the surface part.[12]—— Liposomes were absorbed from their surface region (see Fig. 11).
2) The $EN\%$ of smaller particles is less than that of larger ones.[12]——Smaller liposomes are absorbed more rapidly than the larger ones.

These processes need not occur independently, but may proceed simultaneously. Comparison of B_0 and $EN_R\%$ values of inulin showed that the latter is always larger by almost 10% than the former and this 10% is nearly equal with the rapid disappearance of ^{3}H-DPPC (Fig. 4). This suggests that about 10% of ^{14}C-inulin is absorbed in the first stage as the encapsulated form.

The high $^{14}C/^{3}H$ ratio in the lymph nodes can also be explained by the same mechanisms as in the muscle; *i.e.*, after draining into the lymph nodes, lipid component predominantly flowed out into the efferent lymphatics. The phagocytic uptake of liposomes by histocytes followed by digestion and selective storage of inulin might contribute in part to these phenomena.

Based on the preceding findings, a schematic illustration of drug absorption following *i.m.* injection of liposomes is presented in Fig. 11. In this figure, the width of the arrows represents the presumed contribution of each transport step and the dotted arrows indicate that the transport *via* these routes is relatively negligible. The drug is selectively transported to the regional lymph nodes encapsulated in liposomes, while the free drug is absorbed almost completely *via* the blood capillary route.

The present results demonstrate that liposomes could retain the drug in the injection site and selectively deliver it to the lymphatics. The utility of liposomes as a drug delivery system

in local injection was thus confirmed. As compared with FT-G and HY-G liposomes prepared through pre-condensation, FT-W liposomes gave almost the same lymph node concentration of ^{14}C-inulin. This result raises the possibility that FT liposomes can be used without any pretreatment (such as gel filtration). Furthermore, *EN*% can be increased to more than 80% by the FT method if suitable conditions are selected.[12] The advantage of FT method for preparing liposomes on a manufacturing scale thus seems clear.

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