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In vivo stability of ester- and ether-linked phospholipid-containing liposomes as measured by perturbed angular correlation spectroscopy

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ABSTRACT **To evaluate liposome formulations for use as intracellular sustained-release drug depots, we have compared the uptake and degradation in rat liver and spleen of liposomes of various compositions, containing as their bulk phospholipid an ether-linked phospholipid or one of several ester-linked phospholipids, by perturbed angular correlation spectroscopy. Multilamellar and small unilamellar vesicles (MLVs and SUVs), composed of egg phosphatidylcholine, sphingomyelin, distearoyl phosphatidylcholine (DSPC), dipalmitoyl phosphatidylcholine (DPPC) or its analog dihexadecylglycerophosphorylcholine (DHPC), and cholesterol plus phosphatidylserine, and containing ^{111}In complexed to nitrilotriacetic acid, were injected intravenously in rats. Recovery of ^{111}In-labeled liposomes in blood, liver, and spleen was assessed at specific time points after injection and the percentage of liposomes still intact in liver and spleen was determined by measurement of the time-integrated angular perturbation factor [G22(∞)] of the ^{111}In label. We found that MLVs but not SUVs, having DHPC as their bulk phospholipid, showed an increased resistance against lysosomal degradation as compared to other phospholipid-containing liposomes. The use of diacyl phospholipids with a high gel/liquid-crystalline phase-transition temperature, such as DPPC and DSPC, also retarded degradation of MLV, but not of SUV in the dose range tested, while the rate of uptake of these liposomes by the liver was lower.**

Liposomes have been considered for use as a drug delivery system for many years (1–3) and have in fact recently entered the stage of clinical trials for specific applications (4). In recent years, efforts have been made to optimize the composition, size ranges, and other properties of liposomes relevant for *in vivo* applications (for a review, see ref. 5).

Although small unilamellar vesicles (SUVs) of appropriate lipid compositions may be taken up by nonphagocytic cells, such as hepatocytes (6), the major mechanism of removal of liposomes from the bloodstream involves endocytosis by the mononuclear phagocyte system, especially for large liposomes (7–9). This has led to the recognition that liposomes not only may be considered as a direct drug-delivery system to diseased target cells but also as a vehicle for the creation of intracellular drug depots; thus, in cells other than the ultimate target cells, a sustained release of drug may be effectuated concomitant with degradation of the drug-containing liposomes (10, 11). Also, for this type of application, the degree of intracellular stability [i.e., resistance to (intra)lysosomal degradation] becomes an important factor to consider. Recently, it was found that phospholipids containing an ether instead of an ester link between the alkyl chain and the glycerol backbone are highly resistant to metabolic conversion (12, 13). It has been suggested that such phospholipids may be used to prepare liposomes with an increased resistance to intralysosomal degradation (14).

An attractive method to measure the integrity of liposomes in blood, cells, or even in a whole organ or animal, without the need to recover the liposomes from the system, is perturbed angular correlation (PAC) spectroscopy (15, 16). The angular correlation of the two γ-rays emitted upon the decay of ^{111}In to ^{111}Cd provides information on the tumbling rate of this ion. The high tumbling rate of ^{111}In, chelated in the interior of liposomes [high time-integrated angular perturbation factor, G22(∞)], decreases upon breakdown of the liposomes and the subsequent release of ^{111}In because of the rapid binding of the ion to macromolecules in the surrounding solution. Thus, the fraction of liposomes in the sample that is still intact can readily be measured (15–18).

In view of the possible use of liposomes as an intracellular slow-release drug depot system, we used the PAC spectroscopy technique to compare the *in vivo* rates of degradation in liver and spleen of liposomes of different compositions. We examined the uptake and degradation of both SUVs and multilamellar liposomes (MLVs) of the same compositions. The SUVs were previously shown to be mainly directed to the hepatocytes and the MLV were directed predominantly to the liver macrophages (Kupffer cells) (7–9). All the liposome types investigated contained 50% cholesterol (Chol) to enhance stability in serum (19, 20) and 10% phosphatidylserine (PS) to provide them with a negative charge to prevent aggregation. The balance of the lipid (40%) consisted of either phosphatidylcholine (PC) or beef brain sphingomyelin (SM) or dihexadecylglycerophosphorylcholine (DHPC), an ether-linked analog of dipalmitoyl phosphatidylcholine (DPPC), that was reported to be resistant to intracellular degradation (12–14) and also to increase the stability of liposomes in serum (21, 22).

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine (egg PC), distearoyl phosphatidylcholine (DSPC), and DPPC were purchased from Avanti. Chol (type CH-S), bovine brain PS, bovine brain SM, nitrilotriacetic acid (NTA), Sephadex G-25–50, and Hepes were from Sigma. DHPC was from Bachem (Bubendorf, Switzerland). Carrier-free indium-111 was purchased from Amersham.

Liposomes. For the preparation of liposomes, aliquots of stock solutions in chloroform/methanol (4:1) of either egg

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Abbreviations: Chol, cholesterol; DHPC, 1,2-dihexadecyl-*sn*-glycero-3-phosphorylcholine; DPPC, dipalmitoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; egg PC, egg phosphatidylcholine; MLV, multilamellar vesicle; NTA, nitrilotriacetic acid; PAC, perturbed angular correlation; PS, phosphatidylserine; SM, sphingomyelin; SUV, small unilamellar vesicle.

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PC, DSPC, DPPC, DHPC, or SM; Chol; and PS were mixed in a molar ratio of 4:5:1. The solvent was evaporated under a stream of N_2, whereupon the lipid film was dissolved in cyclohexane and lyophilized. The dried lipid was then hydrated with 1 mM NTA in HN buffer (10 mM Hepes/135 mM NaCl, pH 7.4) and vigorously mixed in a Vortex to form MLVs. For preparation of SUVs, this liposome suspension was sonicated for 60 min with a Branson sonicator (type W225R) equipped with a microtip probe (20% output power) at room temperature or, for DPPC-, DHPC-, and DSPC-containing liposomes, at 50°C. The resulting optically clear SUV suspension was centrifuged for 15 min at 10,000 × *g* to remove probe-derived titanium particles and residual MLVs and then chromatographed on a Sephadex G-25–50 column equilibrated in HN buffer to remove nonencapsulated NTA.

MLVs were prepared by lyophilizing appropriate amounts of lipids from cyclohexane and hydrating the dry lipid with 1 mM NTA in HN buffer, as with the SUV preparation procedure. After vigorous Vortex mixing, the suspension was extruded successively through 0.8- and 0.4-μm polycarbonate membranes (Nuclepore). Extrusion of DPPC- and DHPC-containing MLVs was done above their phase-transition temperatures by immersing the extrusion cell in a 50°C water bath.

To load the liposomes with the radioactive probe, 10 μCi (1 Ci = 37 GBq) of carrier-free ^{111}In HCl per μmol of total liposomal lipid was taken to dryness under a heat lamp and rehydrated with 30 μl of HCl (3 mM) plus 0.5 ml of a 15 mM acetylacetone solution in Tris buffer [10 mM Tris-(hydroxymethyl)-aminomethane/145 mM NaCl, pH 7.6]. To this solution, 2 ml of liposome suspension was added and the mixture was incubated for 60 min at room temperature for the egg PC- and SM-containing vesicles, at 37°C for the DPPC- and DHPC-containing vesicles, and at 50°C for the DSPC-containing vesicles. In this protocol, the presence of the ionophore acetylacetone allows the ^{111}In to traverse the liposomal membrane, whereupon it is chelated in the liposomal interior by the encapsulated NTA. This procedure results in an ^{111}In entrapment efficiency of >80% (23). Nonencapsulated ^{111}In, acetylacetone, and Tris buffer were removed by passing the liposomes over a Sephadex G-25–50 column equilibrated with HN buffer.

Animal Experiments. Eight male Sprague–Dawley rats (190–220 g) per liposome preparation were injected intravenously under light ether anesthesia with 4 μmol of liposome suspension (total liposomal lipid) in 0.5 ml of HN buffer via the penis vein. At 1, 6, 12, and 24 hr after injection, the rats were killed. Blood samples were taken by cardiac puncture, the livers were excised after perfusion through the portal vein with 60 ml of phosphate-buffered saline, and the spleens were extirpated. To determine the amount of liposomes remaining in the blood, we assumed a total blood volume per rat of 6.5% body weight. In the measurements of total organ radioactivity, the decay of ^{111}In was taken into account.

From each rat, ≈1 ml of blood, 1 g of liver tissue, and the spleen were used immediately to determine the absolute ^{111}In uptake and the time-integrated angular perturbation factor [G22(∞)] (17, 18) on the PAC spectrometer at room temperature. To correlate this G22(∞) factor to the percentage intact liposomes, the G22(∞) values of liposomes in HN buffer (0.59 ± 0.02) and of carrier-free ^{111}In in bovine serum (0.19 ± 0.02) were assumed to represent 100% and 0% intact liposomes, respectively.

RESULTS

To assess the solute retention capacity of the liposome preparations in serum, the release of ^{111}In was monitored for 4 days at 37°C (Fig. 1) as a decrease in the time-integrated angular perturbation factor G22(∞), which is equivalent to a decrease in the percentage intact vesicles. As appears from Fig. 1, the resistance of SUVs to serum-induced leakage is strongly dependent on the type of phospholipid used, even in the presence of equivalent amounts of Chol. The use of phospholipids with high gel/liquid-crystalline phase-transition temperatures like DSPC, DPPC, and DHPC [phase-transition temperatures, 53.7°C, 40.6°C, and 42.8°C, respectively (24)] clearly favor good solute retention in serum. This is in good agreement with conclusions reached previously (25).

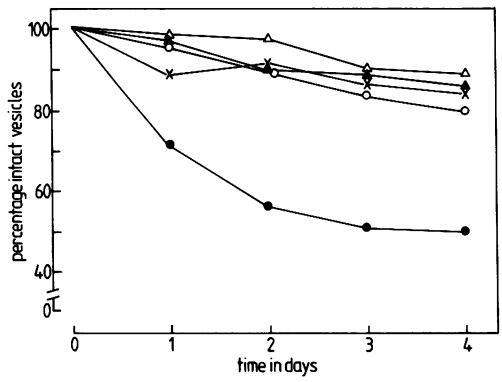

FIG. 1. Serum-induced leakage of liposomes. SUVs (100 μl, 1.0 μmol) were added to 100 μl of rat serum and incubated at 37°C. At the time points indicated, the time-integrated angular perturbation factor G22(∞) was determined, from which the percentage intact vesicles was calculated. Data represent mean of four incubations. Liposome compositions: X/Chol/PS = 4:5:1, where X is egg PC (●), SM (○), DSPC (×), DPPC (△), or DHPC (▲).

Fig. 2 shows the disappearance from blood of ^{111}In after intravenous injections of ^{111}In-labeled SUVs. Of all types of SUVs used, the major fraction is cleared from the bloodstream within 6 hr, although there were slight differences in the elimination rates of the various liposome types tested. SUVs containing phospholipids with higher phase-transition temperatures, such as DSPC, DPPC, and DHPC, are cleared less rapidly from the bloodstream than those containing lower phase-transition temperature phospholipids, like egg PC and SM.

The liver uptake of SUVs was inversely correlated with blood elimination (Fig. 3*A*). The egg PC-containing liposomes

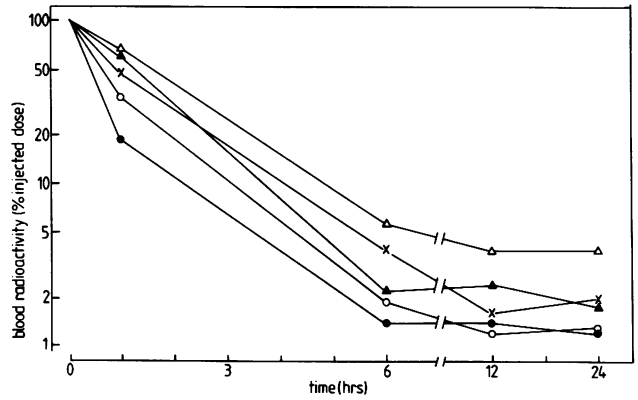

FIG. 2. Blood elimination of SUVs. Male Sprague–Dawley rats were injected intravenously in the penis vein with 2 μmol of ^{111}In-labeled SUVs per 100 g of body weight. The rats were killed 1, 6, 12, and 24 hr after injection and a sample of ≈1 ml of blood was taken from the heart and radioactivity was determined. Total radioactivity remaining in blood was estimated by assuming a total blood volume of 6.5% of the rat's body weight. Each time point represents the average value of two rats. Liposome compositions: X/Chol/PS = 4:5:1, where X is egg PC (●), SM (○), DSPC (×), DPPC (△), or DHPC (▲).

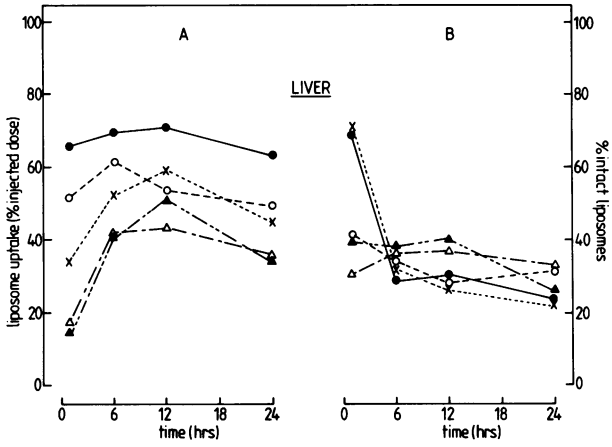

FIG. 3. Uptake and degradation of SUVs in the liver. Rats were injected intravenously with 2 μmol of ^{111}In-labeled SUVs per 100 g of body weight and killed 1, 6, 12, and 24 hr after injection. The liver was perfused with 50 ml of cold PBS, excised, weighed, and cut into small pieces. Total radioactivity and the time-integrated angular perturbation factor G22(∞) were determined in a liver tissue sample of ≈1 g, within 1 hr after death of the animal. From these data, the total liposome uptake (*A*) and the percentage intact liposomes (*B*) in the liver were calculated. Time points represent averages of two rats. Liposome compositions: X/Chol/PS = 4:5:1, where X is egg PC (●), SM (○), DSPC (×), DPPC (▲), or DHPC (△).

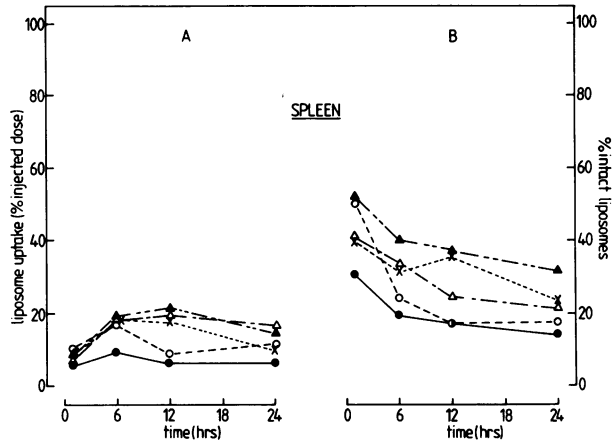

FIG. 4. Uptake and degradation of SUVs in the spleen. Rats were injected intravenously with 2 μmol of ^{111}In-labeled SUVs per 100 g of body weight and killed 1, 6, 12, and 24 hr after injection. The spleen was excised and weighed. Total radioactivity and the time-integrated angular perturbation factor G22(∞) were then determined in the intact spleen. From these data, the total liposome uptake (*A*) and the percentage intact liposomes (*B*) in the spleen were calculated. Time points represent averages of two rats. Liposome compositions: X/Chol/PS = 4:5:1, where X is egg PC (●), SM (○), DSPC (×), DPPC (▲), or DHPC (△).

especially were taken up relatively rapidly, reaching maximal amounts in the liver only 1 hr after injection of the liposomes. The DSPC-, DPPC-, and DHPC-containing SUVs did not reach maximal uptake values until 12 hr after injection. For all these liposome types, the liver-associated fraction decreased between 12 and 24 hr after injection. No difference in liver uptake was observed between the DHPC- and DPPC-containing SUVs.

Fig. 3*B* shows the percentage of intact vesicles in the liver. For DHPC-, DPPC-, and SM-containing SUVs this is only ≈30% within the first hour after injection. For egg PC and DSPC-containing SUVs, however, the fraction of intact vesicles is ≈70% after 1 hr and decreases to 30% between 1 and 6 hr after injection. Between 6 and 24 hr after injection, the proportion of intact vesicles in the liver further decreases only very slowly for all SUV types tested.

Liposome uptake is much lower in the spleen than in the liver (Fig. 4). Clearly, the liposome types that were taken up the least avidly by the liver—i.e., DSPC-, DPPC-, and DHPC-containing SUVs—showed the highest uptake by the spleen. This may be due to the increased spillover from the liver under these circumstances. The percentage of intact vesicles in the spleen varied between 30% and 50% at 1 hr after injection and decreased gradually in the following 24 hr for all liposome preparations (Fig. 4*B*).

As shown in Table 1, the total recovery of liposomal label in liver, spleen, and blood decreases from between 84% and 96% at 1 hr to between 54% and 71% of the injected dose at 24 hr after injection.

Table 1. Percentage total recovery label in blood, liver, and spleen

	Time after injection, hr			
	1	6	12	24
Egg PC	92	80	79	71
SM	96	79	64	78
DPPC	84	64	65	54
DHPC	88	66	76	56
DSPC	92	74	73	63

Rats were injected intravenously with 2 μmol of ^{111}In-labeled SUVs per 100 g of body weight and sacrificed 1, 6, 12, and 24 hr after injection, at which time total recovery of radioactivity in liver, spleen, and blood was determined. Results are averages of two rats.

We do not consider it very likely that the extensive initial release of ^{111}In reflects a proportional chemical degradation of the liposomes in view of our earlier observations on the metabolic conversion of liposomal lipids (26). Physical disturbance—e.g., due to extensive interaction of the liposomal membranes with (lysosomal) proteins—is probably more likely to cause the rapid initial release of ^{111}In. The results of experiments with MLVs are compatible with this latter view. Since MLVs contain several concentric bilayers and as many aqueous compartments, complete release of the encapsulated ^{111}In would require the consecutive degradation of all lipid bilayers.

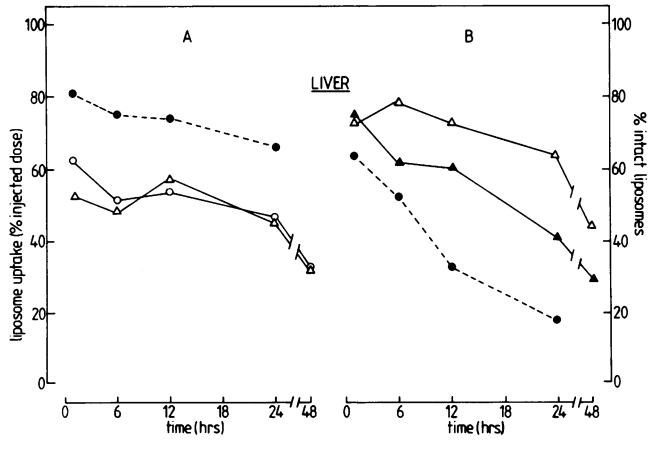

FIG. 5. Uptake and degradation of MLVs in the liver. Rats were injected intravenously with 2 μmol of ^{111}In-labeled MLVs per 100 g of body weight and killed 1, 6, 12, and 24 hr after injection. The liver was perfused with 50 ml of cold PBS, excised, weighed, and cut into small pieces. Total radioactivity and the time-integrated angular perturbation factor G22(∞) were determined in a liver tissue sample of ≈1 g. From these data, the total liposome uptake (*A*) and the percentage intact liposomes (*B*) in the liver were calculated. Time points represent averages of two rats. Liposome compositions: X/Chol/PS = 4:5:1, where X is egg PC (●), SM (○), DPPC (▲), or DHPC (△).

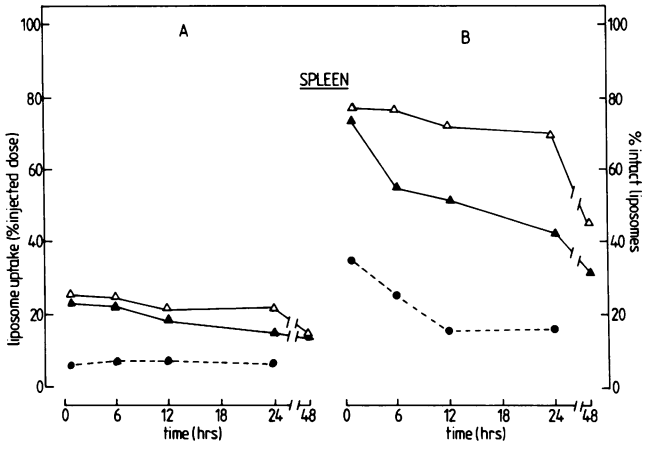

FIG. 6. Uptake and degradation of liposomes in the spleen. Rats were injected intravenously with 2 μmol of ^{111}In-labeled MLVs per 100 g of body weight and killed 1, 6, 12, and 24 hr after injection. The spleen was excised and weighed. Total radioactivity and the time-integrated angular perturbation factor G22(∞) were then determined in the intact spleen. From these data, the total liposome uptake (*A*) and the percentage intact liposomes (*B*) in the spleen were calculated. Time points represent averages of two rats. Liposome compositions: X/Chol/PS = 4:5:1, where X is egg PC (●), DPPC (▲), or DHPC (△).

Fig. 5 presents the liver uptake (Fig. 5*A*) and degradation (Fig. 5*B*) of various types of injected MLVs. Only 1 hr after injection, maximal liver uptake is attained, in contrast to the more slowly eliminated SUVs. Egg PC-containing MLVs are taken up more avidly by the liver than either DPPC- or DHPC-containing vesicles. Similar to what we observed for the SUVs, we noticed also for MLVs a gradual decrease in total label content, particularly of the liver. Also, with respect to intracellular degradation, the egg PC liposomes show a higher rate of degradation than either the DPPC or the DHPC liposomes (Fig. 5*B*). During the first hour after injection, 10–35% of the encapsulated ^{111}In is released in the liver. From then on, label release proceeds more gradually with average rates between 3.0%/hr for DSPC and 0.4%/hr for DHPC during the first 24 hr. The slower phase of the label release presumably reflects metabolic degradation of the liposomes, while the rapid initial phase is probably mostly accounted for by physicochemical effects on the outermost bilayer. The DHPC MLVs are apparently more resistant to breakdown than DPPC MLVs. One day after their uptake by liver cells, only 35% of the ^{111}In label has leaked from the DHPC MLVs, whereas 60% has been released from the DPPC MLVs. After 2 days, these values are 55% and 70%, respectively.

In the spleen, the uptake of egg PC-containing MLVs is much lower than that of DHPC- or DPPC-containing MLVs (Fig. 6*A*). Also, in this organ, just as in the liver, the DHPC MLVs appear to be the most stable liposome type (Fig. 6*B*). In addition, there is an initial rapid release of ^{111}In in the first hour after injection of the liposomes, followed by a second phase of slower release.

DISCUSSION

In recent years, the preparation of stable liposomal slow-release drug-delivery systems has drawn much attention. The use of ether-linked phospholipids to create stable liposomes was suggested several years ago (14). More recently, the use of polymerizable liposomes has also been considered as an alternative strategy for the preparation of liposomes that are resistant to degradation (27–29). However, thus far no studies have been published showing an increased solute retention capacity for polymerized as compared to normal liposomes, and reports dealing with ether phospholipid liposomes mainly focus on the extension of the circulatory half-life and increased resistance against serum-induced degradation (14, 21, 22). None of these reports, however, has examined the actual intracellular stability of these liposomes.

One method to assess the integrity of liposomes involves the relief of self-quenching of carboxyfluorescein encapsulated in liposomes in high concentrations (>30 mM). The observed increase in fluorescence upon leakage of the carboxyfluorescein from the liposomes is a measure of the fraction of the liposomes degraded (30). Although this method is very convenient for measuring liposome stability in buffer and serum, it is less suitable for measuring intracellular liposome breakdown. The use of PAC spectroscopy, on the other hand, has in recent years proven to be a reliable method to simultaneously assess uptake and degradation of liposomes in whole organs and even in small animals, without the need for prior tissue homogenization or label extraction. Hence, this technique was chosen to examine the *in vivo* disposition of liposomes in our studies.

The PAC technique requires that the liposomes contain a relatively large amount of ^{111}In (10 μCi per μmol of liposomal lipid). This high entrapment efficiency could be achieved by loading the liposomes that contained NTA as a chelator in their aqueous compartments with the ionophore acetylacetone (23). Having established first that the liposome compositions used showed no significant leakage of the ^{111}In–NTA complex upon storage for several days in HN buffer at 4°C, we then proceeded to measure the liposomal ^{111}In release upon incubation in rat serum at 37°C. Even though there was a substantial loss of encapsulated ^{111}In from some liposome preparations (as much as 30% for egg PC/Chol/PS SUVs) after 24 hr of incubation in rat serum, this is probably irrelevant with respect to our *in vivo* liposome uptake results. Firstly, it is unlikely that much ^{111}In had leaked from the liposomes during their stay in the circulation, since the liposomes of all compositions used were cleared so fast that within 6 hr after injection the blood compartment was virtually devoid of ^{111}In label. Secondly, the percentage of intact vesicles in the blood, as measured by the G22(∞) parameter, was consistently higher than 90%, indicating that the leakage of ^{111}In from the liposomes in our experiments is low indeed. Thirdly, preliminary experiments showed that nonentrapped ^{111}In had a half-life in blood of ≈3 hr, with the ^{111}In appearing rapidly in the urine. Since the recovery of ^{111}In label in blood, liver, and spleen amounted to at least 80% of the injected dose during the first uptake phase, not much ^{111}In could have escaped from the body during the time the liposomes were in circulation. Therefore, we feel confident that virtually all the ^{111}In remained associated with the liposomes until their uptake by the target cells.

From our results we conclude that, in spite of the reported resistance of DHPC toward lysosomal degradation, in particular against the action of phospholipase A_2 (12, 13), the use of this lipid instead of its ester analog DPPC for the preparation of SUVs does not lead to an increase of intracellular liposomal stability. As was shown, the blood disappearance as well as the uptake by liver and spleen and the *in vitro* serum stability were similar for both the DHPC- and DPPC-containing SUVs. Also, the rate of degradation, as reflected in the percentage intact liposomes in liver and spleen, did not differ appreciably. In MLVs, however, the use of DHPC as a bulk phospholipid instead of DPPC substantially improved the intracellular stability of these liposomes. Both in liver and in spleen, DHPC-containing MLVs were the most stable liposomes tested.

There may be several reasons why the stabilizing effect of DHPC was so much more apparent for MLV than for SUV. Firstly, the degradative capacity of the liver for the types of

SUV tested may be so high that with the liposome doses used no differences in rates of liposome breakdown could be established. Only in the case of a very rapid uptake of large quantities of liposomes, as was the case with the egg PC liposomes, did the liver show any signs of saturation of the liposome degradation capacity. Although a large fraction of the injected dose is still intact in the liver, the total amount of liposomes degraded in the liver is high. We also observed this saturation of liposome degradation in the liver for other liposome compositions at high dose ranges (unpublished observation).

Secondly, MLVs are taken up almost exclusively by the Kupffer cells (7, 8) whereas SUVs of the compositions used predominantly end up in the hepatocytes (6). Thus, an intrinsic difference in degradative capacity between these two cell types could also contribute to a difference in intracellular liposome breakdown. In addition, the number of hepatocytes is ≈10 times that of Kupffer cells, which implies that the amount of lipid taken up per cell is much higher for MLVs than for SUVs. Thus, the intracellular mechanism of liposome degradation may be more likely to become saturated in the case of MLVs than in the case of SUVs.

Thirdly, the difference in intracellular stability between SUVs and MLVs is likely to be related to the difference in the number of aqueous compartments per vesicle. An instantaneous nonchemical destabilization of the membrane of SUVs due to protein–lipid interactions would rapidly release all encapsulated ^{111}In. A similar destabilization of the outermost membrane of a MLV would lead to the release of only a small fraction of the encapsulated ^{111}In, the remainder being released from the inner compartments only upon gradual chemical degradation of the consecutive inner lamellae. The initial rapid release of ≈30% of the entrapped ^{111}In for both the DHPC and DPPC MLVs (Figs. 5 and 6) would seem to support this explanation if it is assumed that 30% represents the label contained in the outermost aqueous compartment (31).

We observed that the radioactivity recovered in the liver as well as in the spleen reaches a maximum between 6 and 12 hr after injection of the SUVs, and within 1 hr for the MLVs. The subsequent decrease in radioactivity reflects an organ redistribution of the label as indicated by the accumulation of radioactivity in the kidneys and at later time points in the urine. No ^{111}In label was excreted in the bile (data not shown). We also noticed that the G22(∞) value is low in the kidney but high in the urine. The ^{111}In secreted by the liver cells into the bloodstream after intrahepatic degradation of the liposomes is probably bound to protein, giving rise to a low G22(∞) value. In the kidneys, the ^{111}In is stripped from the protein, as a result of which the tumbling rate increases [high G22(∞) value]; it is later excreted into the urine.

In conclusion, the use of ether-linked phospholipids offers an advantage over the ester-linked equivalent in terms of increased liposome stability in the case of MLVs but not of SUVs. DHPC-containing MLVs may be useful as a sustained-release drug-delivery system, especially for drugs exerting their therapeutic effect in the liver. After uptake and degradation of such drug-loaded MLVs by Kupffer cells, the entrapped drug may then be released by the cells, thus providing sustained drug availability.

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