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EFFECTS OF LIPOSOME SIZE ON THE DEGRADATION OF BOVINE BRAIN SPHINGOMYELIN/CHOLESTEROL LIPOSOMES IN THE MOUSE LIVER

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The relative rates of degradation of the outer lipid bilayer of large multilamellar and small unilamellar bovine brain sphingomyelin/cholesterol (2:1; mol/mol) liposomes in the livers of Balb/c mice were compared. The rate of the release of entrapped In-111 ions from the aqueous reservoir of small unilamellar liposomes or from the outermost aqueous compartment of multilamellar liposomes was used to monitor the rate of degradation of the exterior lipid bilayer surface of these liposomes. The technique of gamma-ray perturbed angular correlation and a method for loading In-111 ions into the outermost aqueous compartment of liposomes were used in this investigation. It was found that in the liver the exterior lipid bilayer of large multilamellar liposomes was degraded more rapidly than the bilayer of small unilamellar liposomes in vivo. In contrast to the situation for small unilamellar liposomes, the degradative process for large multilamellar liposomes in the liver was not maintained under ischemic conditions. Our results suggest that multiple pathways operate in the degradation of liposomes in the liver. The rate of degradation of liposomes in the liver may depend on accessibility of liposomes to degradative sites.

Introduction

Liposomes have attracted considerable interest as a drug delivery system in recent years [1–4]. A controlled release of liposome contents is one of the most important elements in the rational design of liposomes for the delivery of pharmacologically active agents. Since the release of liposome-encapsulated, water soluble molecule in vivo depends not only on the physical state and chemical composition of the lipid bilayer but also on the interaction of liposomes with tissues and biological fluids, the development of strategies to control the release of liposome-encapsulated molecules requires an understanding of the influence these factors exert on the integrity of the liposome bilayer. It has been shown that the rate of release of liposome contents can be regulated by varying the cholesterol content [5] or glycolipid composition [6], by means of local hyperthermia [7], or by using lipid components which are sensitive to pH [8] or light [9]. In addition, it has been shown that leakage of liposome contents can be induced by plasma [10], lymph [11], and cells or plasma membranes [12]. Yet, the details of the release of entrapped molecules from liposomes taken up by tissues are relatively unknown.

Previously, we have shown [13] that by using the technique of gamma-ray perturbed angular correlation it is possible to continuously monitor the extent of leakage of liposome-entrapped indium-111 in tissues, isolated organs or a living

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animal. Using this technique, it has been shown that the rate of release of liposome content in tissues depends on the lipid composition [6,14] and tissue types [14,15]. Although the exact mechanisms resulting in the release of liposome content in tissues are unknown, the release process appears to be temperature dependent, at least in the liver [15]. Previously, we have shown that bovine brain sphingomyelin/cholesterol (2 : 1; mol/mol) small unilamellar liposomes remained intact in the blood, providing a simple system to study the degradation of liposomes in tissue without the complication of the leakage of liposome content in the blood [15]. Furthermore, because $^{111}In^{3+}$ binds tightly to tissue proteins at the site of the destruction of liposomes and therefore cannot readily redistribute to other tissues or blood, an accurate determination of not only the total amount of liposomes taken up by tissues, but also the percentage of intact liposomes remaining in the tissues, is possible. In continuing the investigation of the various factors which affect the degradation of liposomes in tissues, the present study investigates the rate of release of liposome-entrapped In-111 in mouse liver as affected by the size of liposomes, using bovine brain sphingomyelin/cholesterol (2 : 1; mol/mol) liposomes as a model system.

Materials and Methods

Bovine brain sphingomyelin and cholesterol were purchased from Sigma Chemical Company. Oxine and nitrilotriacetic acid were obtained from Aldrich Chemical Co. Indium-111 chloride was purchased from Med + Physics. The resins, AG 1-X8 (chloride form) was obtained from Bio-Rad. Sephadex G-50 and Sepharose 4B and 2B were purchased from Pharmacia.

The purification of indium-111 chloride and isolation of fresh defibrinated rabbit serum were carried out as described previously [13]. Small unilamellar liposomes were prepared by sonicating the dried thin film of bovine brain sphingomyelin/cholesterol (2 : 1; mol/mol) and 1 mM nitrilotriacetic acid in 0.106 M sodium phosphate, pH 7.4, isotonic buffered solution as described previously [15,16]. Typically, the concentration of liposomes was 20 mg phospholipid per ml buffer solution. The average size of the liposomes was estimated to be 187 ± 42 Å from negative stain electron micrographs of the liposomes, using potassium phosphotungstate as the stain. Untrapped materials were removed by passage of the liposomes over a Sephadex G-50 column, 0.8×35 cm, in 0.9% NaCl, 5 mM sodium acetate, pH 5.5.

Multilamellar liposomes were prepared by bath sonication of 20 mg of dried lipid thin film composed of a mixture of bovine brain sphingomyelin and cholesterol (2 : 1; mol/mol) in one ml of 1 mM nitrilotriacetic acid, 0.106 M phosphate buffer, pH 7.4, or by extrusion through a series of polycarbonate membrane filters with pore sizes ranging from 1.0 to 0.2 μm at 60°C [18]. Sonication was carried out in a model G112 SPIT bath sonicator (Laboratory Supplies Co.) at 60–80 watts in a 55°C water bath, using a glass tube (13×100 mm), for 10–15 min. The liposome suspension was annealed for 1 h at 65°C, cooled, and passed over a Sepharose 4B or 2B column equilibrated in 5 mM phosphate-buffered isotonic saline, pH 7.4 as previously described [13]. The multilamellar liposomes free from unentrapped nitrilotriacetic acid, were collected in the void volume. In all the study, the minimal size of multilamellar liposomes was larger that the exclusion limit of the Sepharose 4B gel.

To encapsulate indium-111, $^{111}In^{3+}$ was delivered to the encapsulated 1 mM nitrilotriacetic acid by means of a mobile ionophore, 8-hydroxyquinoline in a loading procedure described previously [16,17]. In the case of small unilamellar liposomes, In-111 was loaded to the inner aqueous compartment. However, in the case of multilamellar liposomes, In-111 was loaded only into the outermost aqueous compartment. The extent of the leakage of the entrapped cations in the presence or absence of serum was determined by chromatographing on Sepharose 4B and/or by the technique of gamma-ray perturbed angular correlation [13,17]. Only liposomes of proven stability, as confirmed by the lack of release of In-111 in serum at 37°C, were used.

For the biodistribution study, liposomes were injected via the tail vein of Balb/c mice of about 25 g. The lipid concentration was determined by the ferrothiocyanate assay [21]. The injected doses for small unilamellar liposomes and multilamellar liposomes were 35–75 and 1–4 μg total lipid per g

body weight, respectively. The mice were killed at various times post-injection by cervical dislocation and immediate decapitation. In all tissue distribution studies, the radioactivity due to blood background in each tissue sample was subtracted [15].

The following equation was used to assess the percentage of intact liposomes remaining in a sample at time t after the injection using the time-integrated perturbation factor ($\langle G_{22}(\infty)\rangle$) produced by the perturbed angular correlation measurement:

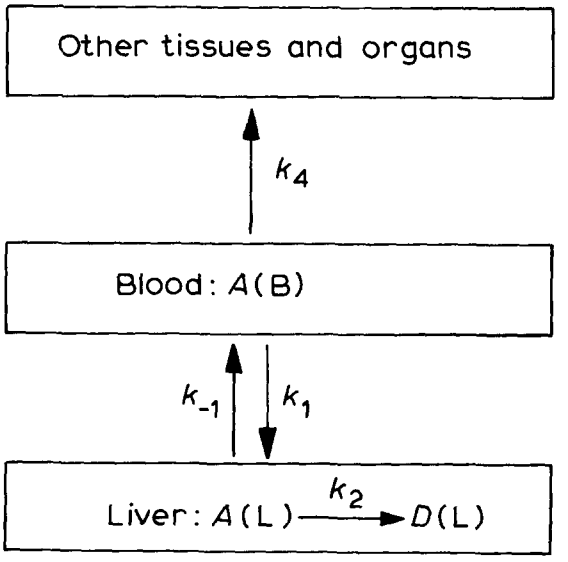
$$\langle G_{22}(\infty)\rangle_t = \langle G_{22}(\infty)\rangle_{\text{intact}}(X_t) + \langle G_{22}(\infty)\rangle_{\text{degraded}}(1 - X_t) \quad (1)$$

where X_t represents the percentage of intact liposomes in a sample at time t after the injection, and $\langle G_{22}(\infty)\rangle_t$, $\langle G_{22}(\infty)\rangle_{\text{intact}}$ and $\langle G_{22}(\infty)\rangle_{\text{degraded}}$ are the time-integrated perturbation factors for liposome-entrapped In-111 in a serum, blood, or liver sample at time t, In-111-nitrilotriacetate encapsulated in intact liposomes, and protein-bound In-111 released from the completely disrupted liposomes, respectively.

Two different experimental approaches were utilized in the perturbed angular correlation studies of the liver degradation of the unilamellar and multilamellar liposomes: in vivo and in vitro degradation studies. To investigate the degradation of liposomes in the liver in vivo, livers removed from mice killed at various times post-injection of liposomes were placed in modified Ringer's solution supplemented with 20% (v/v) rabbit serum, and antibiotics, and monitored immediately in the perturbed angular correlation spectrometer at room temperature [15]. To investigate the degradation of liposomes in the liver in vitro, the liver removed from a mouse killed at 15 min post-injection was placed in the above antibiotic-supplemented Ringer's solution and continuously monitored in a temperature-controlled sample holder for 15–20 h.

To characterize the kinetics of liver uptake and degradation for the liposomes examined, the data collected from tissue distribution studies was combined with the data gathered from perturbed angular correlation studies and analyzed by a least-squares fitting routine assuming a three-compartment model and first-order kinetics in the uptake and degradation [15]. For the determination of the rate of degradation of liposomes in the liver, the three-compartment model presented in Scheme 1

Scheme 1.



was used. The amount of intact liposomes, expressed as the percentage of administered dose in the blood and in the liver is denoted by A(B) and A(L), respectively. The amount of radioactivity released from the degraded liposomes in the liver is represented by D(L). The processes of the release of In-111 from liposomes in the blood and the recirculation of the released In-111 from liver to blood were not considered, since the amount of In-111 released in the circulation was not detectable. Furthermore, the excretion was not considered in the present model because the renal and fecal excretion of radioactivity was less than 0.5% of the injected dose in a 23 h period.

Results

Small unilamellar sphingomyelin/cholesterol liposomes were found to remain intact in vitro during incubation with 50–90% serum for 37°C for 24 h. In vivo, these same liposomes remained intact in the blood for 24 h as well. Similar results were obtained for sphingomyelin/cholesterol multilamellar liposomes which also remained intact in vitro during 37°C incubation in the presence of

serum and during the time course of their presence in the blood in vivo. These findings are based on the perturbed angular correlation measurements since the time-integrated perturbation factor, $\langle G_2(\infty)\rangle$, for both types of liposomes remained constant at 0.59 ± 0.02 indicating completely intact liposomes. On the other hand, the liposomes taken up by the liver were degraded gradually with time. Fig. 1 depicts the percentage of intact liposomes in the livers of mice killed at various times post injection as estimated from Equation 1.

Based on our model and the least-squares kinetics analysis [15], the rate constants for the uptake of liposomes by the liver (k_1) and the degradation of liposomes within the liver (k_2) were estimated. The results of the analysis for an average of two preparations of small unilamellar liposomes and for three different preparations of bath-sonicated large multilamellar liposomes are presented in Table I. Five observations may be made from an examination of the data in this table. First, because of the difference in the size distribution of the three different preparations of the large multilamellar liposomes due to batch variations, the rate constants for the uptake of these liposomes by the liver (k_1) varies. Second, in contrast to the small unilamellar liposomes, the large multilamellar liposomes are taken up rapidly by the liver as indicated by the high values of k_1 [20]. Third, despite the variation in k_1 among the large multilamellar liposomes, the rate constants for the degradation of these large liposomes in the liver (k_2) are not very different. Fourth, the average half-life ($t_{1/2} = 0.693/k_2$) for the in vivo degradation of the small unilamellar liposomes (3.55 ± 0.25 h) is significantly longer than that for the large multilamellar liposomes (1.38 ± 0.22 h). Fifth, it is apparent from an examination of k_1 that once taken up by the liver, intact multilamellar liposomes do not return to any significant extent to the blood; likewise, there is no significant uptake of intact multilamellar liposomes by other tissues and organs compared to liver uptake.

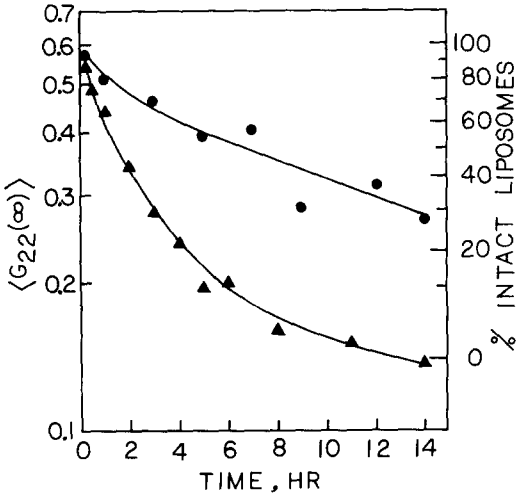


Fig. 1. Percentage of intact liposomes in the livers in vivo at various times post-injection. After various periods post-injection of bovine brain sphingomyelin/cholesterol (2 : 1; mol/mol) small unilamellar liposomes (●) or bath-sonicated large multilamellar liposomes (▲) to mice, the livers were excised for the measurement of time integrated perturbation factor, $\langle G_{22}(\infty)\rangle$. The percentage of intact liposomes was calculated, using the Equation 1 and the $\langle G_{22}(\infty)\rangle$ values of 0.59 and 0.15 for intact and completely disrupted liposomes, respectively.

TABLE I

CALCULATED RATE CONSTANTS

The rate constants were obtained by fitting the tissue distribution and the in vivo degradation data for In-111-loaded sphingomyelin cholesterol (2 : 1, mol/mol) liposomes. The rate constants for the small unilamellar liposomes were calculated from the average of two independent data sets. The rate constants for three different preparations of bath-sonicated large multilamellar liposomes are listed individually because of the lack of similarity in their blood clearance half-life and the time course of tissue distribution.

Rate constant (1/h)	Small unilamellar liposomes	Large multilamellar liposomes		
		Set 1	Set 2	Set 3
k_1	0.024 ± 0.005	1.280	0.160	1.350
k_{-1}	0.016 ± 0.016	0.000	0.000	0.000
k_2	0.195 ± 0.015	0.460	0.645	0.450
k_4	0.023 ± 0.003	0.000	0.030	0.000

Using the rate constants in Table I, it was possible to predict the total amount of radioactivity from intact and degraded liposomes present in the blood and in the liver and the $\langle G_{22}(\infty)\rangle$ values of the liver. Fig. 2 presents a comparison of blood clearance, the liver uptake, and the fraction of total hepatic uptake remaining intact at various times post-injection for small and large multilamellar liposomes. The curves generated from the predicted kinetic parameters derived from the fit-

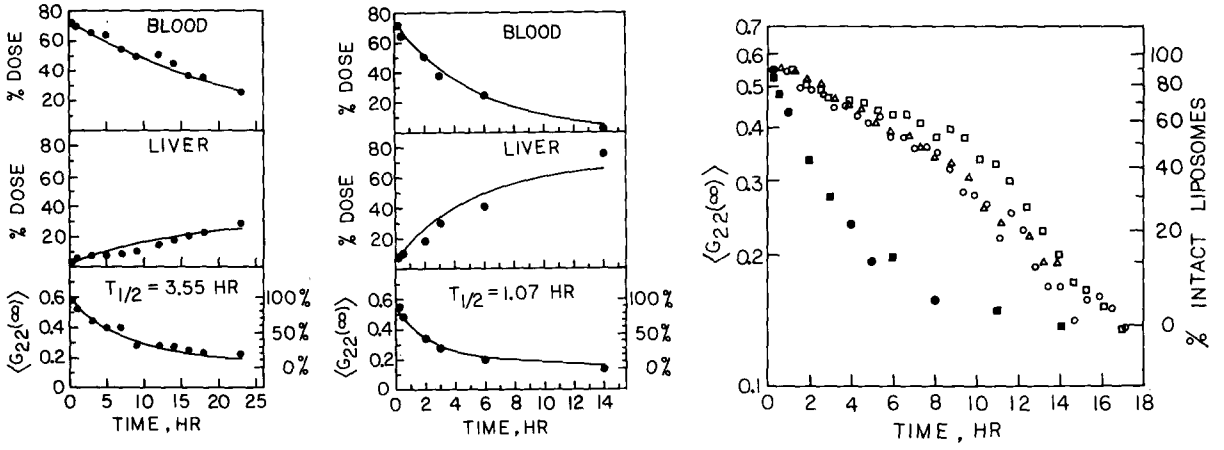


Fig. 2. Blood clearance, liver uptake, and the in vivo hepatic degradation of liposomes. The liposomes were bovine brain sphingomyelin/cholesterol (2 : 1; mol/mol) small unilamellar liposomes (left frame) and bath-sonicated large multilamellar liposomes (right frame). The percentage of intact liposomes in the liver is plotted at the right ordinate, corresponding to the $\langle G_{22}(\infty)\rangle$ designated on the left ordinate and the half-life of the in vivo degradation of each type of liposome is shown in the lower figure of each frame. The curves are generated from the predicted parameters derived from Table I.

Fig. 3. Degradation of the outermost shell of bovine brain sphingomyelin/cholesterol (2 : 1; mol/mol) bath-sonicated large multilamellar liposomes in the liver in vitro and in vivo. The percentage of intact liposomes remaining in three livers under in vitro conditions (○, △, □) was determined. Three different preparations of liposomes were used for the in vitro study. Two different preparations of liposomes were used for the in vivo study (●, ■). In the in vivo study, each point represents the $\langle G_{22}(\infty)\rangle$ of one excised liver.

ting routine are in close agreement with observed data.

In contrast to the similarity in the relative rates of the in vivo ($t_{1/2} = 3.55 \pm 0.25$ h) and in vitro ($t_{1/2} = 3.50 \pm 0.20$ h) degradation of bovine brain sphingomyelin/cholesterol (2 : 1, mol/mol) small unilamellar liposomes in the liver [15], the relative rates of degradation of the exterior bilayer of large multilamellar liposomes of same composition in the liver was very different in the in vitro and in vivo situations (Fig. 3). As can be estimated from Fig. 3, the half-life of the in vitro degradation of the outermost shell of the large bath-sonicated liposomes in isolated livers is 8–9 h. This is about 6-times longer than the in vivo half-life of 1.38 h calculated from the average k_2 of the same type of liposome in Table I. Our results indicated that the phenomenon of the retarded degradation of large liposomes in vitro was not restricted to bath sonicated multilamellar liposomes. Fig. 4 depicts the time course of the in vitro degradation of a typical bovine brain sphingomyelin/cholesterol (2 : 1; mol/mol) multilamellar liposomes prepared by extrusion [18].

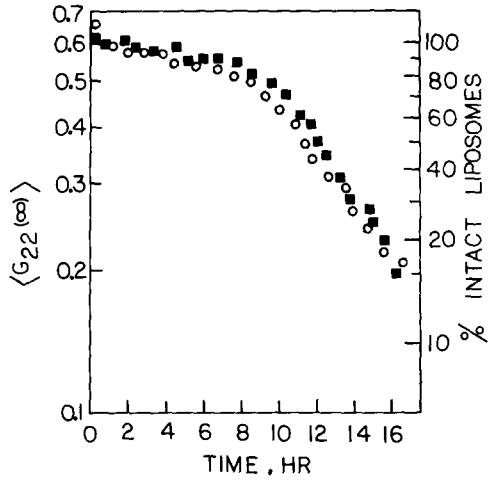


Fig. 4. In vitro degradation of the exterior lipid bilayer of bovine brain sphingomyelin/cholesterol (2 : 1, mol/mol) membrane-extruded large multilamellar liposomes in two livers.

Discussion

In this study, we used probe-sonicated small unilamellar vesicles and bath-sonicated or membrane-extruded multilamellar vesicles to study the

effect of liposome size on the rate of degradation of liposomes in the liver. To compare the degradation rate of small and large liposomes using large multilamellar liposomes, a special strategy was required. The reporter molecule, $^{111}In^{3+}$, was loaded only into the outermost aqueous compartment of the multilamellar liposomes. The degradation of the lipid bilayer for small unilamellar and large multilamellar liposomes is thus comparable. The complication produced by the degradation of successive internal multilamellar liposome bilayer with the concomitant stepwise release of encapsulated reporter molecule is avoided.

The exact size distributions of various types of the multilamellar liposomes used in the present study were not determined. However, the minimal size of the multilamellar liposomes is larger than the exclusion limit of Sepharose 4B. On the other hand, the size of the small unilamellar liposomes (187 ± 42 Å) is smaller than the exclusion limit of Sepharose 4B. The use of Sepharose 4B to select these two populations of liposomal sizes was an arbitrary choice. However, it appears that as indicated by the relatively fast rates of blood clearance of these multilamellar liposomes the choice was quite adequate for the purpose of the present study. In our hands, the preparation of a reproducible size distribution of sphingomyelin/cholesterol (2 : 1; mol/mol) multilamellar liposomes by bath sonication was difficult, consequently there was a variable blood clearance half-life for each preparation. Despite the variation in the apparent size distribution of the multilamellar bath-sonicated liposomes from batch to batch, the rate constant characterizing the in vivo disruption of the outer surface of various batches of large multilamellar liposomes in the liver is consistently and significantly higher than that of the small unilamellar liposomes, the size distribution of which can be controlled very reproducibly by probe sonication and ultracentrifugation. The reasons for this difference will be discussed subsequently.

There are at least two possible mechanistic explanations accounting for the release of In-111 from liposomes taken up by the liver, while at the same time, liposomes present in the circulating blood remain completely intact. First, as originally suggested by Gregoriadis and Ryman [21], it may be that liposomes are phagocytosed and degraded in the lysosomal apparatus of liver cells. Second, it is possible that liposomes are degraded without being internalized into liver cells.

Two lines of evidence suggested that the degradation of both the large multilamellar and small unilamellar liposomes occurred inside the liver cells. First, if liposomes were degraded enzymatically outside the liver cells, the release of some liposome-entrapped In-111 into the circulating blood would be expected. It is known that transferrin-bound In-111 remains in the blood for an extended period of time [22] and such protein-bound In-111 would be characterized by a low $\langle G_{22}(\infty) \rangle$. The result of the perturbed angular correlation study of the blood samples clearly indicated that no In-111 was released or present as a serum protein-bound species during the course of the experiment (23 h). Therefore, degradation of liposomes in vivo must occur at a location sequestered from circulating transferrin. Furthermore, if the liposomes were degraded outside the liver cells by sphingomyelinases, phospholipases or the process of liposome-cell membrane fusion, the rate of degradation of large multilamellar liposomes in the liver in vitro should not be retarded immediately by excising and incubating the isolated liver in culture medium at 37°C (Figs. 3 and 4). Second, the phenomenon of the retardation of the rate of degradation of large multilamellar liposomes in the liver under in vitro ischemic conditions (Figs. 3 and 4) suggests that the degradative process requires normal physiological conditions not present in our in vitro experimental situation. We found previously that the rate of degradation of small unilamellar liposomes in the liver was highly dependent on temperature, and occurred most rapidly at physiological temperature [15]. These two observations are compatible with a phagocytotic and/or an endocytotic process which requires energy and physiological temperature [23].

In fact, the in vitro pattern for the release of In-111 from the outermost aqueous compartment of large multilamellar liposomes in isolated livers at 37°C occurs in a more or less biphasic pattern (Figs. 3 and 4). As clearly shown in Fig. 4, approx. 30% of the liposomally entrapped radioactivity is released and binds to tissue components during the first 9 h. From 9 to 15 h, the degradative process is more rapid and most of the remaining

70% of the entrapped radioactivity is released and bound. Conceivably, in the in vitro liver excised at 15 min after the injection, most of the large multilamellar liposomes taken up by the liver may be only bound to the surface of cells and may not yet have been endocytosed. Under the in vitro conditions of ischemia in the excised liver, the process of endocytosis and/or phagocytosis may have been markedly retarded, resulting in an initial slow phase of the release of liposome content (Fig. 4). DeDuve and Beaufay [24] found that the progressive release of lysosomal enzymes preceded general necrosis, when liver lobes in rats were rendered ischemic by ligation. It is conceivable that after 9–10 h under the in vitro ischemic conditions, the rapid release of liposome-entrapped In-111 shown in Fig. 4 is a result of such release of lysosomal enzymes which rapidly degrade adsorbed liposomes.

It is interesting to note that in the case of bovine brain sphingomyelin/cholesterol (2 : 1; mol/mol) small unilamellar liposomes, the phenomenon of retarded degradation of liposomes in the liver under the same in vitro conditions was not detected. In fact, the degradative rate constants for the small liposomes in the liver in vitro and in vivo were almost identical [15]. This may suggest that the degradative processes of large multilamellar liposomes and small unilamellar liposomes in the liver are via two different pathways perhaps even mediated by different cell types. From an anatomical consideration of the structure of the endothelial lining of the sinusoidal wall, the accessibility of particles, including liposomes, larger than 0.1 μm in diameter, to hepatocytes is expected to be limited [25,26]. It is likely that the large multilamellar sphingomyelin/cholesterol liposomes found in the liver in the present study are predominantly in the Kupffer cells, whereas the small unilamellar sphingomyelin/cholesterol liposomes may be taken up by both the parenchymal cells and Kupffer cells in the liver. Recent studies on the cellular distribution of large unilamellar liposomes or multilamellar liposomes [27] and small unilamellar liposomes [28] in the rat liver appears to support the concept of a differential distribution of liposomes in various types of liver cell.

According to Weibel et al. [29] and Blouin [30], parenchymal cells account for more than 90% of the liver volume; consequently, parenchymal cells have a much greater surface area which could participate in the formation of endocytotic vesicles necessary for liposome uptake. Because of this limited cell-surface area of the Kupffer cells and the rapid accumulation of the large multilamellar liposomes in the Kupffer cells, many of the bound large multilamellar liposomes may not have been internalized into cells at the time of excision (15 min post-injection). On the other hand, since the rate of accumulation of the small unilamellar liposomes in the liver is slower, the process of endocytosis of the small unilamellar liposomes into a much larger pool of cell population of parenchymal and Kupffer cells may not be the ratelimiting step as may be the case for large liposomes. This is offered as an explanation for the retarded rate of degradation for liposomes under in vitro ischemic conditions which occurred only with large multilamellar liposomes and not with small unilamellar liposomes.

Considering the rate of degradation of small unilamellar sphingomyelin/cholesterol (2 : 1; mol/mol) liposomes in the liver in vivo, the finding of a faster rate of release of In-111 from the large multilamellar liposomes in the liver in vivo was unexpected. Using purified phospholipase A_2, Wilschut et al. [31] showed that the rate of enzymatic hydrolysis of a strongly curves liposomal surface was faster than that of the liposomal surface with a relatively low degree of curvature. Although their in vitro result is different from our in vivo finding, it is important to realize our determination of the in vivo rate of release of In-111 from liposomes in the liver represents the net effect of multiple processes, which involve the relative distribution of the liposomes in the parenchymal cells and Kupffer cells, the internalization of bound liposomes into cells via endocytosis or phagocytosis, the fusion of endocytotic vesicles with lysosomes, and the degradation of liposomes by lysosomal enzymes. Consequently, the in vivo rate of degradation of liposomes in the liver depends on the distribution of liposomes into different types of liver cells, and the rate constants for the rate limiting step of these processes. Presumably, the degradative machinery of the Kupffer cell, a specialized phagocytic cell, is more efficient

than that of the non-specialized phagocytic cell, such as parenchymal cell. If the large multilamellar liposomes in the liver are degraded predominantly by the Kupffer cells, and only a small fraction of the small unilamellar liposomes in the liver are degraded by the Kupffer cells, it is quite conceivable that the large multilamellar liposomes are degraded faster than the small unilamellar liposomes. Further work will be needed to verify the above hypothesis.

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