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CELLULAR LOCALIZATION OF STABLE SOLID LIPOSOMES IN THE LIVER OF RATS

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Abstract—Small solid liposomes made from distearoylphosphatidyl choline and cholesterol (molar ratio 2 : 1) showed significant stability in plasma, with a half-life of about 24 hr after intravenous injection in rats. The major cellular uptake of intact liposomes was found in the liver and spleen, peaking after 2–4 hr in the liver and after 24 hr in the spleen. Isolation of parenchymal and non-parenchymal cells from rat livers at various intervals after injection of liposomes showed that both cell types adsorbed liposomal membranes and took up the liposomal contents. Our study has shown that most of the liposomal markers found in the liver shortly (< 40 min) after administration stemmed from the liposomes adsorbed to extracellular binding sites, and that uptake into the cells took place subsequently. In non-parenchymal cells, uptake was rapid and the intracellular level remained rather constant after 40 min and for up to 4 hr. The uptake of liposomes by parenchymal cells was slower, it showed a lag-phase of approx. ½ hr and peaked at 2 hr, whereupon the radioactivity in parenchymal cells dropped. The contents of liposomes behaved in a manner similar to the membranes. It is concluded that, in addition to a rapid uptake of liposomes in non-parenchymal liver cells, there is a significant degree of association with parenchymal cells, provided that the liposomes administered are small (< 100 nm in diameter) and stable.

A number of studies have dealt with the localization of liposomes after intravenous injection in experimental animals. Several factors have been examined and reviewed recently [1–3]. The most important factors with respect to the rate of elimination of liposomes from the blood stream as well as the site and the extent of uptake are: size [4–10], charge [4, 8, 11–14], lipid composition [13–16] and dosage [9, 10, 12, 14, 17, 18]. Liver and spleen have been shown repeatedly to be the main sites of liposomal uptake, whereas the uptake in lungs, intestines and heart is much less.

In a few studies, the cellular or subcellular localization in liver has been investigated by electron microscopy [6, 19–22], cell isolation [6, 12, 23–25] or after subcellular fractionation of liver homogenates [17, 26, 27]. The conclusion drawn from these reports is that the uptake of liposomes, especially of large liposomes, is most rapid in Kupffer cells and the extent of uptake somewhat less in endothelial cells. The uptake in hepatocytes recorded with the types of liposomes used until now was but limited.

Unfortunately most of these studies have been carried out with liposomes of a very heterogeneous size distribution [17–27] and with liposomes known to be unstable in the presence of plasma proteins so that they showed release of entrapped marker molecules and transfer of lipid marker molecules to lipoproteins [28, 29]. Thus is was reported that the distribution of entrapped horseradish peroxidase found in all major liver cell types was not different to that of the enzyme administered without encapsulation [22].

Another study, using bovine serum albumin (BSA) as entrapped marker showed that its uptake in hepatocytes was less than the uptake of phospholipid marker, and the authors concluded that intact liposomes were not taken up in hepatocytes [6]. Others have reached the opposite conclusion from studies using liposomes of a different composition and size [20, 25]. *In vitro*, liposomes are readily taken up in hepatocytes either freshly isolated or grown in culture [30, 31]. Therefore it appeared to us that the cause of the divergent results were the differences in size and stability of liposomes *in vivo*, together with the different accessibility of the major liver cell types due to liver's architecture.

The present report deals with liposomes made by a modified reverse phase evaporation method [31, 33–35]. This method yields liposomes of homogeneous size distribution, as determined by freeze-fracture EM [32], with mean diameters ranging from 40 to 80 nm, depending on the lipid composition, and with an efficiency for entrapment of proteins ranging between 60 and 100%. With a lipid composition of DSPC : CHOL (2 : 1 molar) giving stable liposomes [14, 16, 20, 31, 33, 34] we show that entrapped material can be conveyed to hepatocytes even in quantities exceeding those found in non-parenchymal cells.

MATERIALS AND METHODS

Materials. Distearoyl-sn-phosphatidyl choline (DSPC) cholesterol (CHOL) and collagenase (type I) were obtained from Sigma, Missouri, U.S.A. Di(1-^{14}C)stearoyl-sn-phosphatidyl-choline (^{14}C-DSPC), specific activity 120 mCi/mmol, and sodium boro(^{3}H)hydride, spec.act. 10–15 Ci/mmol, were obtained from The Radiochemical Centre, Amersham, England. Bovine serum albumin (BSA), crystalline A-grade, and pronase (B-grade) were from Calbiochem-Behring Corporation, Switzerland. Tritium labelled BSA was prepared essentially as described in [36], though using (^{3}H)$NaBH_4$ instead of (^{3}H)CH_2O, and the specific activity obtained was 1–5 mCi/mg BSA.

Preparation of liposomes. DSPC : CHOL (2 : 1) liposomes were made according to a recent patent [35], as described in detail earlier [33, 34]. (^{14}C)DSPC was added to DSPC to give a specific activity of about 0.2 mCi/mmol phospholipid, and (^{3}H)BSA was added to unlabelled BSA to give 0.3–1.5 mCi/mg BSA in 10 mg BSA/ml

Reversed micelles were formed in dibutylether : cyclohexane 1 : 1 and then dispersed in 10 mM Tris (pH 7.4). After removal of organic solvents by evaporation, phospholipid suspensions were centrifuged for 30 min at 37,000 *g* to remove the aggregated lipid and then passed through a Sepharose CL-6B column to remove the non-entrapped BSA.

The typical entrapment yield was 60–80%, as determined by rocket immunoelectrophoresis after addition of 1% Triton X-100 to the suspension [37]. The typical mean diameter of liposomes was 70 nm, with more than 90% of the particles with diameters < 100 nm, as determined by freeze-fracture EM [31]. The size of the liposomes was also determined by dynamic light scattering [34]. Generally, the liposomes were concentrated by ultrafiltration on Amicon XM-50 membranes to a phospholipid concentration of 11–21 μmol/ml, and then sterile filtered through a 0.2 μm Nuclepore membrane. The liposomes were stored in 10 mM Tris buffer, pH 7.4, for reasons of stability [34], and brought to isotonicity by addition of 3 M NaCl solution immediately prior to administration to animals.

Tissue distribution. Male Wistar rats from the local stock colony at the Catholic University of Louvain, Belgium, weighing 230–270 g, with ad libitum access to food and water, were injected with DSPC : CHOL liposomes 50 μmol phospholipid/kg body weight. At various intervals after injection, three animals were anaesthetized with ether and as much blood as possible was drawn by heart puncture into heparinized syringes. The following organs/tissues were then sampled: cerebrum, cerebellum, thymus, lungs, heart, diaphragm, liver, kidneys, adrenals, stomach, small intestine and colon freed of their contents, and bone marrow from both femurs [31, 33]. Tissue samples were homogenized in a motor driven Potter homogenizer and then sonicated for 1 min in a Bransonic B-12 sonifier. Total tissue radioactivity was determined after combustion in a Packard Model 306 Oxidizer [33]. Rat serum albumin was determined in plasmas and in supernatants from tissue homogenates by immunodiffusion [31, 38]. True tissue uptake was determined after correction for plasma radioactivity present in tissue, as calculated from the serum albumin content. An insignificant amount of binding of liposomal markers to washed blood cells (< 1% of plasma values) was found for up to 24 hr.

Radioactivity in plasma was counted immediately after sampling and after chromatography of 100–200 μl plasma on 0.7 × 18 cm Sepharose CL-6B columns eluted with 140 mM NaCl, 10 mM Tris pH 7.4 [33].

Liver cell isolation. For these studies, male Wistar rats from the stock at Erasmus University, Rotterdam, The Netherlands, weighing 250–340 g, were injected with DSPC : CHOL (2 : 1) liposomes containing BSA as above. Three minutes post injection, a blood sample of approx. 0.5 ml was taken from the retro-orbital sinus. The animals were anaesthetized with 60 mg Nembutal/kg body weight intraperitoneally. The operation, sampling of blood at the beginning of the preperfusion, 8 min before the sampling of the caudate liver lobe and the start of the collagenase perfusion at time intervals referred to in figures and tables has been described in detail before [39].

Isolation of parenchymal (PC) and non-parenchymal cells by pronase digestion (NPC_1) and by differential centrifugation (NPC_2) followed the procedure outlined in [40]. Centrifugations at 50 *g*, however, were shortened to 30 sec to increase the yield of NPC_2 cells.

Purity and integrity of the isolated cell fractions were determined by measuring the amounts of *L* and M_2 type pyruvate kinase present in postmitochondrial extracts of isolated cells [39, 40]

Radioactivity in cell fractions was determined after combustion of homogenates, as described before [33]. Protein concentration in homogenates was determined with BSA as the standard [41]. Uptake in non-parenchymal cells (NPC_2, method 2 [40]) was corrected for the amount of parenchymal cell protein by pyruvate kinase measurements as described in [39, 40].

RESULTS

Elimination from plasma

After the intravenous injection of this type of liposome in rats the elimination of both the phospholipid membrane and the entrapped labelled BSA was slow (half-life approx. 20 hr) following an initial phase of rapid distribution/tissue uptake. Column chromatography of plasma samples on Sepharose CL-6B (Fig. 1) showed these liposomes to be very stable in plasma, as we have shown before in mice [31] and in rats [31, 33] and as others have shown in mice with sonicated vesicles (SUV) of similar lipid composition [4, 14, 16].

Figure 2 shows the plasma concentration of the two liposome markers as found (1) in intact liposomes, (2) in association with HDL particles or as free BSA, and (3) as low molecular weight metabolites. In contrast to the massive transfer of (^{14}C)DSPC to HDL particles found after the injection of PC : PS (4 : 1) liposomes [33], the amounts

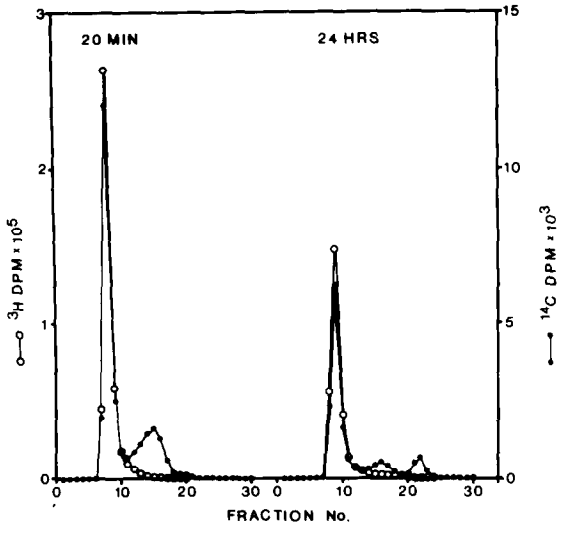

Fig. 1. Representative chromatographic separations of rat plasma on 18 × 0.7 cm Sepharose CL-6B columns after i.v. injection of 50 μmol phospholid of (^{14}C)DSPC : CHOL, (^{3}H)BSA liposomes. 100 μl of plasma applied to the column was eluted with 140 mM NaCl + 10 mM Tris-HCl pH 7.4. Fractions of 0.5 ml collected and used for determination of radioactivity. Intact liposomes eluted around fractions 8–11, labeled HDL around fractions 12–16, intact BSA around fractions 13–17, and low molecular weight metabolites of BSA after fraction 20.

of phospholipid membrane marker transferred from DSPC : CHOL (2 : 1) liposomes were minimal (4–8%) and recognizable only as a tail on the liposome peak (cf. Fig. 1). More than 90% of (^{14}C)DSPC and 2/3 of the (^{3}H)BSA in plasma were recovered as

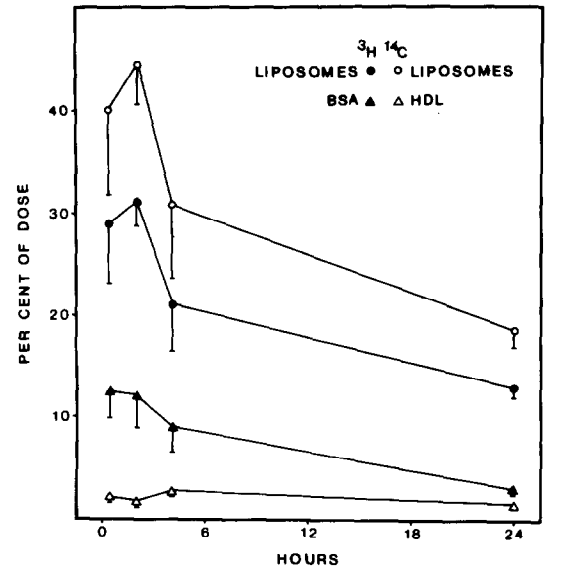

Fig. 2. Distribution of intact liposomes, ^{14}C-labeled HDL, and released BSA in plasma of rats given i.v. injections of DSPC : CHOL (2 : 1), BSA liposomes. Chromatographic separation carried out as stated in Fig. 1. Results are mean values ± S.D. for 3 animals.

intact liposomes for up to 24 hr, at which point 22% of the ^{14}C label and 20% of the tritium dose still circulated in plasma as intact liposomes. The half-lives for the lipid label and the entrapped BSA were 21 and 20 hr when assuming a monoexponential decay and taking into account all observations compared to that of 12 hr in mice [14, 33]. The plasma concentration of liposomes was lower 20 min after injection than 2 hr after (not statistically significant). A similar lower level was seen 30 min after injection to mice [33] but we have no explanation for this observation.

Low molecular weight forms of the phospholipid marker did not appear, while the amount of (^{3}H)BSA metabolites increased to 11% at 24 hr post injection.

Plasma from rats used for the liver cell isolation studies contained similar proportions of intact liposomes, labelled HDL, free BSA and low molecular weight metabolites of BSA. The total amount of radioactivity in plasma was, however, somewhat higher in these studies 20 min after injection and in those from the *in-vivo* stability studies [33]. Taking all data into account, the difference seems to have been due to a low recovery of radioactivity in the plasma from 2 of the 3 rats killed 20 min after injection for the analysis of plasma and tissue distribution of liposome labels.

Net tissue uptake of liposome markers

The correction of tissue uptake data for the contribution of radioactivity due to contaminating blood was based upon the content of rat albumin in tissues and in plasma. Since we have found no binding of liposomes to blood cells, this type of correction takes into full account the liposomes circulating freely in the vascular bed. It does not include liposome markers adsorbed to tissue cells and such material will therefore be accounted for as the net tissue uptake. The relative contribution of binding vs intracellular uptake will be discussed later.

The correction for the amount of blood present in a tissue is carried out individually and will always yield proper values, even with wide variations in the bleeding procedure. However, liver is an exception, since albumin is synthesized in this organ and some intracellular albumin and immunoreactive albumin precursors will inevitably be codetermined with the plasma albumin. Studies on the albumin content in liver homogenates from buffer perfused livers have shown the equivalent of 5 ± 1 μl plasma/g liver after perfusion, i.e. intracellular RSA, vs 57 ± 17 μl plasma/g liver under standard conditions. Under the conditions in this study, with a moderate liver uptake and high plasma concentrations of liposomes, this overcorrection for the plasma content will be 1% of the net liver uptake at the most.

Table 1 shows the relative organ uptake of the liposome markers at 2 hr. Only liver, spleen, heart, kidneys and intestines showed any appreciable uptake in relation to their relative contribution to the body weight. In thymus, stomach and colon we found no net uptake at all. Compared to data published for tissue distributions, not corrected for blood contents [14], our results show a fair correlation only to liver, spleen and kidneys, the organs with the highest amounts of uptake per gram tissue, and

Table 1. Relative uptake of DSPC : CHOL, BSA liposome markers in various tissues of rats

Tissue*	^{14}C	^{3}H
Cerebrum	0.05 ± 0.03	0.08 ± 0.01
Cerebellum	0.17 ± 0.12	0.31 ± 0.24
Heart	0.41 ± 0.27	0.41
Lungs	1.02 ± 0.77	0.95 ± 0.22
Diaphragm	1.93†	1.94†
Liver	11.07 ± 3.77	5.20 ± 2.32
Spleen	30.89 ± 7.44	18.94 ± 7.42
Adrenals	2.16 ± 1.44	1.24 ± 0.85
Kidneys	0.37 ± 0.10	0.41 ± 0.23
Small intestine	0.64†	0.15†

* Other tissues, i.e. thymus, stomach, colon, or the contents of stomach and intestines showed no net uptake after correction for the plasma content.

† 2 animals only.

Accumulation ratios = (dmp/g tissue recovered) : (dmp/g body weight) determined 2 hr after i.v. injection of (^{14}C)DSPC : CHOL, (^{3}H)BSA liposomes. Mean values ± S.D. for 3 animals.

relatively modest correction. Thus the main cause of the indicated liposomal contents in the other organs was blood contamination [14].

Distribution in various organs was studied at various intervals after the intravenous administration of liposomes. Figure 3 gives the data for liver, spleen, kidneys, intestines and plasma recorded 20 min after injection and for up to 24 hr. As mentioned before, the recovery of plasma radioactivity 20 min after injection might have been erroneously low in two animals. The liver showed the most rapid uptake of liposome markers, containing 20% of the (^{14}C) dose and 13% of the (^{3}H) dose already at 20 min. Maximum amounts were found after 2 hr, somewhat later than the time reported for the uptake of multilamellar liposomes [11–13, 15, 17].

The spleen showed a steady uptake of liposomal markers containing 14.4 ± 2.0% of (^{14}C) and 9.5 ± 1.3% of (^{3}H) at 24 hr. This was somewhat unexpected, since a high uptake by the spleen is normally seen with MLV and LUV, and it is more pronounced after charged liposomes than after neutral vesicles [5, 8, 15, 17]. None of these studies were carried out with stable liposomes circulating in plasma for a long period of time, so that the maximum of uptake was found shortly after injection. With unilamellar vesicles of the same size, and the lipid composition of the present study, a less pronounced but slowly increasing uptake was observed in the mouse spleen [14, 42]

The amounts of liposome markers found in kidneys and in intestines were low throughout the 24 hr and probably represented the uptake of liposome degradation products rather than the uptake of intact liposomes [31, 33].

We did not collect urine or breath samples from these animals but studies in mice [31] have shown that ^{3}H from BSA is eliminated mainly via the urine while ^{14}C from DSPC was rapidly eliminated as $^{14}CO_2$ via the lungs (45% within 12 hr).

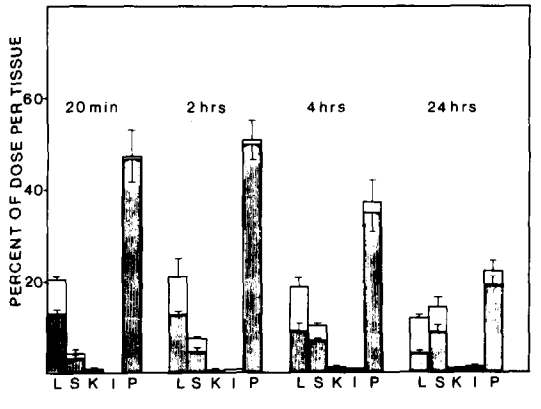

Fig. 3. Tissue distribution of liposome markers in rats in percent of the administered dose of L(^{14}C)DSPC : CHOL (2 : 1), (^{3}H)BSA liposomes. Empty columns = (^{14}C)DSPC derived label; hatched columns = (^{3}H)BSA derived label. Bars indicate S.D. exceeding 0.2% of total dose. Total plasma volume calculated as 42 μl/g body weight. Mean values for 3 animals. L = liver, S = spleen, K = kidneys, I = intestines, P = plasma.

Uptake in various liver cell fractions after injections in vivo

The procedure used to isolate parenchymal (PC) and non-parenchymal (NPC) cells from rat liver yielded a PC fraction containing 100% hepatocytes [37, 38], whereas the NPC fractions isolated by differential centrifugation consisted of a mixture of cells, predominantly endothelial cells (EC) with less Kupffer cells (KC) [37, 43]. NPC_1 cells prepared by the pronase method were free from hepatocytes and contained EC and KC in a ratio of 47 : 53. NPC_2 prepared by differential centrifugation contained less than 4% parenchymal cell protein plus EC and KC cells in a ratio of 7 : 1 [45].

Figure 4 shows the uptake of liposomal markers after intravenous injection of DSPC : CHOL, BSA liposomes in liver, in parenchymal and in non-parenchymal cells. The liver association was calculated on the basis of uptake in the caudate lobe measured immediately before the start of the collagenase perfusion but after 8 min of preperfusion with Ca^{2+}-free Hank's balanced salt solution and on the basis of a relative liver weight of 3.7% [40]. The uptake in non-parenchymal cells is given for NPC_2 cells after correction for the PC cell contamination, as described earlier [40].

Initially, total liver uptake increases with time for up to 2 hr, whereupon a decrease in liver radioactivity can be observed which accounts both for the liposomal lipid and for the protein content. In isolated non-parenchymal cells the uptake is relatively rapid and amounts, already at 40 min, to 66% of the maximum measured at 2 hr. Thereupon the uptake by non-parenchymal cells remains fairly constant. In contrast, the uptake by parenchymal cells is slower and shows an initial lag phase lasting for about 30 min. A maximum of uptake is noted at 2 hr, at which time about 24% of the total lipid and 6% of the ^{3}H-albumin are present in parenchymal cells, followed by a rapid decrease. The combined presence of parenchymal and non-parenchymal cells thus explains the total liver behaviour.

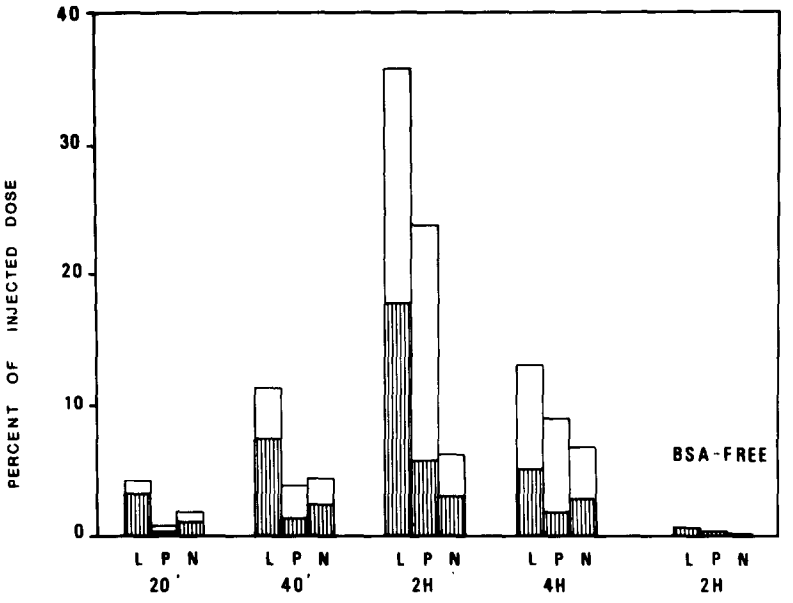

Fig. 4. Uptake of liposomal markers in liver and in isolated parenchymal and non-parenchymal cell fractions after i.v. injection of DSPC : CHOL, BSA liposomes. Mean values for 2 rats with essentially similar uptake. Maximal deviations of uptake from mean values given were 2.9% for both ^{14}C and ^{3}H for total liver uptake. Uptake in liver calculated from a relative liver weight of 3.7%. Parenchymal and non-parenchymal cells were supposed to represent 91.9% and 8.1% of total liver protein. Non-entrapped BSA was given in a dose of 75 μg/kg body weight, corresponding to the amount given in the entrapped form.

With the pronase method, which has been used earlier [20], parenchymal cells are destroyed and the subsequently isolated non-parenchymal cells may contain PC derived particles. Table 2 compares the radioactivity of the liposomal membrane (^{14}C) and the albumin content (^{3}H) of two types of non-parenchymal cells separated from a common PC source by centrifugation only (NPC_2) or by pronase incubation (NPC_1). In pronase-treated cells, the uptake of both liposomal markers in NPC was quite similar to that of cells obtained by differential centrifugation when the cells were isolated 20 min after intravenous injection. At that time, the uptake in parenchymal cells was quite low. However, 2 hr after injection, the pronase-treated cells contained 3–5 times more radioactivity per mg cell protein than the cells isolated by differential centrifugation, the difference being most pronounced for the phospholipid marker. Thus, total recoveries calculated on the basis of NPC_1 cells at 2 and 4 hr exceeded the total amount in liver, calculated from the uptake in the caudate lobe.

Table 2. Uptake of liposomes in liver and in isolated liver cell fractions

	20 min		120 min	
	^{14}C	^{3}H	^{14}C	^{3}H
Liver (after 8 min. perfusion)	4.1	3.2	35.7	17.8
PC	0.7	0.3	23.8	5.8
NPC_1 (pronase incubation)	2.2	0.3	34.6	10.3
NPC_2 (centrifugation only)	1.8	1.0	6.1	3.0

Calculated total uptake of liposomal markers in %-dose in rat liver and in isolated liver cell fractions after intravenous administration of L(DSPC : CHOL, 2 : 1), BSA liposomes containing (^{14}C)DSPC and (^{3}H)BSA at a dose of 50 μmol phospholipid per kg body weight. NPC_1 cells were prepared by pronase treatment. NPC_2 cells were obtained by differential centrifugation and the uptake therein corrected for the presence of parenchymal protein. Total uptakes were calculated as stated in the legend to Fig. 4.

Since this discrepancy between NPC preparations appeared only when the uptake of liposomal markers into hepatocytes became significant, we suggest that the high uptake in NPC_1 is due to *in-vitro* uptake of liposomes set free from broken hepatocytes during the 1 hr of treatment of the mixed cell population with pronase. Such phagocytic activity has been observed as an accumulation of the cell debris in Kupffer cell lysosomes [43] and unpublished observations indicate that *in-vitro* uptake of liposomes by non-parenchymal cells occurs readily. For particles that are not destroyed by pronase, such redistribution leads to an overestimation of the NPC_1 contribution.

In a recent study on the uptake of positively charged liposomes of different sizes in hepatocytes and non-hepatocytes isolated from collagenase perfused mice livers by a Percoll gradient centrifugation, both hepatocytes and Kupffer cells were largely undamaged and took up liposomes [25]. The small liposomes were taken up to a lesser extent in Kupffer cells and accumulated in hepatocytes instead, as seen from the specific uptake per cell. Unfortunately the

Table 3. Effect of size of liposomes on cell specific uptake in rat liver

DSPC CHOL (2:1), BSA diameter (no. of rats)	PC fraction		NPC_2 fraction		Liver (lobe)		Preperfusion		Collagenase perfusion	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
d_z = 112 nm (n = 7)	9.2 ± 1.9	4.1 ± 1.1	24.5 ± 5.0	14.6 ± 2.7	22.8 ± 3.1	16.7 ± 2.3	7.1 ± 1.7	7.1 ± 1.5	1.7 ± 0.4	1.8 ± 0.5
d_z = 75 nm (n = 3)	24.4 ± 2.7	6.6 ± 0.1	6.8 ± 2.8	5.2 ± 2.8	29.8 ± 3.2	17.2 ± 1.6	6.1 ± 0.3	6.0 ± 0.8	2.9 ± 0.3	3.0 ± 0.9

Male Wistar rats were given intravenous injections of DSPC : CHOL (2 : 1), BSA in a dose corresponding to 20 μmol phospholipid per kg body weight and liver cells were isolated 2 hr later. The original liposomes had an average diameter of d_z = 112 ± 5 nm, as determined by dynamic light scattering [34], and they were used either as such or after 60 min centrifugation at 40,000 rpm in a Beckman SW65 rotor [47], which reduced the average diameter to d_z = 75 ± 3 nm at the cost of approx. 65% of the liposome lipid and entrapped BSA. The small liposomes were injected as recovered in 10 mM Tris buffer, pH 7.4. Uptake in percent of administered dose in liver and liver cell fractions, calculated as described in the legend to Fig. 4 (mean ± S.E.). The recovery of liposome labels in the preperfusion and collagenase perfusion effluents is given, too.

total uptake in each fraction was not reported, but a rough estimate from the relative contribution of each cell fraction [43] showed that the total mass of hepatocytes took up as much liposome entrapped material as did the total mass of non-hepatocytes [25].

We have compared the cell specific uptake of DSPC : CHOL (2 : 1), BSA liposomes of different sizes (Table 3). For some unknown reason one liposome batch of this type had a larger mean diameter and, when we used it for cell isolation experiments, the uptake into parenchymal cells was significantly smaller while that in non-parenchymal cells was higher than found in Fig. 4. By ultracentrifugation [47] at 40,000 rpm in a Beckman SW 65 rotor for 1, 2 and 4 hr we could reduce the mean size of these liposomes to 75 ± 3, 71 ± 2, and 66 ± 1 nm (d_z, dynamic light scattering), but the recoveries of the liposomes were only 35 ± 1, 19 ± 1, and 6 ± 0.5%.

The uptake of liposomes centrifuged for 1 hr was higher in hepatocytes than in non-parenchymal cells, while the opposite was true of the original (larger) liposomes.

Because of the loss of liposomes during centrifugation, we injected only 20 mmole of phospholipid per kg body weight to these animals. However, this should have increased the relative uptake in non-parenchymal cells while leaving the relative uptake in parenchymal cells unchanged, as observed in parallel studies [31].

We have measured the uptake in both parenchymal and non-parenchymal cells isolated from the same livers. Believing that the uptake in cells isolated as the NPC_2 fraction represented the *in vivo* uptake in non-parenchymal cells, we found that the sum of PC and NPC_2 uptake did not fully account for the calculated liver uptake. The difference of a few per cent of the injected dose is probably due to detachment of adsorbed liposomes by collagenase during the initial perfusion, which has been shown in parallel studies [31]. Table 3 gives an example from some of our studies.

In comparing the liver uptake determined without perfusion but with correction for plasma content (Fig. 3) with the uptake obtained after perfusion of the liver (Fig. 4) it became evident that part of the liposomes found in the liver shortly (20 min) after injection could be removed by preperfusion with a Ca^{2+}-free Hank's balanced salt solution. This portion probably corresponded to the quantity of liposomes loosely adsorbed to liver cells [27].

GENERAL DISCUSSION

Targeting of liposomes to the liver is not difficult, since liposomes seem to have a natural affinity for this organ, as shown in a number of studies [1–3, 5–21, 25, 31, 33, 42]. Liposomes also accumulate in the spleen, another organ with a high phagocytotic activity [5, 8–10, 14, 15, 18, 31, 33, 42]. The RES involvement is seen more clearly from studies showing that all types of liposomes are taken up, to various degrees, in Kupffer cells, but that only certain types of liposomes are capable of any significant uptake by parenchymal cells [6, 19, 22, 24, 25]. Two

conditions are prerequisite for the transport of entrapped material to hepatocytes. First, the liposomes have to be small enough to pass the fenestrae of the endothelial lining and, second, they have to be stable in plasma.

The normal liposomes used in our study fulfilled both criteria, since > 90% of the vesicles had diameters smaller than the typical size of the endothelial fenestrae, viz. 100 nm, and they remained intact and circulated with a half-life approaching one day (for those not taken up during the initial distribution/capture phase).

Although these liposomes were very stable in plasma, they did not seem to convey the entrapped BSA into the cells in the same proportion as the phospholipid membrane marker. This was observed with the uptake both in total liver and in the isolated cell fractions, where hepatocytes, in particular, showed a low capture ratio for BSA. This could have been due to a higher catabolism of BSA than that of the lipid marker, or it could have been caused by leakage of BSA from liposomes during their interaction with the cell membranes. The latter phenomenon has been described for isolated hepatocytes interacting with 6-carboxyfluorescein containing liposomes [44]

The fact that the relative uptake of BSA was diminishing with time could be explained by both hypotheses. The capacity to degrade albumin is, however, present in both parenchymal and non-parenchymal cells with similar activities expressed per cell [46]. Furthermore, the absorption-induced leakage explanation is strengthened by the observation that the uptake ratio is closer to 1 in the pre-perfusion and collagenase perfusion effluents, the liver lobe and in nonperfused livers, including adsorbed intact liposomes (Table 3, Figs. 3 and 4), than in the perfused liver or subsequently isolated cells.

Therefore, a cellular difference in albumin degradation cannot explain the observed difference in efficiency to deliver ^{3}H-albumin to parenchymal and non-parenchymal cells.

The uptake of the small unilamellar liposomes shows a clear-cut lag phase (approx. 30 min) while no such lag phase is observed with the non-parenchymal cells. Our data thus confirm the observed lag period for the uptake of liposomes by hepatocytes found by electron microscopy after injection of liposomes of heterogeneous size distribution [20–22]. A slower uptake in hepatocytes than in Kupffer cells was also observed with DPPC : CHOL : stearylamine (3.3 : 2.3 : 1) liposomes of approx. 80 nm mean diameter [25]. Our data also show that the size and stability of liposomes are important determinants for a high capture of both the liposomal phospholipids and the entrapped molecules, since we had a higher uptake of BSA with our 70 nm diameter vesicles than with the 112 nm vesicles.

Non-parenchymal liver cells are exposed directly to blood and can therefore interact with the liposomes immediately after injection. In order to reach the hepatocytes, however, the liposomes have to pass through the fenestrae in the endothelial cell lining, and since the size of the liposomes is close to that of the fenestrae, the time needed to reach equilibrium between the blood sinusoid and the space of Disse may delay the interaction with hepatocytes. No such lag phase was observed when the liposomes employed in this study were incubated *in vitro* with cultured hepatocytes [31] or with freshly isolated hepatocytes (unpublished data).

It is also possible that liposomes are initially taken up by non-parenchymal cells and subsequently transported to parenchymal cells, as suggested earlier [6, 23]. However, such a mechanism does not explain why liposomes of a size < 100 nm are able to transport significant amounts of entrapped protein to the parenchymal cells (as shown here) while bigger liposomes in general are not [23–25, 31]. Therefore we conclude that, in view of the liver architecture, the liposomes needed to transport significant amounts of entrapped protein to parenchymal cells must be < 100 nm in diameter. The use of such small liposomes for the transport of metabolic effectors to cells exerting important liver functions, such as glucose synthesis and drug detoxification, may open the possibility of modulating these important processes.

REFERENCES

1. D. A. Tyrell, T. D. Heath, C. M. Colley and B. E. Ryman, *Biochim. biophys. Acta* **457**, 259 (1976).
2. H. K. Kimelberg and E. Mayhew, *Crit. revs. Toxicol.* **6**, 25 (1978).
3. G. Gregoriadis, in *Drug Carriers in Biology and Medicine* (G. Gregoriadis), pp. 287–341. Academic Press, London (1979).
4. R. L. Juliano and D. Stamp, *Biochem. biophys. res. Comm.* **63**, 651 (1975).
5. P. Sharma, D. A. Tyrell and B. E. Ryman, *Biochem. Soc. Trans.* **5**, 1146 (1977).
6. F. H. Roerdink, E. Wisse, H. W. M. Morselt, J. Van der Meulen and G. L. Scherphof, in *Kupffer Cells and Other Liver Sinusoidal Cells* E. Wisse and D. L. Knook, pp. 263–272. Elsevier/North-Holland Biomedical Press, Amsterdam.
7. G. H. Hinkle, G. S. Born, W. V. Kessler and S. M. Shaw, *J. Pharmac. Sci.* **67**, 795 (1978).
8. V. J. Richardson, K. Jeyasingh, R. F. Jewkes, B. E. Ryman and M. H. N. Tattersael, *J. Nucl. Med.* **19**, 1049 (1978).
9. Y. J. Kao and R. L. Juliano, *Biochim. biophys. Acta* **677**, 453 (1981).
10. R. M. Abra and C. A. Hunt, *Biochim. biophys. Acta* **666**, 493 (1981).
11. G. Gregoriadis, *FEBS Lett.* **36**, 292 (1973).
12. T. Tanaka, K. Taneda, H. Kobayaski, K. Okumura, S. Muraniski and H. Sczaki, *Chem. pharm. Bull.* **23**, 3069 (1975).
13. A. Surolia and B. K. Bachhawat *Biochim. biophys. Acta* **497**, 760 (1977).
14. M. R. Mauk and R. C. Gamble, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 765 (1979).
15. G. Gregoriadis and D. E. Neerunjun, *Eur. J. Biochem.* **47**, 179 (1974).
16. D. S. Deshmukh, W. D. Bear, H. M. Wisniewski and H. Borckerhoff, *Biochem. biophys. res. Comm.* **82**, 328 (1978).
17. G. Gregoriadis and B. E. Ryman, *Eur. J. Biochem.* **24**, 485 (1972).
18. R. L. Souhami, H. M. Patel and B. E. Ryman, *Biochim. biophys. Acta* **674**, 354 (1981).
19. A. W. Segal, E. J. Wills, J. E. Richmond, G. Slavin, C. D. V. Black and G. Gregoriadis, *Br. J. exp. Path.* **55**, 320 (1974).

20. Y. E. Rahman and B. J. Wright, *J. Cell Biol.* **65**, 112 (1975).
21. T. DeBarsy, P. Devos and F. Van Hoof, *Lab. Invest.* **34**, 273 (1976).
22. E. Wisse, G. Gregoriadis and W. T. Daems, in *Reticuloendothelial System in Health and Disease: Functions and Characteristics* (Eds. S. M. Reichard, M. R. Escobar and H. Friedman), pp. 237–245. Plenum Publishing Corporation, New York, (1976).
23. F. H. Roerdink, J. Dijkstra, G. Hartman, B. Bolscher and G. Scherphof, *Biochim. biophys. Acta* **677**, 79 (1981).
24. P. Ghosh, P. K. Das and B. K. Bachhawat, *Archs biochem. Biophys.* **213**, 266 (1982).
25. Y. E. Rahman, E. A. Cerny, K. R. Patel, E. H. Lau and B. J. Wright, *Life Sciences* **31**, 2061 (1982).
26. G. Gregoriadis and B. E. Ryman, *Biochem. J.* **129**, 123 (1972).
27. G. Gregoriadis, D. Putman, L. Louis and D. Neerunjun, *Biochem. J.* **140**, 323 (1974).
28. G. Scherphof, F. Roerdink, M. Waite and J. Parks, *Biochim. biophys. Acta* **542**, 296 (1978).
29. G. Gregoriadis and C. Davies, *Biochem. biophys. res. Comm.* **89**, 1287 (1979).
30. D. Hoekstra, R. Tomasini and G. Scherphof, *Biochim. biophys. Acta* **542**, 456 (1978).
31. C. Gotfredsen, Thesis, Catholic University of Louvain, Brussels, Belgium (1982).
32. P. Guiot, P. Baudhuin and C. Gotfredsen, *J. Microscopy* **120**, 159 (1980).
33. C. F. Gotfredsen, S. Frøkjær, E. L. Hjorth, K. D. Jørgensen and M.-C. Debroux-Guisset, *Biochem. Pharmac.* **32**, 3381 (1983).
34. S. Frøkjær, E. L. Hjort and O. Wørts, in *Optimization of Drug Delivery Systems* (Eds. H. Bundgaard, A. Bagger Hansen and H. Kofod), Munksgaard, Copenhagen (1982). pp. 384–397.
35. Batelle Memorial Institute, British Patent Appl. No. 2001929 A (1979).
36. R. H. Rice and G. E. Means, *J. Biol. Chem.* **246**, 831 (1971).
37. L. D. Lee, H. P. Baden and C. K. Cheng, *J. Immunol. Meth.* **24**, 155 (1978).
38. G. Mancini, A. O. Carbonara and J. F. Heremans, *Immunochemistry* **2**, 235 (1965).
39. Th. J. C. Van Berkel, J. K. Kruijt, R. G. Slee and J. F. Koster, *Archs biochem. Biophys.* **179**, 1 (1977).
40. Th. J. C. Van Berkel and A. Van Tol, *Biochim. biophys. Acta* **530**, 299 (1978).
41. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
42. M. R. Mauk, R. C. Gamble and J. D. Baldeschwieler, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4430 (1980).
43. D. L. Knook and E. C. L. Sleyster in *Kupffer Cells and Other Liver Sinusoidal Cells* (Eds. E. Wisse and D. L. Knook), pp. 273–288. Elsevier/North-Holland Biochemical Press, Amsterdam (1977).
44. A. J. B. M. Van Renswoude, Ph.D. Thesis, State University of Groningen, pp. 65–85.
45. H. Jansen, Th. J. C. Van Berkel and W. C. Hülsmann, *Biochim. biophys. Acta* **619**, 119 (1980).
46. A. Van Tool and Th. J. C. Van Berkel, *Biochim. biophys. Acta* **619**, 156 (1980).
47. Y. Barenholz, D. Gibbes, B. J. Litman, J. Goll, T. E. Thompson and F. D. Carlson, *Biochemistry* **16**, 2806 (1977).