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# Liposome Disposition *In Vivo* VI: Delivery to the Lung

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**Abstract** □ The effect of negatively charged liposome components and vesicle size on the time course and dose dependency of liposome disposition in mice was studied with a view to optimizing liposome delivery to the lung. The disposition of large multilamellar liposomes was followed using $^{125}$I-labeled *p*-hydroxybenzamidine phosphatidyl ethanolamine. Of the three negatively charged liposome compositions studied (phosphatidyl choline–X–cholesterol–α-tocopherol, molar ratio: 4:1:5:0.1; X = phosphatidyl serine, dipalmitoyl phosphatidic acid, or phosphatidyl glycerol), phosphatidyl serine liposomes resulted in the greatest accumulation in lungs. Lung levels decreased up to 95 h postdose, at which time 6% of the liposome dose present at 2 h still remained. The disposition of phosphatidyl serine-containing liposomes was independent of dose for the range 0.04–21 μmol/animal. When liposomes containing phosphatidyl choline were prepared using a variety of extrusion and dialysis conditions, a strong link between liposome size and lung accumulation was revealed. A maximum lung accumulation of 30.9% of the administered dose was achieved with no detectable gross pathological lung lesions up to 24 h postdose.

**Keyphrases** □ Liposomes—multilamellar, phosphatidyl serine, disposition *in vivo*, delivery to the lung, mice □ Disposition—multilamellar liposomes, *in vivo*, delivery to the lung, mice, phosphatidyl serine □ Phosphatidyl serine—multilamellar liposome disposition *in vivo*, delivery to the lung, mice

Liposomes may act as drug carriers (1, 2) but some degree of target specificity is necessary to maximize the therapeutic index of the drug. Intravenously administered liposomes generally become associated with organs of the reticuloendothelial system, mainly the liver and spleen (3, 4).

The lungs, because of their susceptibility to disease, *e.g.*, metastatic cancer (5), are a suitable target organ for attempts to localize drugs. Liposome preparations in common use generally do not accumulate in the lung to any significant extent after intravenous administration (6, 7). It is known, however, that liposome doses containing vesicles of ≥1-μm diameter exhibit improved localization in the lungs compared with smaller diameter preparations (8–10). This effect has been attributed to simple mechanical trapping in the capillary bed of the lungs (9). Liposomes bearing either positive (11) or negative (6, 10) surface charge accumulate in the lungs to a greater extent than neutral liposomes of similar size. However, there is some evidence that positively charged liposomes containing stearylamine may be toxic *in vivo* (12). Attempts have been made to deliver liposomes to the lungs *via* routes other than a distant intravenous site, *e.g.*, the ear vein of rabbits (13) and intratracheal instillation (14). Such approaches can have only specialized applications.

We have examined the effects of negatively charged liposome components and vesicle size on the time course and dose dependency of liposome disposition with a view to optimizing liposome delivery to the lungs. These results will be utilized for directing antitumor drugs to the lungs.

## EXPERIMENTAL

**Chemicals**—Purified egg yolk phosphatidyl choline and phosphatidyl glycerol were prepared as previously described (15). Phosphatidyl serine[1], sodium dipalmitoyl phosphatidate[2], cholesterol[2], and α-tocopherol[2] were chromatographic grade. The method of Szoka and Mayhew (16) was used to synthesize the $^{125}$I-labeled *p*-hydroxybenzamidine phosphatidyl ethanolamine (45 mCi/mg), subsequently referred to as the $^{125}$I-marker. All other chemicals were at least reagent grade. The phosphate-buffered saline (pH 7.4) contained 92 mM sodium chloride, 43 mM anhydrous dibasic sodium phosphate, 11 mM monobasic sodium phosphate monohydrate, 100 USP U of penicillin, and 100 μg of streptomycin/mL. All buffer-containing solutions were routinely filtered through 0.22-μm pore size filters[3]. Dipalmitoyl phosphatidic acid was prepared by chloroform extraction of the sodium phosphatidate in 0.4 M HCl in 20% (v/v) methanolic aqueous solution.

**Preparation of Liposomes**—Liposomes were prepared as described previously (4). Three lipid compositions were used: (A) phosphatidyl choline, dipalmitoyl phosphatidic acid, cholesterol, and α-tocopherol; (B) phosphatidyl choline, phosphatidyl serine, cholesterol, and α-tocopherol; and (C) phosphatidyl choline, phosphatidyl glycerol, cholesterol, and α-tocopherol. In each case the molar ratio was 4:1:5:0.1. Sufficient lipid together with ~0.05 μCi of $^{125}$I-marker per experimental animal was dried and suspended in buffer by mechanical agitation, yielding large multilamellar liposomes. In most cases these liposomes were subsequently extruded through polycarbonate membranes[4] having 8-, 5-, 3-, 2-, and 1-μm diameter pore sizes to generate populations having different mean diameters (17).

Liposomes were dialyzed at 4°C in the dark against frequent changes of buffer, for ~2 d. Dialysis was carried out in 1-mL dialysis cells fitted with 25-mm polycarbonate membranes[5] with a variety of pore sizes (specified below) to remove traces of dialyzable iodine-125 and some liposomes of smaller diameter than the membrane pores (17).

The final total lipid concentration was estimated by phosphorus assay (18) of extracted samples (19) and then corrected for the presence of non-phosphorus-containing lipids. Vesicle diameters were examed by electron microscopy following negative staining (4).

[1] Avanti Biochemicals Inc., Birmingham, Ala.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Millipore Corp., Bedford, Mass.
[4] Bio-Rad Labs, Richmond, Calif.
[5] Nucleopore, Bio-Rad Labs, Richmond, Calif.

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**Table I—Effect of Extrusion Pore Size and Dialysis Treatment on the Various Liposome Batches**

| Batch | Extrusion Pore Size, μm | Dialysis Pore Size, μm | Total Lipid, μmol/mL | Loss of Liposome Material on Dialysis, %[a] |
|---|---|---|---|---|
| 1 | 8 | 5.0 | 18.8 | 54 |
| 2 | 8 | 0.8 | 27.1 | — |
| 3 | 5 | 3.0 | 19.8 | 42 |
| 4 | 5 | 0.8 | 31.0 | — |
| 5 | 3 | 2.0 | 22.0 | 12 |
| 6 | 3 | 0.8 | 25.5 | — |
| 7 | 2 | 1.0 | 28.6 | 0 |
| 8 | 2 | 0.8 | 27.3 | — |
| 9 | 1 | 0.8 | 18.4 | 26 |
| 10 | 1 | 0.2 | 25.0 | — |

[a] Expressed as total radioactivity remaining after dialysis *versus* larger pore size membrane divided by total radioactivity remaining after dialysis *versus* smaller pore size membrane.

***In Vivo* Disposition Studies**—All experiments used male ICR mice[6] weighing 19–23 g. At the end of an experimental period, mice were lightly anesthetized with ether, and a 1-mL blood sample was rapidly removed from the jugular vein into an heparinized syringe. Livers, spleens, and lungs were subsequently removed, weighed, and stored at −20°C together with the remaining carcass. The bladder and contents were discarded prior to storage. Four experiments were performed. The mean weight of lungs sampled during these experiments was 0.16 ± 0.03 g ($n$ = 83).

*Experiment 1*—To assess the effect of liposomal lipid composition on disposition, three batches of liposomes (A, B, and C) containing the $^{125}$I-marker were prepared. Each batch was divided, and one-half extruded through an 8.0-μm pore size polycarbonate membrane. All six doses thus prepared were dialyzed *versus* 0.8-μm pore size polycarbonate membranes. Final total lipid concentrations were: (A) 32.5 μmol/mL, extruded 28 μmol/mL; (B) 45 μmol/mL, extruded 45.5 μmol/mL; and (C) 43.5 μmol/mL, extruded 36 μmol/mL. Groups of three mice each received 0.2-mL iv injections of liposome suspension *via* a tail vein and were sacrificed 4 h later.

*Experiment 2*—To establish the time course for the disposition of composition B liposomes, liposomes were prepared at 115 μmol of total lipid/mL and dialyzed *versus* 0.8-μm pore size polycarbonate membranes. Mice each received 0.2-mL iv injections, and groups of three mice were killed after 2, 5, 9.2, 24, and 94.8 h.

*Experiment 3*—To study the dose dependency of composition B liposomes, liposomes were prepared as described above at 105 μmol of total lipid/mL. Doses of 105, 33.5, 17, 13, and 0.2 μmol/mL were generated by serial dilution with buffer. Groups of three mice each received 0.2-mL iv injections and were sacrificed 4 h later.

*Experiment 4*—The effects of extrusion pore size and dialysis treatment on disposition were investigated for composition B liposomes. A batch of the liposomes was prepared as described above at a concentration of ~25 μmol/mL. The liposomes were then extruded twice at each pore size through a sequential series of 8.0-, 5.0-, 3.0-, 2.0-, and 1.0-μm pore size polycarbonate membranes. After each extrusion a sample was retained and, in this way, five batches of liposomes of different size were created. Each batch was then divided in half and dialyzed as follows: 8.0-μm pore size extruded, dialyzed *versus* either 5.0- or 0.8-μm pore size membranes; 5.0-μm pore size extruded, dialyzed *versus* either 3.0- or 0.8-μm membranes; 3.0-μm pore size extruded, dialyzed *versus* either 2.0- or 0.8-μm membranes; 2.0-μm pore size extruded, dialyzed *versus* either 1.0- or 0.8-μm membranes; and 1.0-μm pore size extruded, dialyzed *versus* either 0.8- or 0.2-μm membranes. Final dose concentrations are given in Table I. Each of 10 groups of three mice received intravenous doses of one of these preparations and were sacrificed 3 h later.

**Analysis of Total Organ Radioactivity**—Whole liver, spleen, lungs, 0.05-mL $^{125}$I-labeled doses, and ~0.8 mL of blood were assayed for radioactivity by placing them in vials and counting in a γ-radiation counter[7]. Carcasses were cut into conveniently sized pieces and then assayed as above. Values for sample (cpm) were converted to percent of administered dose and a mean was calculated for the three animals in each group. Carcass values were corrected for blood volume remaining after sampling (4). Total radioactivity remaining *in vivo* was estimated by summation of the liver, spleen, total blood volume, lungs, and carcass values.

[6] Simonsen Laboratories Inc., Gilroy, Calif.

[7] Gamma 300; Beckman Instruments Inc., Mountain View, Calif.

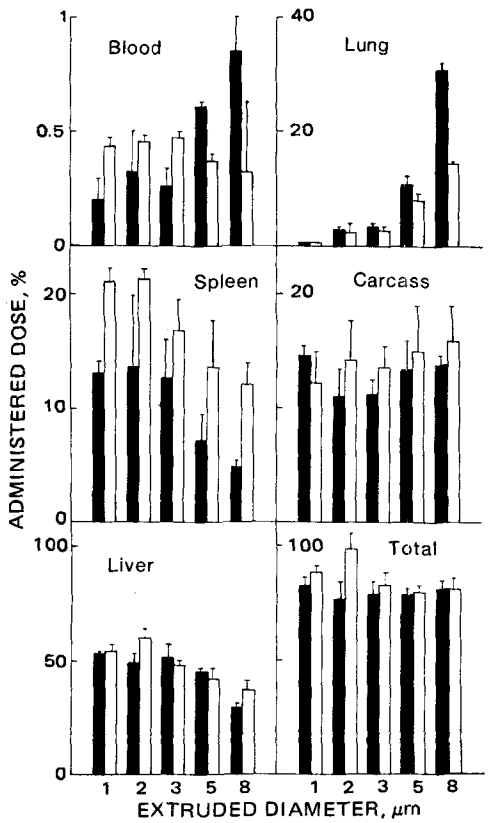


**Figure 1**—*Percentages of the administered dose (iodine-125) in blood (per milliliter), liver, lungs, spleen, carcass, and total remaining* in vivo *3 h after administration of composition B liposomes containing the $^{125}$I-marker and prepared according to Table I. The histograms represent the mean of three mice for each extrusion pore diameter. Shaded bars represent liposomes dialyzed* versus *a membrane of pore size close to their extrusion pore size; clear bars represent liposomes dialyzed* versus *a membrane of pore size much smaller than their extrusion size. The vertical lines show one* SD.

**Pathology**—To examine the effect of liposomes on lung structure, groups of three 24-g mice[8] were injected intravenously with doses of 20.4 μmol of total lipid/animal (846 μmol/kg) of composition B liposomes. Three untreated mice served as controls. Two doses were prepared: one (lung-accumulating) was extruded through 8.0-μm pore size membranes and dialyzed against a 5.0-μm pore size membrane and the other (non-lung-accumulating) was extruded at 1.0-μm pore size and dialyzed against a 0.8-μm pore size membrane. Groups of mice were killed 1, 5, and 24 h postdose; their lungs were removed, washed in saline, and fixed overnight in 10% formalin. The lung samples were then dehydrated, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (20). Sections from coded animals were examined under the light microscope for signs of damage; the nature of the treatment group was unknown at the time of examination.

## RESULTS

**Effects of Liposomal Lipid Composition on Disposition**—The results of this experiment are given in Table II. All three compositions gave similar overall patterns of disposition, with the majority of the radiolabel residing in liver and carcass. Liposomes containing phosphatidyl serine accumulated in the lungs to a level four times that of phosphatidic acid-containing liposomes. Phosphatidyl glycerol-containing liposomes gave an intermediate level. This shift in disposition towards the lungs was at the expense of spleen and blood levels. All three doses were equally stable as judged by total radioactivity remaining *in vivo*. The extrusion treatment had no effect on radiolabel disposition. Electron micrographs taken of negatively stained liposomes failed to detect any differences in size distribution between the doses, but highlighted their extremely heterogeneous nature. Liposomes containing phosphatidyl serine (composition B) were chosen for all further experiments.

[8] CD-1; Charles River Breeding Laboratories, Wilmington, Mass.

**Table II—Liposome Disposition *In Vivo* 4 h Postdose**

| Composition (mole ratio)[a] | Dose[b], μmol/animal | Disposition, % of Administered Dose[c] | | | | |
|---|---|---|---|---|---|---|
| | | Blood[d] | Liver | Spleen | Lungs | Total *In Vivo*[e] |
| PC/PA/CH/α-T | 6.5 (U) | 4.0 ± 2.1 | 23.2 ± 3.6 | 12.2 ± 4.4 | 4.8 ± 0.5 | 73.8 ± 5.3 |
| (4:1:5:0.1) | 5.6 (E) | 4.0 ± 2.0 | 25.0 ± 6.6 | 12.9 ± 3.2 | 4.6 ± 0.9 | 83.4 ± 4.4 |
| PC/PS/CH/α-T | 9.0 (U) | 1.0 ± 0.2 | 25.6 ± 1.4 | 9.3 ± 1.9 | 18.1 ± 2.1 | 81.0 ± 3.4 |
| (4:1:5:0.1) | 9.1 (E) | 1.2 ± 0.3 | 30.3 ± 3.6 | 9.7 ± 1.2 | 16.9 ± 1.0 | 86.6 ± 1.1 |
| PC/PG/CH/α-T | 8.7 (U) | 5.7 ± 2.9 | 31.7 ± 4.0 | 11.1 ± 0.7 | 9.5 ± 2.9 | 89.3 ± 5.4 |
| (4:1:5:0.1) | 7.2 (E) | 3.6 ± 1.9 | 34.7 ± 4.4 | 16.5 ± 4.1 | 7.8 ± 1.5 | 94.0 ± 4.5 |

[a] Key: (PC) phosphatidyl choline; (CH) cholesterol; (α-T) α-tocopherol; (PA) dipalmitoyl phosphatidic acid; (PS) phosphatidyl serine; (PG) phosphatidyl glycerol. [b] Liposomes were prepared and dialyzed without extrusion (U) or were extruded through an 8-μm pore size membrane prior to dialysis. Quantitation was *via* the $^{125}$I-marker. [c] Mean ± *SD*. [d] Per milliliter. [e] Calculated by summation of values for total blood volume, whole liver, spleen, lungs, and carcass.

**Time Course for Disposition of Composition B Liposomes**—Results are displayed in Table III. All organ levels of radioactivity were maximal at 2 h postdose and declined continuously up to 94.8 h postdose. The values for total radioactivity remaining *in vivo* reflected a net loss of radioactive label during this period.

**Dose Dependency**—Table IV gives the results for composition B liposomes. In the dose range of 0.04 to 21 μmol of total lipid/animal (1.7–871 μmol/kg) there was no apparent dose effect for blood, liver, or lung 4 h after dosing. However, there was a consistent increase in the percent associated with spleen for increasing dose. The *in vivo* stability of the dose (as estimated by total radioactivity remaining *in vivo*) was unaffected by dose.

**Effect of Extrusion Pore Size and Dialysis Treatment on Disposition of Composition B Liposomes**—Increasing the extrusion pore size led to an increase in accumulation of the radiolabel in the lungs and a decrease in the spleen (Fig. 1). Putative liposome size had little effect on levels in the blood, liver, carcass, or on total radioactivity remaining *in vivo*. The disposition of doses dialyzed *versus* membranes of a pore size close to that through which they were extruded (doses 1, 3, 5, 7, and 9; Table I) was markedly higher in the lungs than those doses dialyzed *versus* small pore size membranes (doses 2, 4, 6, 8, and 10; Table I). The reverse effect was noted in the spleen. A maximum lung accumulation of 30.9% of the administered dose (193.1 %/g wet weight) was achieved with 8.0-μm pore size extruded liposomes subsequently dialyzed *versus* a 5.0-μm pore size membrane. This contrasted with 0.5% of the dose (3.1%/g wet weight) for 1.0-μm pore size extruded liposomes dialyzed *versus* a 0.2-μm membrane.

Electron microscopic examination of these doses revealed, once again, a great heterogeneity of the vesicle size. In the fields examined, the largest diameter of a liposome noted for dose 1 (Table I) was 9.5 μm; for dose 3, 1.3 μm; for dose 5, 5.7 μm; and for dose 10, 2.0 μm.

**Pathology**—Light microscopy of stained lung sections from mice receiving one of the two liposome doses (20.4 μmol of total lipid either lung-accumulating or non-lung-accumulating) and the control group revealed no significant pathological features either within or between groups.

## DISCUSSION

We have utilized a liposomal lipid label (the $^{125}$I-marker) in order to follow the disposition of liposome doses *in vivo*. Abra *et al.* (7) have demonstrated that the use of such a label gives results equivalent to those obtained when inulin is used as an aqeuous space marker at times <5 h postdose. An important caveat is that blood levels are elevated for the lipid marker due to lipid exchange. We may therefore reasonably assume that radioactivity detected in organs other than blood, at least at 3–5 h postdose, for the protocols used here are indicative of the presence of liposomal lipid in the form of liposomes. We further assume, based on prior results (7), that we are observing a delivery of both liposomes and their contents at these early times. The question of subsequent liposome stability *in vivo*, which may be vital in the tailoring of liposomal properties to the pharmacokinetics of the encapsulated drug, cannot be answered with this kind of label. The degree to which tissue levels of the $^{125}$I-marker reflect intact liposomes, free $^{125}$I-marker, or its metabolites after ~5 h postdose is unknown.

It is immediately apparent that, of the negatively charged liposome compositions tested, composition B (phosphatidyl choline, phosphatidyl serine, cholesterol, α-tocopherol in the molar ratio of 4:1:5:0.1) gave the highest liposome levels in the lungs for a given preparation protocol. Fidler *et al.* (10) also found that phosphatidyl serine-containing liposomes accumulated in the lungs to a greater extent than phosphatidic acid-, ganglioside-, or dicetylphosphate-containing vesicles. The available data do not allow us to specify a mechanism accounting for this lung accumulation. Liposomes prepared identically but of different composition may in fact have different mean effective diameters. Phosphatidyl serine-containing liposomes may thus, on average, be larger than other negatively charged liposomes even though we were unable to detect such differences owing to the heterogeneity of the preparations. There may also be a specific, but unknown, interaction between phosphatidyl serine and the lung vasculature.

In the absence of any lung pathology, it seems unlikely that the larger liposomes actually form classical microemboli that block capillaries. It is more likely that liposomes bind to the capillary walls without completely blocking blood flow through that capillary. This binding may be dependent on two related variables, the transit time of liposomes through lung capillaries and the binding affinity between liposomes and lung capillary endothelial cells. As diameter increases, transit time (in the absence of binding) decreases. As diameter increases, the area available for endothelial cell contact increases and so the apparent binding constant (between liposomes and endothelial cells) increases per liposome. Thus, for some, but not all compositions, a critical diameter may be reached where prolonged attachment to endothelial cells will occur without capillary blockage. If this description is reasonable, then it follows that this critical diameter is smallest when the negative phospholipid is phosphatidyl serine, all other variables being constant.

There is no evidence that phosphatidyl serine-containing liposomes are toxic at doses of up to 20.4 μmol/mouse (864 μmol/kg). The $LD_{50}$ of intravenously administered liposomes composed solely of phospholipids and cholesterol has been estimated to be ~18 mmol/kg (21, 22).

The time course of accumulation and retention of the $^{125}$I-marker in the lungs is such that only 6% of the dose present in the lungs at 2 h remains by 95 h postdose. This remnant represents some 6% of the total liposome dose remaining *in vivo* at 95 h. We can not say if the $^{125}$I-marker remains associated with intact liposomes at that time. Our assumption is that by 95 h considerable loss to the tissues has occurred. The fate of liposomes and their contents delivered to the lung can include binding and prolonged erosion, lysis and release of contents extracellularly, uptake by the local tissues *via* endocytosis, and uptake of leaked liposomal contents by local diffusion or pinocytosis.

The only evidence for dose dependency is that spleen levels of the $^{125}$I-marker increase with increasing dose, consistent with previous studies of dose dependency using a similar dose range of smaller lipo-

**Table III—*In Vivo* Disposition of Composition B Liposomes[a]**

| Time Postdose, h | Disposition, % of Administered Dose[c] | | | | |
|---|---|---|---|---|---|
| | Blood[b] | Liver | Spleen | Lungs | Total *In Vivo*[d] |
| 2 | 1.3 ± 0.2 | 24.2 ± 1.8 | 10.3 ± 1.7 | 16.5 ± 6.3 | 70.7 ± 7.4 |
| 5 | 0.9 ± 0.2 | 23.2 ± 2.8 | 8.4 ± 1.8 | 15.3 ± 1.7 | 65.6 ± 4.4 |
| 9.2 | 0.3 ± 0.01 | 22.4 ± 2.3 | 7.7 ± 2.3 | 12.1 ± 0.5 | 52.5 ± 1.0 |
| 24 | 0.4 ± 0.01 | 19.7 ± 1.2 | 6.1 ± 1.3 | 7.5 ± 0.8 | 44.0 ± 0.4 |
| 94.8 | 0.1 ± 0.02 | 9.6 ± 0.5 | 2.5 ± 0.4 | 1.0 ± 0.02 | 17.4 ± 0.4 |

[a] Mice received 23.0 μmol of total lipid each. Liposomes were unextruded, but were dialyzed against a 0.8-μm pore size membrane. Quantitation was *via* the $^{125}$I-marker. [b] Per milliliter. [c] Mean ± *SD*. [d] Calculated by summation of values for total blood volume, whole liver, spleen, lungs, and carcass.

Table IV—Effect of Dose on *In Vivo* Levels of Composition B Liposomes 4 h Postdose [a]

| Liposome Dose μmol of total lipid/animal | Disposition, % of Administered Dose [c] | | | | |
|---|---|---|---|---|---|
| | Blood [b] | Liver | Spleen | Lungs | Total *In Vivo* [d] |
| 21 | 1.3 ± 0.5 | 24.2 ± 2.8 | 8.2 ± 1.6 | 23.5 ± 7.3 | 80.7 ± 15.5 |
| 6.7 | 1.2 ± 0,2 | 24.5 ± 1.3 | 7.1 ± 0.6 | 18.7 ± 1.1 | 69.8 ± 3.0 |
| 3.4 | 1.1 ± 0.4 | 25.8 ± 3.9 | 5.6 ± 1.6 | 15.0 ± 1.5 | 68.8 ± 2.3 |
| 2.6 | 1.3 ± 0.2 | 25.6 ± 1.8 | 4.4 ± 1.0 | 16.4 ± 1.9 | 67.4 ± 3.0 |
| 0.04 | 1.7 ± 2.0 | 31.7 ± 7.4 | 2.6 ± 0.9 | 15.8 ± 3.2 | 77.5 ± 4.5 |

[a] Mice received intravenous doses of liposomes that were unextruded, but dialyzed against a 0.8-μm pore size membrane. Quantitation was *via* the $^{125}$I-marker. [b] Per milliliter. [c] Mean ± *SD*. [d] Calculated by summation of values for total blood volume, whole liver, spleen, lungs, and carcass.

somes of composition A (4). The absence of a significant dose effect in other organs may be due, in part, to the presence in our doses of a whole range of liposome sizes which are able to interact with different organ binding sites (23).

The delivery of liposomes to the lungs clearly depends on liposome size, and in the absence of more definitive data, this may be due in some part to a requirement for microembolism formation to arrest liposomes in the lung capillaries (9, 10, 24). The composition data discussed above offer an alternative mechanism. Those liposome doses having a size distribution weighted toward the larger vesicles by dialysis against large pore size membranes (indicated by substantial losses of lipid during dialysis, see Table I) accumulate in the lung particularly well. Yet there is no evidence that such trapping in lung at doses of 846 μmol/kg results in any gross pathological damage. Our maximum delivery to lung, 30.9% of the administered dose or 193.1%/g wet weight, which was achieved with liposomes prepared by protocol 1 (Table I) represents the highest published figure for lung disposition that we are aware of.

We intend to utilize these techniques in the future to deliver antitumor drugs to a lung tumor model.

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