Is Stability a Key Parameter in the Accumulation of Phospholipid Vesicles in Tumors?

Ronald H. Wallingford* and Lawrence E. Williams

Division of Medical Physics, Radiological Sciences, University of California at Los Angeles, Los Angeles; and Department of Radiology, City of Hope National Medical Center, Duarte, California

Phospholipid vesicle (liposome) stability in human plasma was determined using perturbed angular correlation (PAC) with ^{111}In as the aqueous phase marker. Using compositions given in earlier tumor imaging studies, liposomes were made with either egg lecithin (EL) or distearoyl phosphatidylcholine (DSPC) as the dominant phospholipid. With fresh human plasma at 37°C, EL vesicles lysed much more rapidly with survival half times being 7 hr at 6.7 mg lipid/ml plasma. DSPC liposomes had a half-time of 130 hr independent of lipid concentration. No lysis occurred with plasma previously stored for 1 wk at 5°C. The addition of 143 USP units of heparin per 4.5 ml sample decreased both half-times by two orders of magnitude. We conclude that EL vesicles exhibit plasma survival times much shorter than those found with DSPC liposomes; this may preclude the former agents from having significant tumor accumulation in vivo.

J Nucl Med 26:1180–1185, 1985

Phospholipid vesicles (liposomes) of various compositions have been developed to expedite targeting of pharmaceutical materials to animal and human tissues (*1*, *2*). The artificial membrane is often presumed to remain intact during this process; in fact, membrane integrity may be considered to be a significant aspect of the targeting strategy (*1*). In several recent examples, vesicles made of distearoyl phosphatidylcholine (DSPC) and cholesterol (CH) have been shown to accumulate in murine tumors in vivo. These small (60 nm) unilamellar vesicles (SUVs) incorporated indium-111 (^{111}In) chelated to nitrilotriacetic acid (NTA) in the aqueous phase. Uptakes of ~20% injected dose per gram (% ID/g) were obtained in EMT6 (*3*), colon 51 (*4*) and Lewis Lung Carcinoma (LLC) (*5*). In the case of EMT6 (*4*), time-course studies revealed continued accrual of ^{111}In at the tumor site and consequential improvement in the tumor/blood ratio out to times as long as 72 hr postinjection.

Received Nov. 5, 1984; revision accepted June 14, 1985.

For reprints contact: L. E. Williams, Dept. of Radiology, City of Hope National Medical Center Duarte, CA 91010.

* Present address: Department of Astronomy and Physics, California State University, Northridge, CA 91330.

Earlier biodistribution studies done with vesicles made with egg lecithin (EL) did not exhibit comparable accumulations in murine tumors (*6*, *7*). Maximal values were ~6% ID/g for LLC (*6*). These magnitudes, being similar to those obtained with gallium-67 (^{67}Ga) citrate (*8*), were sufficiently encouraging, however, to initiate human imaging trials using vesicles containing EL, CH and phosphatidic acid (PA). In the initial study, by Segal et al. (*9*), no obvious tumor visualization was observed in the scintillation camera images of the two patients receiving vesicles encapsulating ^{111}In bleomycin. Richardson et al. (*10*) reported similar results on 14 patients who were injected with the same composition vesicles which had a technetium-99m (^{99m}Tc) membrane label. Neither the size nor the stability of either agent was documented, although the sonication procedure was described in the latter case (*10*).

In view of the extended uptake intervals observed for SUV deposition in the EMT6 tumors, it might be predicted that the EL vesicles may have been relatively unstable—at least in plasma. This would explain their reduced accumulation in murine lesions and the lack of success in the human trials. For this reason, we attempted stability evaluations using perturbed angular

correlation measurements (PAC) of EL- and DSPC-containing vesicles in human plasma. Data were taken with both fresh and stored plasma as well as in the presence of heparin in order to determine the effects of these parameters on membrane integrity.

MATERIALS AND METHODS

PAC analysis

Perturbed angular correlation studies using ^{111}In as the gamma-cascade emitter† have been described elsewhere (*11*, *12*). Our use of this method followed from the manifest murine tumor targeting observed with SUVs containing [^{111}In]NTA. Although other techniques have been reported (*13*, *14*), we believe that angular correlation studies permit the most direct measurement of the movement of ^{111}In out of the encapsulated phase and into the surrounding human plasma. As the membrane began to break down, i.e., release [^{111}In]NTA, the relatively weak strength of the chelator ($K_1 \cong 10^{15}$) led to a very rapid exchange of the indium ion to transferrin ($K_1 \cong 10^{31}$) and possible other iron-binding proteins in the plasma. Because of the large difference in the masses and hence rotational rates of NTA and protein, different angular correlations $W(\theta, t)$ resulted upon gamma counting of these two molecular species. When a mixture of In-NTA and In-transferrin was present, the resultant correlations were a linear combination of those attributable to each molecular type considered separately. In this way, taking data at angular separations (θ) of 90° and 180°, time-averaged angular correlations were determined and used to calculate an averaged perturbation factor $< G_{22}(\text{meas}) >$. The fraction of intact vesicles was then determined from a linear sum of the form (*11*)

$$< G_{22}(\text{meas}) > = f < G_{22}(\text{NTA}) > + (1\text{-}f) < G_{22}(\text{protein}) >. \quad (1)$$

The two limiting perturbation factors were measured using intact vesicles ($< G_{22}(\text{NTA}) >$) and those lysed with isopropanol ($< G_{22}(\text{protein}) >$) in the presence of plasma. Given these values, the above equation was solved for f, the intact fraction of liposomes. Measurements were taken at frequent intervals to determine the time course of liposome membrane stability. A personal computer, interfaced to the four NaI(Tl) detectors, acquired the correlated gamma events at predefined intervals and performed the G_{22} (meas) temporal averaging.

Vesicle preparation

Vesicle compositions generally were taken from the literature. Egg lecithin liposomes had the molar ratio of 7:2:1 for EL:CH:PA (*9*, *10*), while the neutral SUVs were in the proportion DSPH:CH = 2:1 (*3*). A small amount of the ionophore A23187, 0.004 mole to 1 mole of CH, was included in each mixture. Lipids were dissolved in chloroform:methanol 3:1 solvent and then evaporated to dryness under Ar gas. Further drying of the solvent was achieved by placing the sample in a vacuum overnight prior to ultrasonic agitation.

In these experiments, 0.6 ml of of 1m*M* NTA in PBS was added to the various evaporated samples. Sonication was carried out with a 3 mm Ti probe at 20 kHz for ~10 m. The probe was operated at a 50% duty cycle and a power level of 57 W. All sonications were performed under Ar gas so as to minimize oxidation of the resultant membranes. By including a small amount of tritiated cholesterol oleate in the sample, it was found that 75 to 80% of the lipids were incorporated into vesicles.

Separation of SUVs from residual molecules and excess NTA was achieved by a simultaneous column/centrifugation process using Sephadex G-5080 beads. The centrifuge tubes, eluted with 100 μl of PBS, were spun at 450 g for 10 m. The process was performed twice on each preparation.

Labeling with 250 μCi of ^{111}In was done using the method of Mauk and Gamble (*15*). Vesicles were incubated in [^{111}In]Cl_3 for 60 m at 60°C (EL) or 80°C (DSPC). Termination occurred upon adding excess 10m*M* EDTA and passing the mixture over a G-5080 column. The EDTA chelated excess ^{111}In outside of or on the lipid bilayer. Approximately 80% of the tracer was incorporated inside the vesicles in the incubation process. This was a factor of 40 larger than the amount encapsulated by agitation of an aqueous solution in the presence of phospholipids. Such an enhanced encapsulation efficiency was necessary before PAC measurement of these small samples was possible.

Vesicle sizing

Quasi-elastic laser light scattering (*16*) was performed on diluted (25/1) vesicle samples. Intensity (I) autocorrelations were obtained from photomultiplier tube outputs at time (t) intervals of $\Delta t, 2\Delta t, \ldots, 64\Delta t$ where Δt was a digital channel width. The autocorrelation C(t) was related to the scattering object's rate of movement by the equality

$$C(t) = A \exp(-2K^2 Dt) + < I^2 >, \quad (2)$$

where D is the particle's diffusion coefficient and K is a geometric factor dependent upon the incident light's wavelength. Assuming an Einstein-Stokes relationship, the hydrodynamic radius is inversely proportional to D. Physically, this means that as the radius becomes larger, D decreases and the autocorrelation is relatively enhanced. By repeating the intensity measurements, the mean and distribution of the vesicle radius parameter were obtained. A test of the system's calibration was provided by comparing its results with those of electron micrographs of the DSPC preparations.

Plasma samples

In the following description, fresh plasma refers to that used within 1 hr of withdrawal whereas old plasma implies storage for 1 wk at 5°C. Samples were maintained in either [Na]citrate or heparin during an experiment. In the former case, 0.5 ml of 0.105*M* [Na]citrate (3.2% concentration) was added to 4.5 ml of plasma. When heparin was used, 143 USP units were added to the plasma, i.e., the concentration employed for hematologic storage. This level may be comparable to some found regionally in the lung and liver (*17*).

Mixtures of plasma, citrate, or heparin and vesicles were placed at the geometric center of the four NaI (Tl) detector PAC array. A water jacket around the outside of the mixture permitted temperature control. A differential water flow system was used to maintain the temperature at 37 ± 0.2°C during the 60 hr course of an experiment.

RESULTS AND DISCUSSION

Limiting G_{22} values were found to be 0.54 ± 0.02 for intact and 0.13 ± 0.02 for completely lysed DSPC:CH = 2:1 vesicles. These two constants were used in Eq. (1) when calculating f, the intact liposome fraction, at various times during an experiment. Perturbation factors for egg lecithin SUVs were 0.49 ± 0.02 and 0.11 ± 0.02, respectively. The differences between corresponding mean values were not significant ($p > 0.30$) using a Student's t-test.

Egg lecithin vesicle stability-versus-time graphs are given in Figure 1. These curves at 6.7, 8.3, and 10.0 mg lipid/ml of plasma, appeared to have two components. The more rapid was depleted within ~10 to 20 m and, because of count-rate limitations, could not be sampled sufficiently and rapidly to estimate a half-time. The slower component, which amounted to ~80% of the total curve in all cases, decayed with a half-time dependent upon plasma concentration. This value varied between 30 and 13.5 hr for 10.0 and 6.7 mg/ml, respectively. Because of limited amounts of activity in the 0.2 ml PAC sample, we were not able to continue diluting the SUVs with plasma. A clinical level might be estimated at 1 g of lipid per patient; i.e., 5 l of whole blood. This is equivalent to 0.4 mg/ml of plasma using a 50% hematocrit. It is difficult to extrapolate our EL data to this low ratio, but we can predict that the effective half-time of the intact vesicles should be substantially less than 7 hr in that case.

Vesicles made with the 2:1 molar ratio of DSPC:CH exhibited a quite different stability-versus-time behavior. As shown in Fig. 2, the opening of these microcapsules was a linear process with a half-time well beyond 65 hr. By extrapolation , we estimated a value of ~130 hr for the decay of half of the SUV sample. Moreover,

TABLE 1
Survival Half-Times of Vesicles in Human Plasma

Vesicle type	Concentration of Lipid/plasma (mg/ml)	Survival Half-times (hr) With citrate buffer	Survival Half-times (hr) With heparin*
EL:CH:PA 7:2:1	6.7	7 ± 2	
	8.3	13 ± 3	0.12 ± 0.02
	10.0	20 ± 3	
DSPC:CH 2:1	4.2 or 8.3	130 ± 10	1.30 ± 0.20

* 143 USP units in 4.5 ml of plasma.

variation in the lipid/plasma ratio by a factor of two had no effect on the breakdown of the membrane. Table 1 contains a list of the $T_{1/2}$ values for vesicle lysis. This activity was defined as the time for one-half of the total vesicle sample to break open. In the case of EL SUVs, $T_{1/2}$ corresponded to an effective half-time involving both rapid and slow compartments. Errors shown in the table are estimates based on the calculated standard deviations of f given in the figures.

Data shown with solid circles in both Figs. 1 and 2 reveal that leakage of vesicle content did not occur for

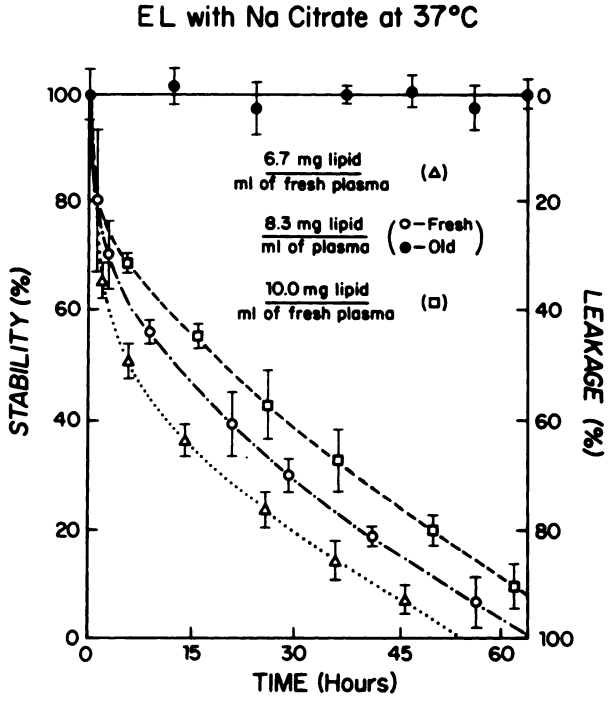

FIGURE 1
Stability of egg lecithin vesicles as opposed to time measured in human plasma at 37°C. Molar composition was PC:CH:PA = 7:2:1. Sodium citrate was used as stabilizer. Old plasma refers to that stored for 1 wk at 5°C. An average of two experimental runs is shown and errors are calculated standard deviations based on Eq. (1)

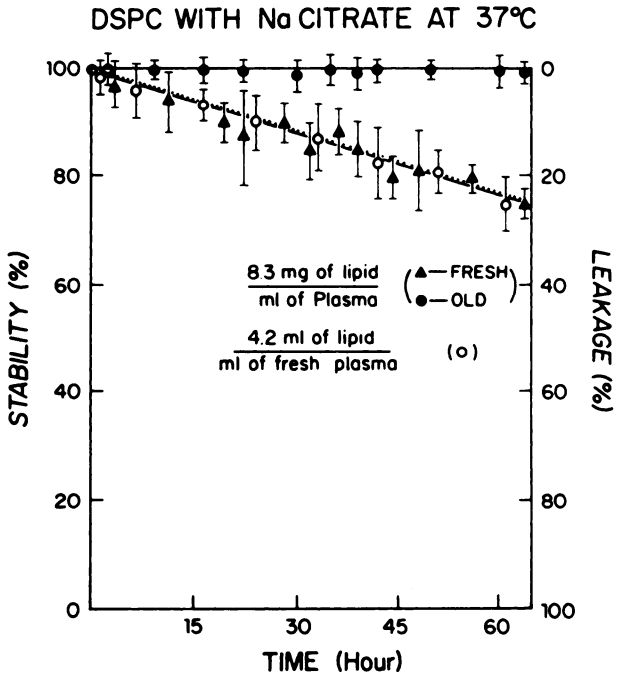

FIGURE 2
Stability of DSPC:CH = 2:1 vesicles as opposed to time in Na Citrate-stabilized human plasma at 37°C. See Fig. 1 for other details

older human plasma that was refrigerated for 1 wk at 5°C prior to incubation. We surmise that one or more active agents present in the fresh sample had been denatured or precipitated during the interval. When controlled lysis of the two vesicle types was performed with stored plasma, the resultant G_{22} (protein) values were found to be equivalent to those found with fresh plasma. Thus, we can rule out any change in the indium-binding capabilities during refrigeration.

The addition of heparin had a striking lytic effect on both types of vesicles. Half-times measured with 143 USP units (~1 mg) of sodium heparin per 4.5 ml of plasma (38 mg lipid) were reduced by approximately two orders of magnitude (Fig. 3). A recent report (*18*) has described much less marked decreases for multilamellar vesicles immersed in PBS at 5:1 lipid to heparin weight ratios. Apparently, the presence of human plasma greatly augmented the anticoagulant's lytic effect on the artificial membrane even when the lipid-to-heparin ratio was essentially eightfold higher.

Comparison of our stability data with earlier measurements must be made indirectly since previous results involved nonhuman plasma and, in general, vesicle types not appropriate for tumor imaging. Several PAC stability experiments, using high purity phospholipids, have, however, demonstrated extended survival times in rabbit plasma of uncertain history (*12*) or after s.c. injection in whole mice (*19*). In fresh mouse plasma, Senior and Gregoriadis (*14*) had estimated stability half-times to be greater than 50 hr for DSPC:CH = 1:1 SUVs while pure EL vesicles were shown to lyse within a few hours of incubation. Thus, while the superior lifetimes of the high purity SUVs were established, values for tumor-imaging agents in human plasma were previously not known.

Quasi-elastic light scattering measurement yielded mode and full width at half maxima values 33 ± 5 nm and 64 ± 6 nm for the diameters of the EL and DSPC vesicles, respectively. The latter result was in good agreement with previous electron microscopy of DSPC liposome samples (*3*). Although Richardson et al. (*10*) did not directly measure vesicle size, one can estimate by comparing sonication regimens, that their liposomes would probably have been comparable to or smaller than those synthesized here. Previously, McDougal et al. (*13*) have shown that increased sonication leads to enhanced stability relative to buffer-mediated dialysis. Scherphof et al. (*20*), however, have demonstrated the relatively enhanced susceptibility to plasma-induced lysis of smaller SUVs. In any event, we believe it is unlikely that the vesicles used by Richardson et al. (*10*) were substantially more stable than the ones tested here.

One demonstrable difference between our EL liposomes and those reported earlier (*9*, *10*) was the use of A23187 as a loading ionophore. Without this or some other equivalent membrane constituent, we would have been unable to achieve the high specific activities required by PAC. While it is conceivable that any ionophore could change membrane stability, it was unlikely at the low levels used here. Essentially one atom of A23187 per 10^4 phospholipid molecules was included in the membrane; the relative fraction of ionophore/ cholesterol being the same in either SUV type.

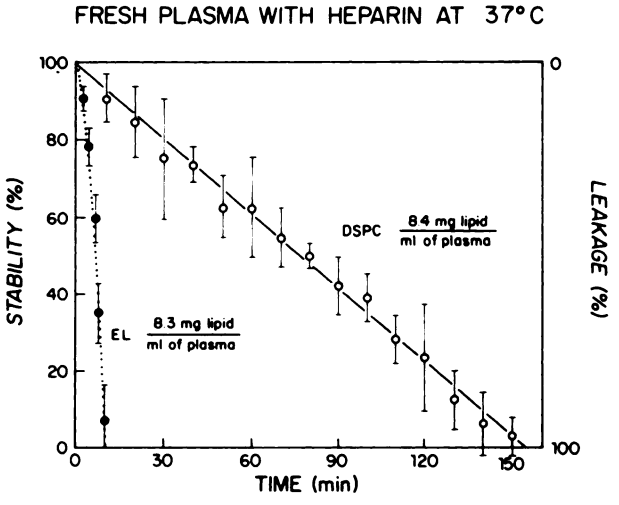

FIGURE 3
Effect of heparin on vesicle stability. 143 USP units of heparin per 4.5 ml of human plasma were used in these trials

A possible explanation for the reduced plasma stability of the EL SUVs could be the variable length of the fatty acid chains in the constituent phospholipid molecules. The actual distribution of lengths depends upon the specific source of lecithin. Solid to liquid phase transition temperature for our samples was ~−20°C. On the other hand, DSPC is a highly purified phospholipid having an 18 carbon chain and a correspondingly increased phase-transition temperature of 55°C.

Our results have several implications for tumor-imaging trials. Long-term accumulations observed in murine studies, with vesicles targeting over times as long as several days, would imply that stable liposomes such as those made of DSPC:CH should be relatively superior imaging agents. This superiority appears to be borne out in comparing animal biodistributions obtained with DSPC (*3*) and EL vesicles (*6*). Human tumor uptake, if it is similar to the animal models, would also be expected to be enhanced with high purity phospholipid membranes. This occurrence could explain the lack of success in human trials (*9*, *10*), although variation in the membrane material, the method of labeling, and possibly vesicle size cannot be excluded as contributing factors.

Establishment of the ultimate importance of stability awaits confirmation of our results at physiological concentrations of lipid in plasma. We were unable to achieve such low values because of the amount of activity required in PAC analysis.

The lytic effect of heparin seen in our work may imply a mechanism for the breakdown of liposomes in certain tissues. For example, the previously demonstrated lysis of DSPC:CH = 2:1 SUVs by the EMT6 tumor (*4*) might be attributable to mast cells in the neovasculature (*21*). Breakdown of vesicles in the liver has also been measured using PAC techniques (*22*) and could be caused, at least in part, by endogenous heparin. One could predict that patients receiving anticoagulant therapy would exhibit such greatly reduced vesicle integrity in vivo as to preclude targeting beyond times of a few hours even for high purity liposomes. In this regard, it is important to recognize that the presence of fresh plasma enhances the lytic action of heparin as compared to buffer (PBS) (*18*).

FOOTNOTE

† In these discussions, we refer to ^{111}In, the parent radionuclide; it is actually the daughter, cadmium-111 that emits the cascade gammas.

ACKNOWLEDGMENTS

The authors thank Vestar Research Incorporated for the use of their facilities. Experimental assistance was offered by Ms. Bonnie Blamick and Mr. Su-Ming Chiang. Additional advice was provided by Drs. George Tin and Ronald Gamble of Vestar. Professor H. Rodney Withers of UCLA supplied insight on the effect of plasma concentration. This work was part of a MSc thesis in Medical Physics (R.H.W.) submitted to the University of California at Los Angeles.

REFERENCES

1. Gregoriadis G: The carrier potential of liposomes in biology and medicine. *N Engl J Med* 295:704-710, 765-770, 1976
2. Yatvin MB, Lelkes PI: Prospects for liposomes. *Med Phys* 9:149-175, 1982
3. Proffitt RT, Williams LE, Presant CA, et al: Tumor-imaging potential of liposomes loaded with In-111-NTA: Biodistribution in mice. *J Nucl Med* 24:45-51, 1983
4. Williams LE, Proffitt RT, Lovisatti L: Possible applications of phospholipid vesicles (liposomes) in diagnostic radiology. *J Nucl Med Allied Sci* 28:35-45, 1984
5. Williams LE, Patel KR, Tin GW, et al: Comparison of murine abscess and tumor uptake of small unilamellar vesicles (SUVs). *Clin Nucl Med* 8: P41, 1983
6. Gregoriadis G, Neerunjun DE, Hunt R: Fate of liposome-associated agent injected into normal and tumour-bearing rodents. Attempts to improve localization in tumour tissues. *Life Sci* 21:357-370, 1977
7. Richardson VJ, Jeyasingh K, Jewkes RF, et al: Possible tumor localization of Tc-99m-labeled liposomes: Effects of lipid composition, charge and liposome size. *J Nucl Med* 19:1049-1054, 1978
8. Larson SM, Rasey JS, Allen DR, et al: A transferrin-mediated uptake of Gallium-67 by EMT6 sarcoma. II. Studies in vivo (BALB/c mice): Concise communication. *J Nucl Med* 20:843-846, 1979
9. Segal AW, Gregoriadis G, Lavender JP, et al: Tissue and hepatic subcellular distribution of lipsomes containing bleomycin after intravenous administration to patients with neoplasms. *Clin Sci Mol Med* 51:421-425, 1976
10. Richardson VJ, Ryman BE, Jewkes RF, et al: Tissue distribution and tumor localization of ^{99m}Technetium-labelled liposomes in cancer patients. *Br J Cancer* 40:35-43, 1979
11. Leipert TK, Baldeschwieler JD, Shirley DA: Applications of gamma ray angular correlations to the study of biological macromolecules in solution. *Nature* 220:907-909, 1968
12. Hwang KJ, Mauk MR: Fate of lipid vesicles *in vivo*: A gamma-ray perturbed angular correlation study. *Proc Natl Acad Sci USA* 74:4991-4995, 1977
13. McDougall IR, Dunnick JK, McNamee MG, et al: Distribution and fate of synthetic lipid vesicles in the mouse: A combined radionuclide and spin label study. *Proc Nat Acad Sci USA* 71:3487-3491, 1974
14. Senior J, Gregoriadis G: Is half-life of circulating liposomes determined by changes in their permeability? *FEBS Lett* 145:109-114, 1982
15. Mauk MR, Gamble RC: Preparation of lipid vesicles containing high levels of entrapped radioactive cations. *Anal Biochem* 94:302-307, 1979
16. Pecora R: Laser light scattering and macromolecular Brownian motion. *Nature Phy Sci* 231, 73-85, 1971
17. Brozovic M, Bangham DR: Standards for heparin. In *Heparin, Structure, Function and Clinical Implications*, Bradshaw RA, Wessler S, eds. New York, Ple-

num Press, 1974, pp 163-170
18. Patel HM, Parvez N, Field J, et al: Lytic effect of heparin on liposomes: Possible mechanism of lysis of red blood cells by heparin. *Bios Repts* 3:39-46, 1983
19. Mauk MR, Gamble RC, Baldeschwieler JD: Vesicle targeting: Timed release and specificity for leukocytes in mice by subcutaneous injection. *Science* 207:309-311, 1980
20. Scherphof GL, Damen J, Wilschut J: Interactions of liposomes with plasma proteins. In *Liposome Technology*, Vol. III, Gregoriadis G, ed. Boca Raton FL, CRC Press, 1984, pp 205-224
21. Kessler DA, Langer RS, Pless NA, et al: Mast cells and tumor angiogenesis. *Int J Cancer* 18:703-709, 1976
22. Hwang KJ, Luk K-FS, Beaumier PL: Hepatic uptake and degradation of unilamellar sphingomyelin/cholesterol liposomes: A kinetic study. *Proc Natl Acad Sci USA* 77:4030-4034, 1980