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Evaluation of liposomes as a drug delivery system

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EVALUATION OF LIPOSOMES AS A DRUG DELIVERY SYSTEM

by

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DISSERTATION

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To My Parents

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Mark E. Bosworth
December, 1980

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List of Abbreviations

Abbreviation	Meaning
RES	Reticuloendothelial System
FP	French press
Tc	Phase Transition Temperature
PBS	Phosphate Buffered Saline
TLC	Thin Layer Chromatography
PC	Egg Phosphatidyl Choline
CH	Cholesterol
PA or DPPA	Dipalmitoyl Phosphatidic Acid
a-T	alpha-Tocopherol

Abstract

The objective of this study was to characterize the *in vivo* disposition of liposomes and liposome-entrapped markers. This was done in several distinct sets of *in vitro* and *in vivo* experiments. Initial studies evaluated the most commonly used methods of liposome preparation, with the finding that almost all of them are insufficiently standardized to give easily reproducible liposome preparations. One previously reported method (French press extrusion) was found to give very small liposomes reproducibly. The initial set of *in vivo* studies was done in rabbits. Liposome plasma levels were first monitored by using ^{14}C-cholesterol and ^{3}H-dipalmitoylphosphatidyl choline labels. It was found that these labels did not measure liposome levels accurately (possibly due to lipid exchange) and so ^{14}C-inulin was chosen as a water soluble marker for all subsequent *in vivo* studies. It was found that the plasma kinetics were a function of the liposomes' physical properties, particularly surface charge and size. The importance of the influence of liposome size led to the development of a method to improve the sizing aspect of liposome preparation. It was found that dialyzing liposomes with straight bore pore polycarbonate membranes gave narrower, more reproducible size populations, especially when the liposomes had already been extruded through the same type of membrane. The final set of *in vivo* studies, done in mice, focused on delineating the major liposome disposition factor, which is their interaction with the reticuloendothelial system (RES), particularly in the liver and spleen. This RES disposition was found to be dose dependent and qualitatively similar to that seen for other colloids. The data also suggested that liposomes may stay bound extracellularly for several hours before being taken up by cells. Further studies in mice showed that the dose effect is not a simple function of the number of administered liposomes, and that the liposome size may control this effect. Finally, liposome stability, as measured by *in vitro* tests, correlated well with *in vivo* stability, as measured by tissue levels of the ^{14}C marker. This work provides a foundation for rigorous development of clinically useful liposome drug delivery systems.

CHAPTER I: GENERAL INTRODUCTION

Purpose of this study. During the past decade liposomes have emerged as a promising new in vivo drug delivery system. The purpose of this work is to characterize some of the important processes in the liposomes' in vivo disposition, so that their potential as a therapeutically efficacious system can be optimized.

Available literature. The first extensive physical characterization of liposomes was reported by Bangham in 1965 (24). Since then there has been an explosion of literature dealing with liposome physical properties and their use as model membrane systems. Their use as drug carriers brought on a second explosion of in vivo work. There are several good reviews now, dealing with both physical (1-4) and biological (5-14) properties. The liposome literature is so extensive and diverse that it is beyond the scope of this thesis to give a rigorous critique of all the relevant publications. Instead, important findings will be summarized, and criticism of the literature as a whole as it relates to this work will be given.

Organization of this thesis. The work reported here was done in several distinct yet interrelated subject areas, which are reported in separate chapters. Chapter II covers the previously developed basic liposome preparation procedures used here and some of our own qualitative and quan-

titative observations concerning them. The succeeding chapters each have sections with additional introductory or methodological material relevant to the topic. Finally, a general conclusions chapter summarizes the important findings from this work and addresses the subject of future research in this area.

Previous work done to define and characterize liposomes. When dry phospholipid comes in contact with excess aqueous solution the molecules spontaneously arrange themselves in what is known as the lipid bilayer (2,25). Liposomes are spherical bilayer shells with a single entrapped aqueous compartment, or a multi-concentric bilayer structure with alternating aqueous and lipid layers (Fig. 1) (2,3,28). The bilayer forms because of the phospholipid's amphiphilic nature (Fig. I-2) and dual solubility characteristics (25): that is, the polar head group tends to associate with the aqueous environment, and the water tends to be excluded from the nonpolar side chain environment. Many other lipid types can be accommodated in the bilayer structure (e.g. fatty acids, lysolecithin, cholesterol, alkanes) but a minimum amount of phospholipid must be present to maintain the bilayer structure (25). In fact the bilayer (as opposed to the globular type of micelle formed by detergents or bile salts) is the more stable phospholipid micelle form because of the three-dimensional geometry of the phospholipid molecules (25). When other amphiphiles such as detergents

(which favor the globular micelle) are added in excess to a liposome suspension the bilayer structure is lost and the phospholipids are incorporated into the detergent micelles (25).

Liposome size can cover a broad range, typically 0.1-10 microns for a liposome batch prepared by simple aqueous dispersal of the dried lipid (6). Under specialized preparation conditions they can be as large as 25 microns (6) or as small as 200 angstroms (e.g. after sonication), which is thought to be the limiting smallest size due to molecular packing and bilayer curvature constraints for liposomes that small (2).

Drugs and other molecules of interest may be included in the liposome. For nonpolar compounds, this may be accomplished by having them present with the phospholipid, in the dry state, before the aqueous dispersal. When the liposomes form, the compound is then part of the bilayer (2). Polar compounds are entrapped by being dissolved in the aqueous solution used to disperse the lipid. The liposomes, however, do not entrap 100 percent of the total aqueous volume, and thus less than 100 percent of the polar compound is entrapped in the liposomes (2).

Another important liposome property is the phase transition temperature, or Tc. When heated or cooled pure phospholipids, and liposomes composed of a single pure lipid,

undergo a sharp change in physical properties at a characteristic temperature (the Tc). The Tc value depends on phospholipid properties such as fatty acid chain length and degree of unsaturation, and the head group type (1,2,38,39). It is analogous to a melting temperature, and the bilayer properties above (the "liquid crystal" state) and below ("solid" or gel state) are distinguished by changes in such measurements as NMR, ESR, and fluorescence depolarization, which detect increases in intramolecular motion and lateral diffusion rates of molecules in the monolayer above the Tc (1)(often called the "fluidity" of the bilayer). The transition itself is endothermic (as reflected in differential scanning calorimetry measurements (1)) and is the point of maximum permeability of the bilayer (32). Liposomes composed of two very similar phospholipids such as dipalmitoyl and distearoyl phosphatidyl choline also exhibit sharp transitions; mixtures of dissimilar lipids, though, can exhibit more than one endothermic peak or much broader and less well defined single transitions (2). The presence of cholesterol above approximately 33 mole percent effectively eliminates the transition (6). The Tc is important in liposome preparation because the lipids must be above the Tc for liposomes to be formed (1-3).

Liposome permeability to various substances has been investigated in great detail (31-37). In brief, the intact bilayer is least permeable to large, polar substances (espe-

cially ions) and most permeable to nonpolar substances, which can partition into the nonpolar region of the bilayer (2). Bilayer permeability is also a function of composition (31,35); the presence of cholesterol, which has a condensing effect on the bilayer above its Tc, can reduce liposome permeability to a great extent (31,33). The liposomal lipids are also subject to oxidation in unprotected conditions (i.e., presence of oxygen and light (49-51)), and the products of lipid oxidation often cause increased liposome permeability (36,49-51). It has been found, however, that by taking the precaution of incorporating small amounts of an antioxidant such as alpha-tocopherol into the liposome (Ø.1-1.Ø mole percent) that gross oxidative degradation (as monitored by leakage of an entrapped marker) can be prevented over the time course of most experiments (several days) (41,49); under protected conditions (nitrogen, dark, refrigeration) these liposomes can be stable for many weeks or months (41). The stability of liposomes in biological fluids is discussed below.

A great variety of lipid types can be accommodated in the liposome bilayer, and many different combinations have been used in studies (8). The different types of nonpolar side chains impart differences in bilayer fluidity and permeability. Polar head groups can have net electrical charges and thus can impart a net surface charge to the liposome (2).

Interactions of liposomes with biological systems. The interest in liposomes as drug carriers had one of its origins in cell culture studies. Papahadjopoulos and coworkers (82) wanted to use them to promote cellular fusion (as surrogate viruses) and in the process they and others found that liposomes could be taken up by the cells (fusion and active endocytosis being the primary mechanisms (85,86)), and thus that liposome-entrapped agents could be delivered to cells (83,84). This naturally promoted interest in their use in vivo , which led to studies that examined the effect of liposome entrapment on the disposition of many different compounds (8). In brief, two significant findings emerged which led investigators to believe that liposomes had potential as target-specific intracellular delivery vehicles: first, the liposome "confers" its pharmacokinetics on the entrapped compound, which in most cases means that the compound's kinetics are greatly altered (106); second, after an intravenous dose liposome-entrapped agents were found to localize to a significant extent (up to 90 percent of the dose) in organs that contain cells of the reticuloendothelial system (RES)(100,101,104,105). This system is responsible for the removal of colloids, both endogenous and exogenous, from the blood, and the colloidal nature of liposomes appeared to explain their RES localization. It was thus postulated that liposomes could eventually be targeted to other sites in the body to effect intracellular delivery.

It was also found that certain liposome types are unstable in blood, as measured in *in vitro* leakage rate tests (42,44-48). However, it is now known that with appropriate lipid compositions, particularly high levels of cholesterol, liposomes can be made to be very stable (44).

Evolution of the objectives of this project. At the time this work was started, expectations for liposomes as a delivery tool had reached their highest level, particularly in the anti-cancer area, in which there were reports of increased efficacy of liposome-entrapped agents in animal model tumor systems (99,107). However, there were also some disturbing trends in the literature. First, although the volume of *in vivo* work was increasing rapidly, there was a great lack of uniformity in the experimental protocols: 1) preparation procedures (especially sizing) were very crude and unstandardized and 2) each investigator had a favorite liposome composition, entrapped compound, dose, etc. A few studies attempted to rigorously examine the effect of liposome physical properties on disposition (79,97,102) but the crudeness of the existing preparation procedures made the results suspect. Second, tissue distribution data were steadily being accumulated, all of which showed RES localization of liposomes. This led to the assumption that liposomes, as other colloids, were intracellularized by RES cells, and that tissue levels of entrapped label could be taken as an indication of the intracellular delivery of that

label by the liposomes. The RES disposition of liposomes had not been rigorously investigated, though (i.e. dose behavior, dependence on liposome size), and we suspected that there might be some crucial differences between liposomes and other colloids in this regard. A full discussion of this point is developed in Chapter V.

The objectives of this work were, then, to 1) examine the effect of liposome physical properties on their plasma kinetics in order to verify some previously reported results (79) and to evaluate different liposome marker compounds, 2) develop techniques to improve the size homogeneity of liposome preparations and 3) characterize the RES handling of liposomes, since control of this will be crucial in making them a versatile drug delivery vehicle.

Important literature reports since the inception of this study. By and large most of the in vivo work reported since this study began has been phenomenological and has only repeated and confirmed the earlier findings that liposomes affect the disposition of the entrapped agent and that they localize in RES tissues; the only differences have been new compounds and new animal systems. Some investigators have attempted to use present knowledge to optimize therapeutic efficacy in animal model disease systems (the work of Papahadjopoulos in the cancer area (94,98) and of Alving in the leishmaniasis area (90,91) are good examples) but characterization of important disposition processes such as RES

uptake has not been done. Reports in the areas of liposome toxicity and immunological properties have confirmed that the use of naturally occurring lipids results in no problems (6,52), but that liposomes may have significant adjuvant properties for certain synthetic molecules incorporated into the bilayer (6,88).

There has been great progress, however, in using certain specialized liposome properties to achieve target-specific delivery. The above-mentioned work in leishmaniasis is an example for an obvious liposome target--the liver. Other investigators have been able to target the lung fairly selectively: either by i.v. administration of large liposomes that apparently lodge there in the capillaries (75), or by intratracheal administration, which was shown to increase pulmonary and reduce systemic effects of entrapped anticancer drugs (80,81). The latter route also has potential as a means of delivering lung surfactant lipids for lung surfactant deficiency diseases (109). Another delivery strategy was reported by Weinstein and coworkers (89,92,93), who designed liposomes with phase transitions slightly above body temperature, and then applied hyperthermia to localized tumors so that the liposomes could selectively release their contents (the hyperthermia brings the liposomes to their Tc so that they leak) at the tumor site. Another approach has been to use liposomes as a therapeutic intermediate: it has been found that

entrapping lymphokines (macrophage activating factors) increases their ability to stimulate the tumoricidal activity of macrophages in vitro (78). Finally, a physical technique has been reported (perturbed angular correlation (95)) that apparently makes possible the measurement of intact liposome levels in vivo and should help elucidate some of the basic liposome disposition processes.

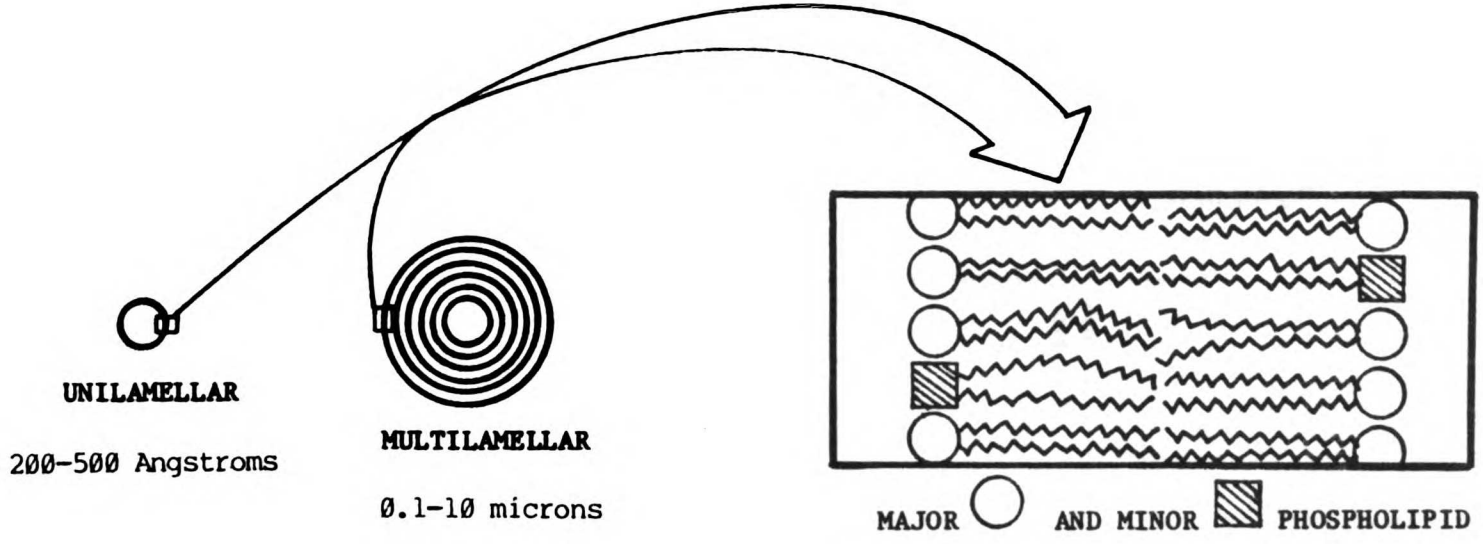
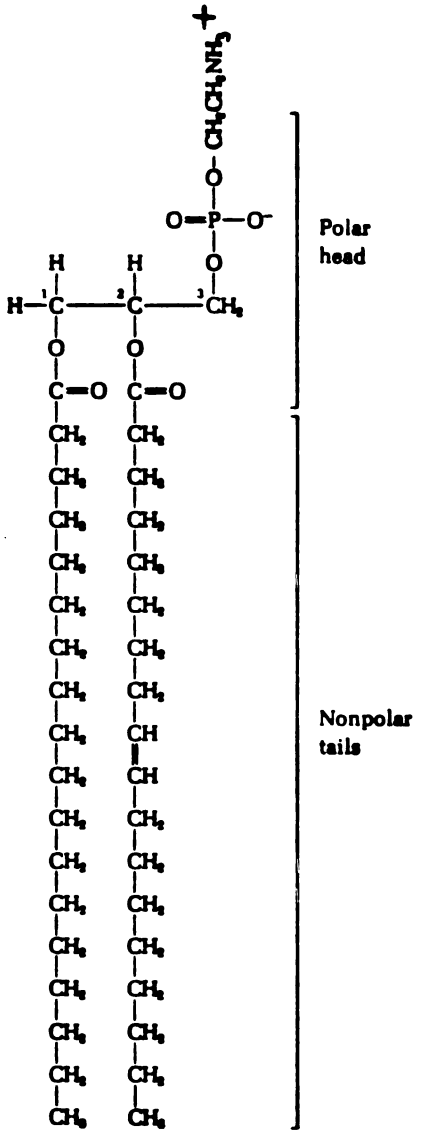


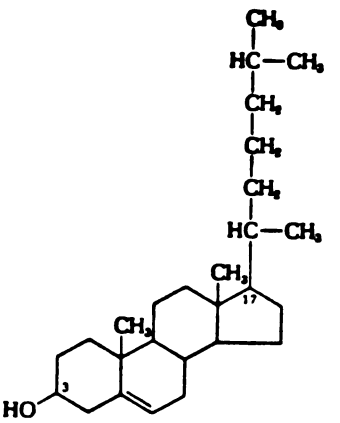
Figure I-1. Diagram of the liposome lamellar structure and blowup showing the bilayer configuration.

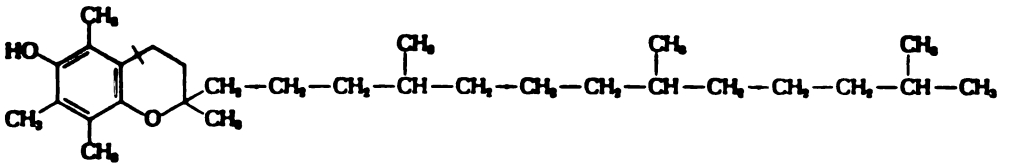
Figure I-2. Structure of phosphatidyl choline showing the polar and nonpolar regions.



Phosphatidyl choline

Cholesterol





Vitamin E (α-tocopherol)

Figure I-3. Structures of cholesterol and alpha-tocopherol.

CHAPTER II: METHODS OF LIPOSOME PREPARATION

Methods of Preparation: Materials and Methods.

Materials. Egg yolk phosphatidyl choline (PC), sodium dipalmitoylphosphatidate (PA), cholesterol (CH), DL-alpha-Tocopherol (a-T) (all chromatographic grade); and inulin (from dahlia tubers), Sodium Bisulfite, and Sodium Sulfite (all reagent grade) were obtained from Sigma Chemical Co. (St. Louis, Missouri). Egg PC was also obtained in purified form from the laboratory of D. Papahadjopoulos, U.C.S.F. Sodium Phosphate (dibasic anhydrous) and Ammonium Molybdate were obtained from J.T. Baker Co., Phillipsburg, N.J. ("Baker Analyzed Reagent"). Sodium Chloride, Sodium Phosphate (monobasic monohydrate), Sulfuric Acid concentrated, Hydrochloric Acid (concentrated), Chloroform, Methanol, and Sucrose were obtained from Mallinckrodt Chemical Co., St. Louis, Missouri (all "Analytical Reagent" grade). 1-amino- 2-naphthol-4-sulfonic acid was obtained from Fisher Chemical Co. (Pittsburgh, P.A.) (Fisher certified chemical). U-^{14}C-sucrose, carboxyl-^{14}C-inulin, 4-^{14}C-cholesterol and 9,10-^{3}H-dipalmitoyl phosphatidylcholine (all chromatographically pure) were obtained from New England Nuclear (Boston, MA); prior to use phosphatidic acid was obtained by chloroform extraction of a solution of sodium phosphatidate in 0.4 N HCl with 20% methanol.

Tests for purity. Lipid purity was assessed by thin layer chromatography using Adsorbosil-5 TLC plates (Applied Science, State College, PA.). The following solvent systems were used: $CHCl_3$:MeOH:H_2O = 65:25:4 (v:v) for PC, DPPC; hexane:ether:acetic acid = 70:40:3 for CH; $CHCl_3$:MeOH:NH_4OH (aq) = 65:25:4 for PA. Spots were visualized in an iodine chamber or by sulfuric acid charring (26). The radiopurity of the ^{14}C-CH and ^{3}H-PA were checked by scanning the TLC plates (run with cold carrier) with a TLC plate radioscanner device. Radiopurity of the ^{14}C-inulin was checked by running it (with cold carrier) on a Sephadex LH-20 column (Pharmacia Fine Chemicals, Sweden), 1X30 cm, eluted with a phosphate-saline buffer (see below).

Quantitation of lipids. Stock solutions of phospholipids were quantitated by a modification of a colorimetric assay for inorganic phosphate by Fiske and Subba-Rowe (27). It is performed as follows:

Reagent Solutions:

a) reducing reagent: 10 gm Na Bisulfite /L

2 gm Na Sulfite /L

168 mg 1-2-4 acid /L

b) Molybdate reagent: 4.4 gm NH_4 Molybdate /L

14 ml conc. Sulfuric Acid /L

Procedure:

a) Hydrolyze sample (typically about 0.05 -0.1 mg phospholipid = about 100 nmoles PO_4) in 0.2 ml conc. perchloric acid for at least 2 hr. at 180°C.

b) Cool to room temperature and add 1.25 ml reducing agent; mix and add 1.25 ml molybdate reagent; mix again.

c) Boil for 10 min. in a water bath.

d) Cool and read the absorbance at 700 nm versus a reagent blank. A standard curve is made up using sodium phosphate solutions as standards.

Cholesterol stock solutions are quantitated gravimetrically (after solvent drying); otherwise by measuring ^{14}C-CH as a tracer (see below).

Buffer composition, osmolality. The following phosphate-buffered saline (PBS) was used for all liposome batch preparations:

Sodium Phosphate, monobasic	1.53 gm/L
Sodium Phosphate, dibasic	6.29 gm/L
Sodium Chloride	5.57 gm/L
Pen-Strep Solution*	2ml/L

* (10,000 units/ml penicillin + 10,000 mcg/ml streptomycin)

The pH of this solution is 7.3-7.4 and the osmolality approximately 290 mOsm (as measured on a clinical osmometer, courtesy of the Clinical Laboratory at U.C.S.F.).

Solvents and solvent filtrations. Double distilled water was used in all experiments. All solvents used were filtered. Chloroform and methanol were filtered through a Millipore FG 0.2 micron pore size filter fitted in a suction filtration apparatus (Millipore Co., Bedford, Mass.). All aqueous solutions were filtered through Nuclepore "Unipore" polycarbonate membranes of 0.05 or 0.10 micron pore size, and a polyester post filter (Bio-Rad, Richmond, CA), fitted in a Millipore 25mm diameter stirred filtration cell with reservoir (Millipore Corp.) by positive nitrogen pressure.

Preparation of "Mechanically Dispersed" liposomes. The basic liposome preparation (MD), which results in large multilamellar vesicles, is made as follows: the desired lipids, in chloroform, are dried onto the walls of a round bottom flask using a rotary evaporating device with vacuum, using a mild warm water bath (30-40^{o}C) for the flask. Drying is completed by applying the house vacuum to the flask at room temperature for 0.5-1 hours. An effort is made, during "roto-vaping", to distribute the lipid over as much area of the flask as is possible. An aqueous solution (usually PBS containing a marker compound) is added to the flask, the flask is sparged with nitrogen and stoppered, and then it is gently agitated by hand (with intermittent rest) until all

of the lipid is visibly dispersed from the walls of the flask. This dispersal, as aforementioned, results in the formation of the liposomes. During the dispersal, which takes 1/2 - 1 hr to complete, the flask is occasionally immersed in a warm water bath (40°C), not exceeding 5 min. total. Radioactive marker compounds are incorporated into the liposomes by two methods. ^{14}C-CH and ^{3}H-PA are dried along with the other lipids from the chloroform solution. The water-soluble markers (^{14}C-sucrose and ^{14}C-inulin) are dissolved in the PBS which is used to disperse the dried lipid. In the former case it is assumed that all of the marker is incorporated into the lipid bilayer, while in the latter case the marker remains in the aqueous solution. Since only part of the total aqueous solution is entrapped in the liposomes, only part of the water-soluble marker will be entrapped. The liposomes are allowed to sit at room temperature for another hour before additional steps are performed on them.

Lipids used in these studies. Egg yolk phosphatidyl choline was chosen as the principal phospholipid in these studies because: it is the most commonly used lipid for liposome studies and thus well characterized; it is relatively inexpensive; it has properties, including a low Tc, that make it easy to work with at room temperature. It also has a net neutral charge at physiological pH, which means that the liposome surface charge can be altered by adding in only a

small amount of a charged lipid (2). In addition to egg phosphatidyl choline the only other lipids used here are cholesterol and alpha-tocopherol (Fig. I-3), dipalmitoyl phosphatidyl choline (all with net neutral charge) and dipalmitoyl phosphatidic acid, which carries a net negative charge at ph 7.4. Alpha-tocopherol was used as an antioxidant, as described in Chapter I. The liposomal lipids are restricted to these because it was felt that it would be advantageous to study a few types thoroughly and to use them consistently throughout all the studies. Thus, the lipids used and their abbreviations:

Egg Phosphatidyl Choline	PC
Dipalmitoyl Phosphatidyl Choline	DPPC
Dipalmitoyl Phosphatidic Acid	DPPA or PA
DL-alpha-Tocopherol	a-T
Cholesterol	CH

Preparation of extruded liposomes. "Extruded" liposomes are prepared by the procedure of Olson et al (64): The "mechanically dispersed" liposomes are placed in the 25 mm Millipore stirred ultrafiltration cell fitted with a Nuclepore polycarbonate membrane and polyester postfilter. These membranes are "straight-bore" pore membranes that are available in 8.0, 5.0, 3.0, 2.0, 1.0, 0.8, 0.6, 0.4, 0.2, 0.1,0.08,0.05, and 0.03 micron pore sizes. The liposomes are forced through the membrane with positive nitrogen pressure at room temperature. The liposomes can be extruded

sequentially through membranes of decreasing pore size (e.g., 1.0, then 0.8, 0.6, 0.4, 0.2), and afterwards bear the label of the smallest pore size through which they have been extruded (e.g. "0.2 micron-extruded liposomes").

Preparation of French press (FP) liposomes. This procedure, originally developed by Hamilton and Goerke at U.C.S.F., has been reported recently by them and others (61,62). MD liposomes are placed in a French pressure cell (Aminco, Silver Spring, Md.) and as much of the air is expelled from the cell as possible before closing the orifice valve to the cell's interior. Both cell sizes (40ml or 5ml capacity) were used, depending on the liposome batch size. The cell is then placed in a Carver Type C press (F.S. Carver Inc., Menomonee Falls, Wis.), and the liposomes are extruded at 20 ml/min. at room temperature. The press gauge pressure is kept constant throughout the run; this is done by regulating the cell's orifice size and varying the press pumping rate (done by hand). All French press runs are done at 20,000 psi (inside the cell); this means that, because the surface areas of the cells' pistons aren't equal to 1 square inch, that the poundage applied by the press (i.e. psi read on the press pressure gauge) is 13,000-15,000 psi for the large and 2500-3000 psi for the small French press. After this procedure, which is always done more than once on a liposome batch (comments later), the liposomes are centrifuged at low speed for ten min. on a lab bench centrifuge to remove

rubber particles that shear from the French press piston O-rings during the extrusion.

Column runs with liposomes and markers. Various column procedures were carried out with the liposomes and their marker compounds. Sephadex G-50-80, G-75-40, G-200-120, and LH-20 (as powders), and Sepharose 4B (pre-swelled in a .02 percent Sodium Azide solution) gels were obtained from Pharmacia Co. (Sweden). Biogel A-150m was obtained as pre-swelled gel in sodium azide from Bio Rad. The powders were allowed to swell for appropriate times in .02 % sodium azide. "Econo-columns" of various dimensions were obtained from Bio-Rad. Prior to packing the columns, the swelled gels were degassed as follows: they were placed in a flask in a 40°C water bath, placed under house vacuum for two hours, and swirled intermittently. The elevated temperature makes the gel less viscous. The columns were packed at room temperature by pouring in the gels, allowing the column to run and the gel to settle by gravity. Two to three column volumes of the sodium azide were run through to stabilize and pack the gel bed. Prior to liposome runs, always done at room temperature, the columns were equilibrated with at least 4-5 column volumes of PBS. After use, they were reequilibrated with the sodium azide solution. When samples of liposomes or marker compounds were run on the columns the sample volume was kept low enough to avoid overloading the column yet high enough to cover the entire bed area. For 1 cm diameter

columns the typical sample volumes were Ø.1 to Ø.4 ml. Column void volumes (except for the LH-2Ø and A-15Øm) were determined by measuring the elution volume of Ø.2 ml of a 2mg/ml Blue Dextran (Sigma, St. Louis, MO, average MW=2,ØØØ,ØØØ) solution. Column eluate was collected in "by-drop" fractions with a Gilson (Middletown, Wis.) fraction collector using 13x1ØØ mm test tubes.

Dialysis of Liposomes and markers. Dialyses were carried out on liposomes and markers using two types of procedures, always done at 5°C:

a) Dialysis Bags: dialysis tubing (12,ØØØ MW cutoff) was purchased from VWR Scientific. Collodion dialysis bags (75,ØØØ MW cutoff) were purchased from Schleicher and Schuell (Keene, N.H.). The 12,ØØØ MW tubing came in dry form and was prepared for dialysis as follows: it was boiled in 1% sodium carbonate for 1 hour, then rinsed and soaked in distilled water for 1/2 hour. The 75,ØØØ MW bags came in 3Ø% Ethanol. They were also prepared for dialysis by soaking in distilled water. Just prior to dialysis of liposomes both bag types were rinsed in PBS. For 12,ØØØ MW dialysis, sections of appropriate length of the tubing were cut, clamped off at one end, and the sample placed in the bag; the other end was then clamped and the bag placed in an appropriately-sized container of PBS. The clamps caused it to float. For 75,ØØØ MW dialysis the sample is simply placed into the bag (cone-shaped) and the bag opening

clamped with forceps. The forceps are then placed over the opening of an appropriately-sized beaker and PBS added to the beaker such that the PBS level is above the liquid level in the bag. In both cases the PBS dialysate is stirred with a magnetic stirring bar and electric stirrer. The beakers are also covered lightly with aluminum foil.

b) Dialysis cells: Dialysis cells (Technilab, 1 ml capacity on each side) were obtained from Bel Art Products (Pequannock, N.J.). These were fitted with the aforementioned 25mm Nuclepore polycarbonate membranes. The membranes were wetted first, then placed over one half of the cell; the other cell half was added and the bolts tightened hand tight. Samples and dialysate solution (usually PBS) were added to their respective sides through the entry ports with glass transfer pipets. The ports were then taped shut. For dialysis, the cells were placed on a horizontal shaker and shaken at 1.5-2 cycles/sec., with 5 cm amplitude.

Sampling of solutions. Solutions were quantitatively sampled using a 10 microliter syringe (Hamilton, Reno, Nev.) or Eppendorf pipets of varying sample capacities (Brinkman, Westbury, N.Y.). When it was desired to measure the contents of the sample side of a dialysis cell, the following procedure was used: a 10 microliter sample was first taken, then the entire cell weighed, the sample side contents were withdrawn with a transfer pipet and the cell reweighed. Thus amounts (concentration X volume) were measured in all

dialysis cell studies. In dialysis bag studies only concentrations (by syringe sampling) were monitored.

Measurement of Radioactive Markers. Radioactive marker compounds were quantitated by two methods:

a) Direct Counting. Markers in aqueous media (i.e. PBS, water, or plasma), organic media (stock solutions of labeled lipids), or as dry compound (by evaporating the above solvents) were combined with 3.5 ml PCS scintillation counting fluid (Amersham, Arlington Heights, Ill.) in 7ml polypropylene screw-cap mini-vials or with 10-15 ml PCS in 25 ml screw-cap glass counting vials (VWR Scientific, Pittsburgh, PA.). Miscibility was checked visually (up to 0.4 ml of aqueous solution could be added to 3.5 ml PCS without phase separation). Counting was done using a Packard Tri-Carb scintillation counter (Packard Instrument Co., Downer's Grove, Ill.) in the external standard channels ratio mode. To correct for variable quenching in samples, quench curves were set up: constant amounts of isotope counts were put in several vials with PCS, and then varying amounts of chloroform were added as quenching agent. Plots of relative efficiency vs. external standard ratio were then made.

b) Sample combustion. Animal tissue samples (e.g. blood or liver) were prepared for counting by combustion. Tissue aliquots were weighed and placed in combustion cups with pads (Packard) and allowed to air dry at room tempera-

ture overnight. They were then combusted in a Packard tissue combustion apparatus using Carbo-Sorb and Permafluor V (Packard) as carbon dioxide trapper and scintillator, respectively, for ^{14}C samples (10 ml of each were used per sample). The isotope and solvents were machine-collected in the 25 ml glass vials, and then counted on a Packard Tricarb counter as mentioned previously. Quench curves were constructed to correlate counting efficiencies in PCS and the combustion solvents. Samples containing known amounts of radioactivity were combusted to evaluate combustion efficiency.

Methods of Preparation: Results and Discussion.

Assay standard curves: Figures II-1 and II-2 show a typical standard curve for the phosphate assay, and quench curves for ^{14}C in PCS and in combustion solvents. Curves for ^{3}H were similar in nature.

Column runs with liposomes and markers. In the early work we re-confirmed a fact that had already been well known: that column procedures could be used to separate liposomes from nonentrapped marker, and liposomes of different size from each other. All liposome types and sizes, when applied to a Sephadex column, elute entirely in the void volume because their minimum size is approximately 200 angstroms, and molecular weight 5,000,000 (6). Fig. II-3 shows the combined results of Sephadex G-50-80 runs of ^{14}C-inulin and

^{14}C-sucrose. It shows that these markers are clearly separated from the void volume (i.e., liposomes) and from each other (two distinct peaks are also obtained when the two are run together). This latter fact was used as a crude test of the purity of the inulin; since inulin's molecular weight is approximately 5,000, there should be no molecules as small as sucrose present. Thus, the column method can be used to separate free from entrapped marker, and also to measure the fraction of marker entrapped in liposomes when both free and entrapped marker are present. However, we and others (69) have found that the percent recovery of liposomal lipid from a column run (as quantitated with labeled lipid) can be as low as 50 percent and can depend on the number of times the column has been used. Another disadvantage of the method is that a sample dilution effect takes place. Because of these problems, column procedures are used here only to determine fraction entrapped. When the liposomes are to be used for other purposes the nonentrapped marker is removed with dialysis whenever possible.

Different sized liposomes can also be separated on columns. However, the largest available column molecular weight exclusion limits (e.g. 150,000,000 for the Biogel A-150m) will not allow size separation of liposomes above approximately 0.15 microns in diameter; samples of 0.2 micron-extruded liposomes were run on the A-150m column and eluted completely in the void volume. Very small liposomes

are retained (see next section).

Characteristics of French press liposomes. Figure II-4 shows the results of a study in which French Press liposomes labeled with ^{14}C-cholesterol were chromatographed on a Sepharose 4B column. The liposomes were composed of PC/CH = 7/3 (molar ratio), made at 5 mg PC/ml. The liposomes in curve A had one pass through the cell, the ones in B three passes (cell pressure was 20,000 psi for both runs). These results show that multiple passes reduced the fraction of void volume liposomes-that is, reduced the average liposome size; the location of the retained peak is the same for both, indicating that the average size of the column retained liposomes did not change. This is in accordance with the notion that liposomes have a smallest limiting size. This shift toward small size is accompanied by a characteristic change in turbidity of the liposome suspension. The original mechanically dispersed preparation is milky white, and the three-pass French press liposomes are translucent and almost optically clear. These column retention and optical properties depend on the lipid concentration. At low lipid concentrations the preparation becomes clear after just one pass, while for higher concentrations (20 mg/ml) two or three passes are required. This is probably because the cell does have a certain "dead space" - a volume of liposomes that isn't extruded - and this volume would be expected to contribute more to the turbidity if the

concentration was higher, which is also the reason why more than one pass is needed. The properties of French press liposomes have also been observed to depend on the extrusion pressure. It was found by us and others (62) that MD liposomes extruded at low pressure (about 6,000 psi) were larger than these done at 20,000, as judged by Biogel A-150 M retention (Fig. II-5) and electron microscopy (62). We have also observed, as have others, that when liposomes are extruded at high pressure the suspension is often warm (30-35^{o}C) when it exits the press, due no doubt to the large energy input of the extrusion process. On the whole the process is a very easy one, and much preferable to other methods that are used for obtaining these small vesicles, i.e. sonication, which is not as reproducible and which can result in lipid degradation (6), and detergent dialysis, which is too sensitive to lipid composition and a host of other factors (4). Therefore in applications where small unilamellar vesicles (as 20,000 psi FP liposomes are thought to be (2)) were needed, French press liposomes were used, with 20,000 psi operating pressure and three passes (or cycles) for the liposome batch.

Qualitative aspects of liposome preparation. Several aspects of the liposome preparation procedure involve steps that are not easily quantifiable, yet are probably very important. The first is the way in which the lipids are dried from chloroform in the round bottom flask. Ideally

the lipid should be dried to a uniform and continuous film, over as great an area as possible (2). In practice this isn't possible. The film must be relatively thick, i.e. confined to a small enough area, so that the aqueous solution used to disperse it can be agitated conveniently; the film contour is often irregular and discontinuous just because of the crude nature of the rotovap process. This could conceivably affect the way in which the aqueous solution disperses the lipid. There have been no thorough studies on this matter, but there is evidence that suggests that lipid layer uniformity and rate of solvent agitation may affect the size of the MD liposomes (72). In spite of this there are great differences in the methods different investigators use to disperse the lipid; at one extreme is a preliminary exposure of the dried lipid to water-saturated nitrogen, followed by extremely delicate agitation (72); at the other, the use of vigorous vortexing (64) and even sonication (6) for dispersal. The dispersal process itself (i.e. time needed to disperse all the lipid) is variable, and even when the agitation rate is kept relatively constant, certain lipid compositions (usually the ones with higher cholesterol) disperse much more slowly than others. This might be due to the differences in fluidity caused by cholesterol, and is expected to be temperature dependent (see below). The process of lipid dispersal (and liposome formation) is one of hydration - the process of the water-lipid head group interaction replacing the head group-head

group interaction in the dried lipid (2). As others have noted (2), this process need not be complete when the lipid is entirely dispersed from the walls of the flask. Chunks of lipid several bilayers thick are probably taken off the lipid film during the agitation, leaving some of the lipid unhydrated. For this reason most liposome investigators prefer to let their preparations incubate at the dispersal temperature for at least an hour after dispersal to complete the "hydration" process. Again, there have been no thorough quantitative studies done on this aspect of liposome preparation, but it is almost certainly true that the aforementioned size heterogeneity in MD liposome preparations is a result of the lack of uniformity in these various steps. In the work reported here the liposome batches were allowed to "equilibrate" at room temperature for an hour after dispersal before any other procedures (e.g. extrusion through membranes) were done on them.

As with any other colloidal suspension, a relevant concern with liposomes is whether or not they separate out of suspension. Liposomes are capable of both creaming and sedimenting, depending on the conditions. For sedimentation the effective liposome density must be greater than that of the surrounding medium; this is sometimes possible at room temperature, depending on the liposome type, but is often facilitated when the centrifugation is done at $5^{\circ}C$ due to the increase in lipid density compared to the aqueous solution. Increased liposome density can also be achieved by

entrapping compounds of high density. For creaming, the liposomes' density must be less than that of the medium; this is done by simply placing the liposomes in a higher density solution (e.g. sucrose, see Chapter V). Liposomes can also separate out without the aid of centrifugation. This is the case especially for neutral liposomes - i.e. liposomes that have a net neutral surface charge, such as pure egg PC liposomes - and not so for liposomes that have a net charge. In the former case the liposomes will settle out of suspension visibly. This is probably an aggregation or flocculation effect, due to lack of net charge, similar to that seen for other colloids (111) and is readily reversible by mild agitation.

Multilamellar liposomes are known to be osmotically sensitive structures (29), principally because they act as a semipermeable membrane; water can diffuse across intact bilayers readily, ions with extreme difficulty (2). The only precaution that was taken in this regard in these studies was to make sure that the liposomes used didn't undergo extreme osmotic shock by exposure to media of greatly different ionic strength. As for the osmotic contribution that the liposomes themselves make, it is negligible: a sample of FP liposomes made at 20 micromoles/ml lipid in buffer with the composition PC/PA/CH/a-T = 4/1/1/.05 (molar ratio) showed no detectable increase in osmolality compared to buffer alone when measured on the clinical osmometer. This

result was expected based on calculations of the number of liposomes (particles) that would be present.

Extrusion of liposomes through membranes. As mentioned before, the MD liposomes can be extruded through the Unipore membranes. The MD liposomes were always extruded through a 2.Ø or 1.Ø micron pore size membrane first, even though the final extrusion (after a sequence of membranes) might be with a Ø.2 micron membrane. This is because the liposome size distribution is made smaller with each decrement in membrane pore size, so that pre-extrusion through the larger pore sizes makes extrusion through the smaller ones easier. When done this way the pressure required for a "typical" liposome batch (e.g. 2Ø micromoles/ml lipid) is 1-1Ø psi for extrusion down through the .6 micron membrane, 1Ø-15 psi for the .4 and 2Ø-3Ø psi for the Ø.2. If MD liposomes are not initially put through the larger sizes, much higher pressure is needed. In the paper describing the method Olson et al (64) note that these extrusions are done with 1ØØ% recovery of lipid. In the present work it was found that this was true for most liposome batch sizes extruded. For most membrane sizes only when very small amounts of lipid (less than Ø.5 mg) were extruded was the recovery less than 1ØØ%. This was assessed by recovery of ^{3}H-DPPC and ^{14}C-CH in the extrudates. Only for the case of extrusion of Ø.4 micron-extruded liposomes through a Ø.2 micron membrane was the recovery less than 1ØØ% for all lipid amounts (typically

50-70%). In all experimental preparations, the liposomes were extruded twice through the final membrane used, to ensure that the liposomes were sized correctly.

Dialysis of water soluble markers. The dialysis rates of ^{14}C-inulin and the ^{14}C-sucrose out of various dialysis bags and across the Unipore membranes were measured. The various dialysis half-lives were: 3-4 hours for inulin in the 75,000 M.W. collodion bag; one hour for inulin with Unipore membranes with pore sizes 0.2 microns and larger; 8-10 hours for inulin with Unipores smaller than 0.2 microns; one hour for sucrose in a 12,000 M.W. dialysis bag (diameter 5-8 mm). These results indicated that 1) when sucrose was used as the encapsulated marker, the nonentrapped sucrose could be removed easily by overnight dialysis 2) when inulin is used, the dialysis bag method is too slow for overnight dialysis. The liposomes should be "cleaned up" using either the dialysis cell-Unipore membrane method (which has the complicating factor that liposomes can also dialyze across (Chapter IV)) or a column procedure. When the Unipore can't be used (i.e. for FP liposomes, as they will dialyze across most sizes) and the column dilution effect is unwanted, the only solution is to dialyze in the 75,000 MW bag for 48 hours. (This was done in the mouse studies).

Encapsulation efficiencies and effect of dispersal temperature. As mentioned before, one of the variables in the liposome dispersal process is the temperature. It is known

that the lipid must be dispersed at a temperature greater than its phase transition temperature, but how much greater is not a well-answered question. The data in Table II-1 are the encapsulation efficiencies (that is, volume of aqueous solution entrapped per mole of lipid), as measured by the dialysis bag technique, of several liposome types. The liposomes were PC/DPPA/CH/a-T = 4/1/1/.05 made at 10 micromoles lipid/ml. MD and 1.0 micron-extruded sizes were studied, with the entire dispersal-extrusion process taking place at RT, 40°C, or 60°C. The results were unexpected, as it was anticipated that the entrapment efficiency would rise with temperature, the rationale being that higher temperature would enhance the "hydration" process. Instead, what actually happened was that the gross dispersal- i.e. removal of lipid from the flask-was greatly speeded (from 0.5 hours at RT to 4 min. at 40°C and 2 min. at 60°C). This probably meant that the lipid's lowered viscosity and higher malleability at the higher temperatures caused more non-hydrated lipid to be "scooped" off the flask. It is commonly held (4) that the temperature of dispersal should be above that of the phase transition of the lipid with the highest Tc, even if it is a minor component, for the liposomes to form "perfectly". In this case the DPPA has its Tc at 52°C (egg PC at approximately -13°C). However, we routinely disperse our lipid at room temperature (because of the elevated temperature effect on encapsulation), and use occasional brief periods at 40-50°C (as mentioned before) in

deference to the high Tc of DPPA.

It can also be seen that the encapsulation efficiency is lower for the 1.0-micron extruded liposomes. This decrease in efficiency with size holds true for all large multilamellar liposomes (.1 micron and up) (64) - there is a moderate decrease in encapsulation efficiency with size; however, for the small unilamellar liposomes (FP or sonicated) the efficiency is much lower because of the increased ratio of lipid to entrapped volume in these liposomes (2). For FP preparations with the same composition and concentration as the above - mentioned preparations, efficiencies of ten-fold lower magnitude were commonly seen. The efficiencies given here are typical for the encapsulation of polar, water-soluble compounds. However, for compounds which are nonpolar (and can partition into the bilayer) or that can bind strongly to the bilayer (as for some proteins) the encapsulation efficiencies can be much higher (30). Thus the entrapment efficiency depends on both the liposome type and the nature of the entrapped compound, and can be used as supporting evidence that a particular liposome type has been prepared.

Dialysis of liposomes using Unipore membranes. See Chapter IV.

Statistical procedures used in these studies. In all cases where two sample means are compared for statistical differ-

ence, one-tailed t-tests were done at the p = .05 significance level. Differences that are statistically significant will be referred to as "significant".

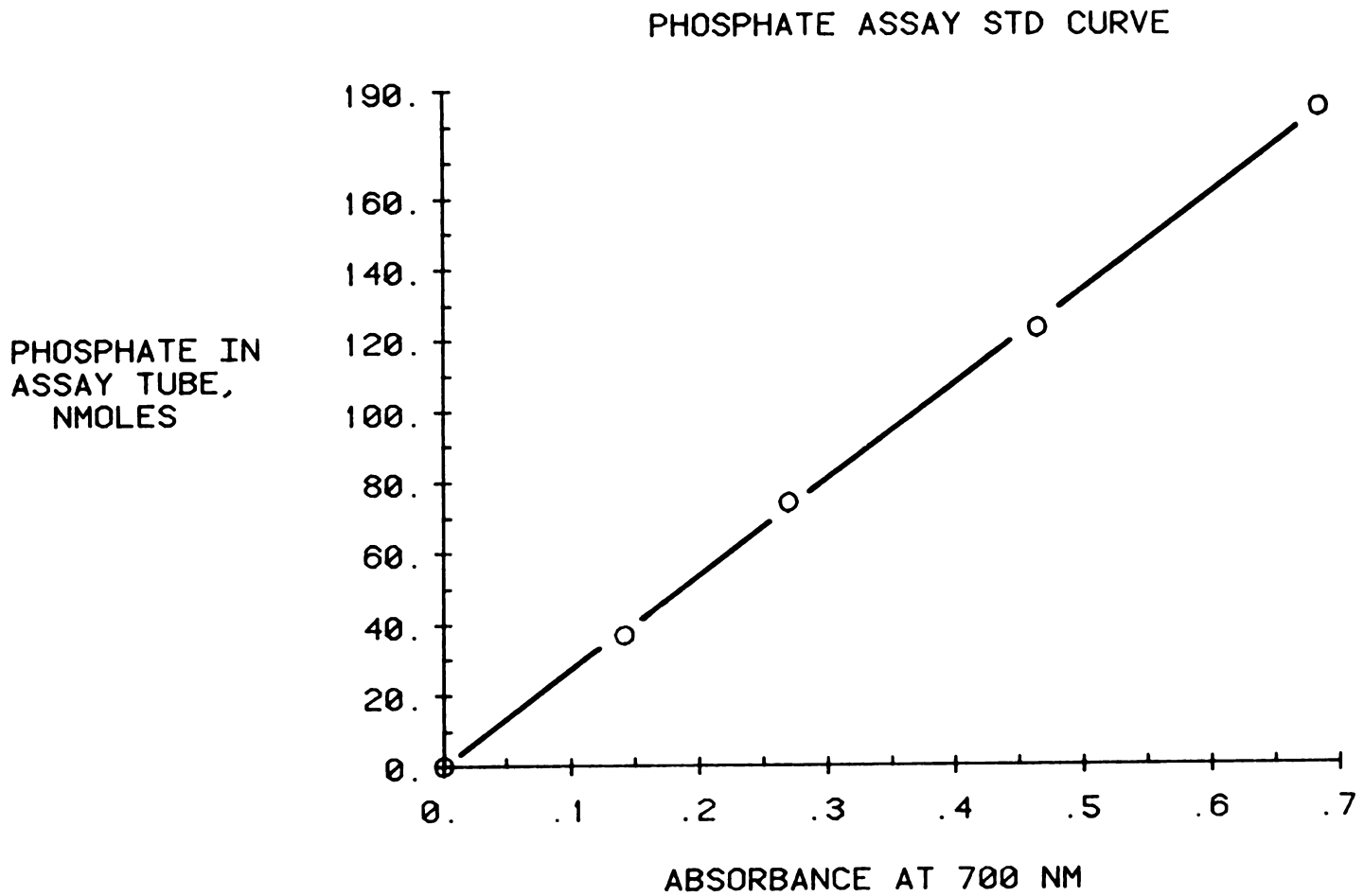


Figure II-1. Typical standard curve for the phosphate assay used in phospholipid quantitation. Each point is the average of duplicate determinations; in each case the range of values is covered by the height of the symbol.

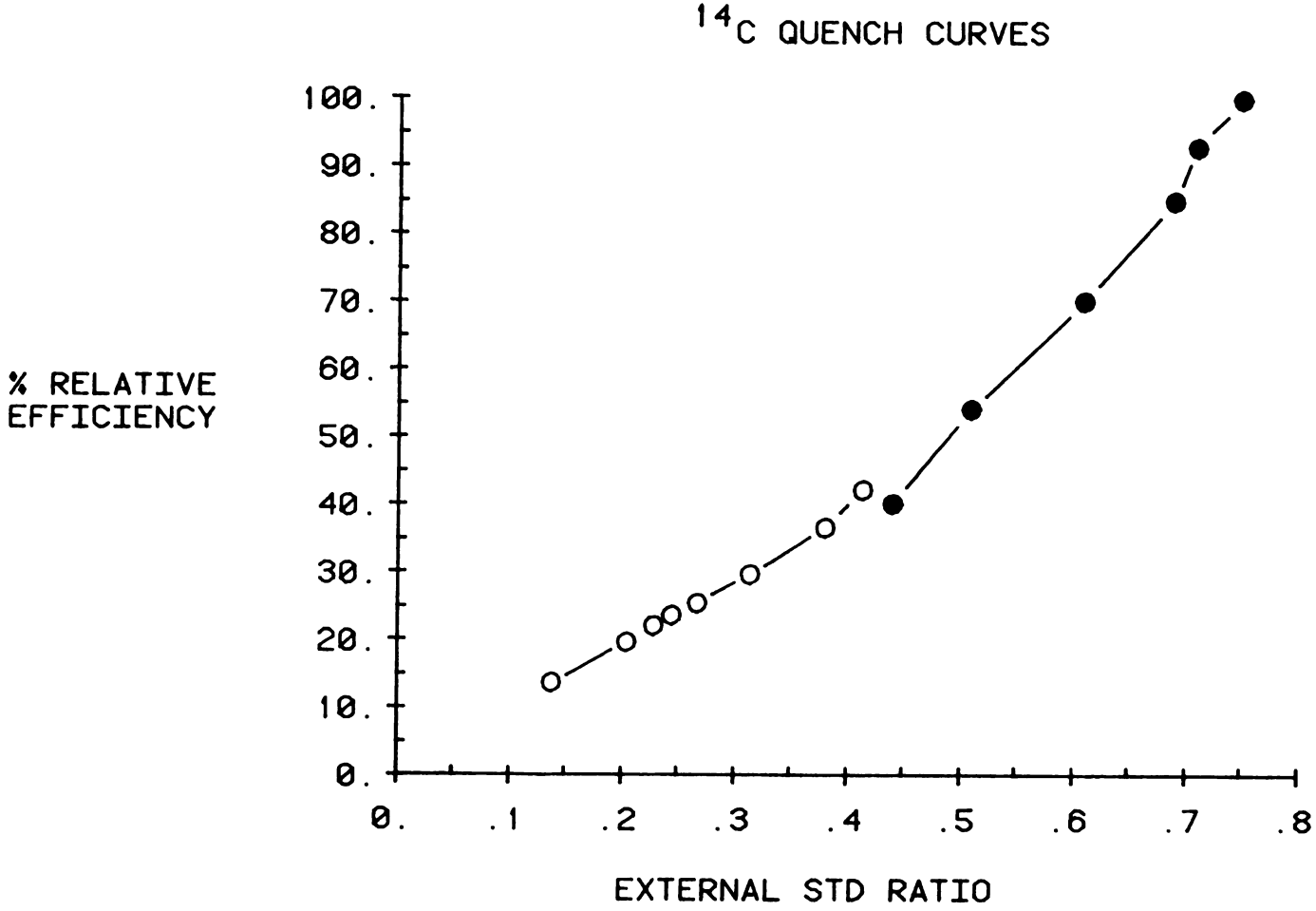


Figure II-2. Quench curves for the ^{14}C label in two scintillation counting solvent systems: open circles are for combustion solvents, closed circles are for PCS.

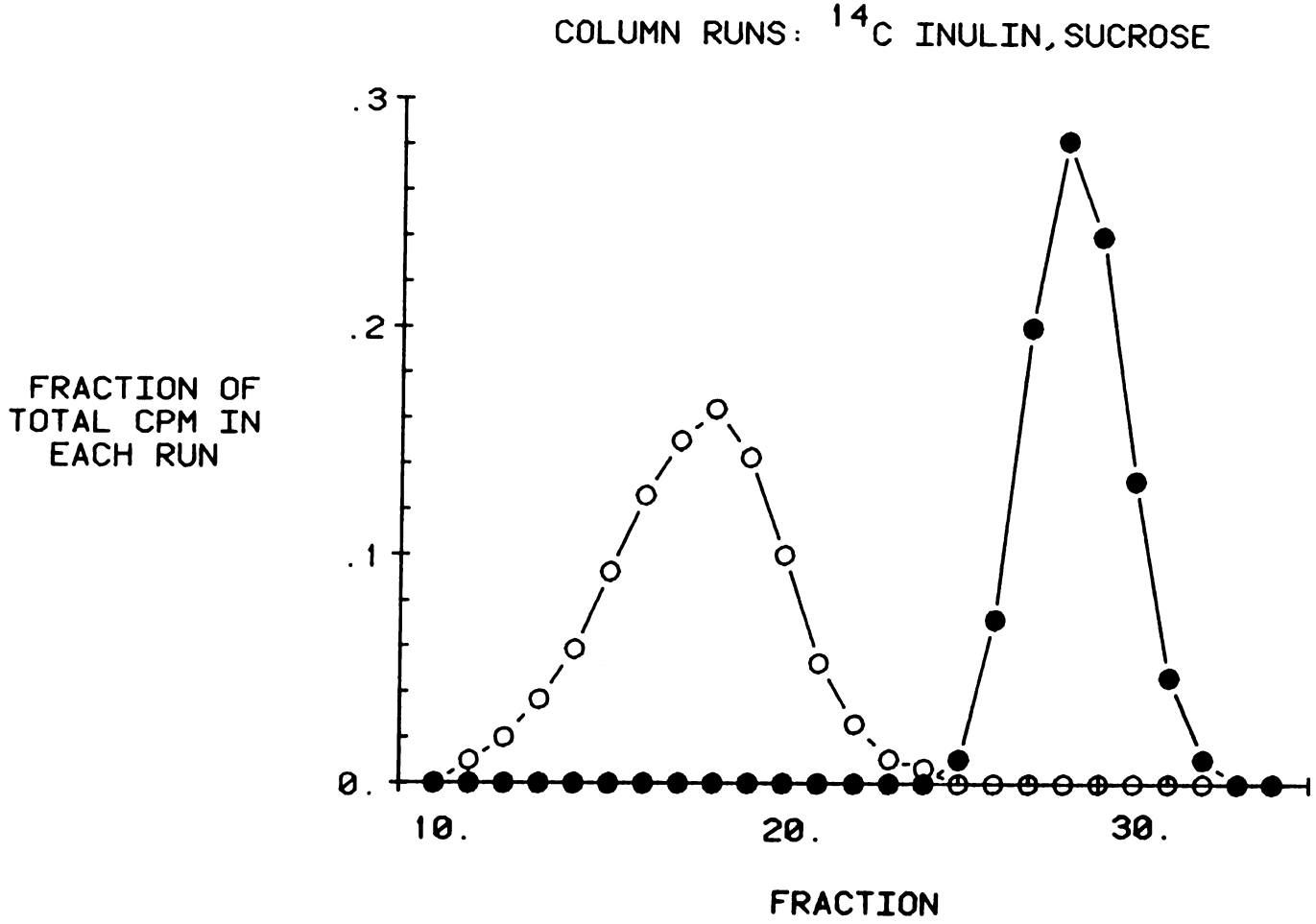


Figure II-3. Elution profiles of 14C-labelled inulin (open circles) and sucrose (closed circles) on a Sephadex G-50-80 column. Fraction number 9 is the void volume.

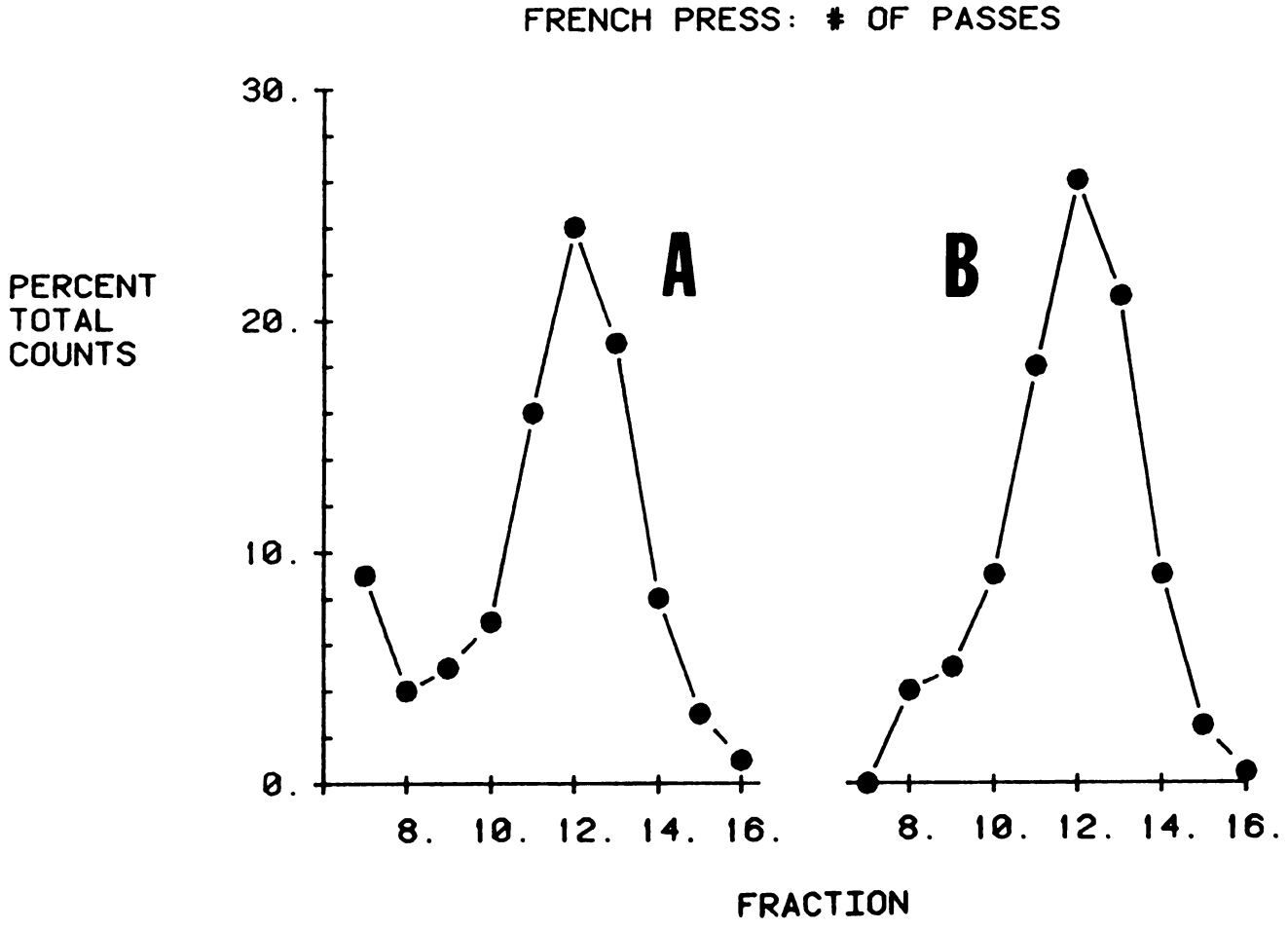


Figure II-4. Effect of number of passes through the French press on liposome Sepharose 4B elution patterns. Liposomes were made at 5mg PC/ml and were of the PC/CH = 7/3 composition, labeled with ^{14}C-cholesterol. Curve A is for one pass, curve B for three passes. Fraction number 7 is the void volume.

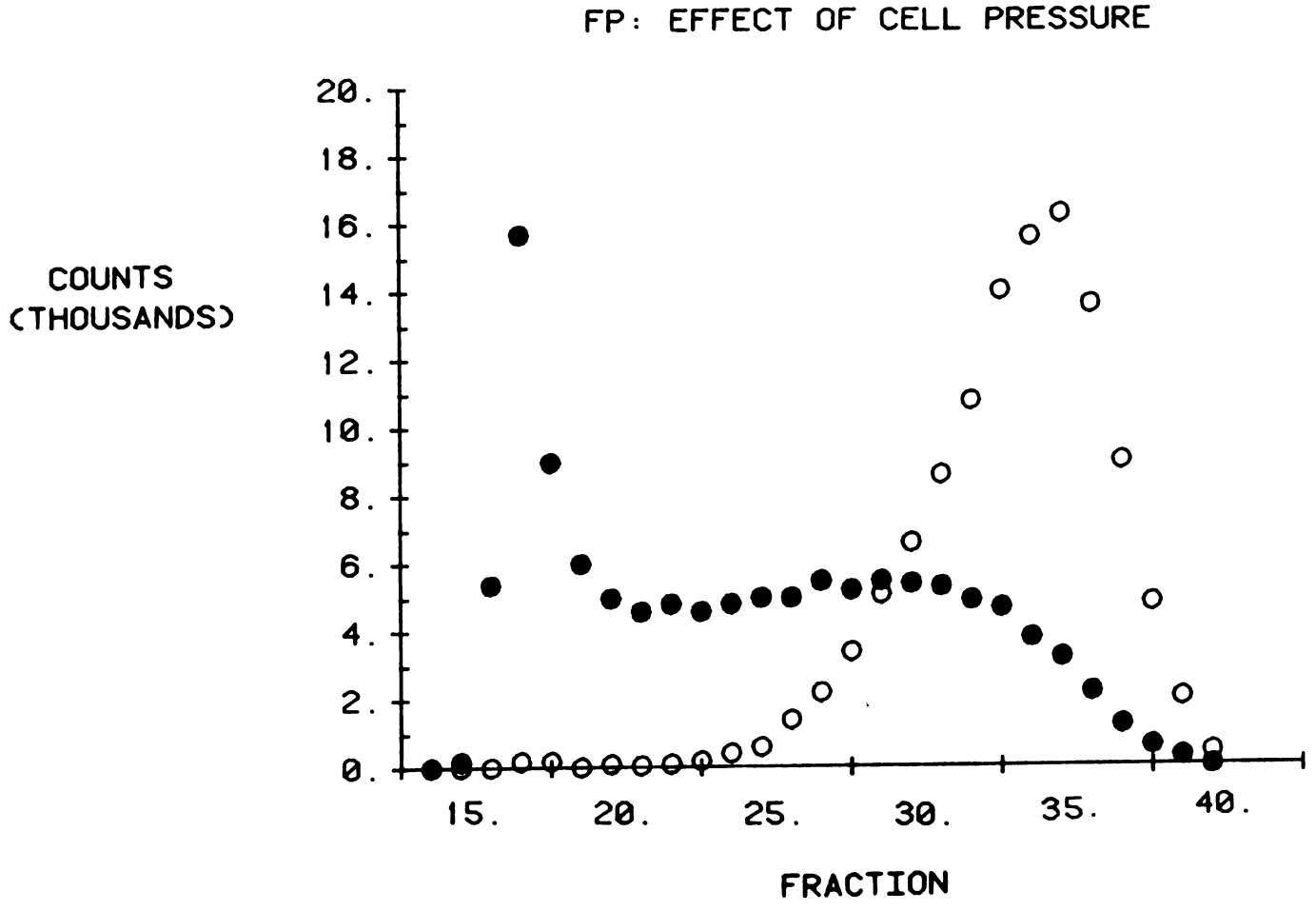


Figure II-5 Effect of French press operating pressure on liposome Biogel A-150m elution patterns. Liposomes were made at 10 micromoles lipid/ml and were of the PC/PA/CH/a-T = 4/1/1/0.05 composition, labeled with ^{14}C-cholesterol. Closed circles represent liposomes extruded at 6000 psi, open circles for 20,000 psi. Fraction number 17 is the void volume.

Table II-1: Entrapment efficiency data for liposomes prepared at RT, 40°C, or 60°C. Values are expressed as liters of aqueous solution entrapped per mole of lipid. In this experiment these numbers also equal the percent of the total aqueous solution entrapped by the liposomes. All values are the average of duplicate determinations.

Liposome Type	RT	40°C	60°C
Mechanically Dispersed (MD)	8.3	7.1	5.8
1.0-extruded	6.6	6.9	5.5

CHAPTER III: EFFECT OF THE LIPOSOMES' PHYSICAL PROPERTIES ON THEIR PLASMA KINETICS IN RABBITS

Introduction.

In the early investigations of the interaction of liposomes with cultured cells it was found that the nature of the interaction (i.e. stable adsorption to, fusion with, or endocytosis of the liposome by the cell) depends on the physical properties of the liposomes (85). Papahadjopoulos and coworkers found that cultured cells would bind a variety of liposome types, but that delivery of the liposome-entrapped agents into the cells depended on liposome properties such as surface charge and the fluidity of the bilayer (83). It is thus very likely that liposome physical properties play an important role in governing their fate *in vivo*, especially in light of the fact that the *in vivo* fate of other colloidal materials is known to depend on particle size and surface charge (16).

Very few of the *in vivo* studies done previously have examined the effects of liposome physical properties on their fate. The purpose of this study is to examine the influence of liposome size and surface charge on their plasma kinetics in rabbits. In addition, the effect of the choice of liposomal marker compound (i.e. whether it's an aqueous space or lipid bilayer marker) on these results is studied.

Materials and Methods.

Chemicals. All chemicals and reagents were as in Chapter II.

Rabbits. The rabbits used were adult male New Zealand Whites, weighing 3.6-4.4 kg. They were housed in the Animal Care Facility at U.C.S.F. and received a diet of Purina rabbit chow #5321 and water ad libitum up until, and then after, the studies.

Experimental Set-up. During the studies the rabbits were placed in restrainers (Plas-Labs, Lansing, Michigan) and given access to water. They were then catheterized in a marginal ear vein with an 8-inch 22-gauge Intracath catheter (Deseret Pharmaceutical Co., Sandy, Utah). The catheter was taped to the ear and maintained by periodic infusion of normal saline with 50 units/ml heparin (Liquaemin^{R} Sodium, 1000 USP units/ml; Organon, West Orange, N.J.) in Monoject Disposable Tuberculin Syringes (1cc, 5cc, Sherwood Medical Industries Inc., Deland, FL.). Blood sampling was done with a Luer 3-way nylon stopcock (Bio-Rad, Richmond, CA) connected to the catheter. One syringe wetted with the heparin-saline was used to collect the blood and another syringe, filled with the heparin-saline, was used to flush the catheter.

Preparation of Liposomes. Mechanically dispersed (MD) or French press (FP) liposomes containing a) a lipid label

(^{14}C-cholesterol or ^{3}H-DPPC) b) ^{14}C-inulin or c) ^{14}C-sucrose were prepared as in Chapter II. Nonentrapped marker was removed with Sephadex column chromatography immediately prior to use.

Rabbit Studies. Doses were given intravenously in Ø.5-1.Ø minute infusions. Dose volumes were 1.Ø-1.8 ml. Blood samples were taken with time and kept at room temperature in 13X1ØØ mm culture tubes (Pyrex, McGaw Park, Ill.) until they could be spun down to obtain the plasma (less than 15 min.). Blood samples ranged from Ø.4 ml (early times) to 1.5 ml (later times), and care was taken not to remove more than 5-7 percent of an animal's blood volume over the 24 hours. The following studies were done:

a) Rabbits were given neutral French press liposomes (PC/CH = 7/3 molar ratio composition) at 1Ø mg lipid/kg, dual-labeled with ^{14}C-cholesterol and ^{3}H-DPPC.

b) Rabbits were given neutral French press liposomes (PC/CH = 7/3 composition) at 1Ø mg lipid/kg, labeled with ^{14}C-inulin (present at 5mM in the dispersal buffer).

c) Rabbits were given negative French press liposomes (PC/PA/CH = 7/1/3 composition) at 1Ø mg lipid/kg, labeled with ^{14}C-inulin as in b) above.

d) Rabbits were given neutral liposomes (PC/CH = 7/3 composition), either French press or MD, labeled with

entrapped ^{14}C-sucrose (at 5mM in the buffer).

e) Rabbits were given either free ^{14}C-inulin or free ^{14}C-sucrose in buffer (5 mM).

Treatment of Blood samples. Blood samples were centrifuged at low speed and aliquots of the plasma were counted for radioactivity as in Chapter II. Hematocrit was estimated by measuring plasma and red cell fraction volumes by pipetting (all values fell within the Ø.3-Ø.35 range).

Results.

Figures III-1 and 2 show the results of the studies using the dual lipid-labeled neutral French press liposomes. Fig. III-1 reveals a multiphasic falloff curve for both the ^{14}C-cholesterol and the ^{3}H-DPPC labels. A total of six rabbits received this liposome type, but two of them developed upper respiratory distress during the experiment. The plasma data for these two differed markedly from those of the healthy animals in that the healthy rabbits all showed two distinct log-linear phases for the ^{3}H label, while the unhealthy rabbits exhibited just one. The biphasic nature of the curves for the healthy rabbits is lost in the averaging, and so this information is given in Table III-1. This observation suggested that the liposomes' disposition may depend on the animal's state of health, and thus the data for the sick rabbits was not included. In addition, one healthy rabbit's catheter failed at 5 hours. Figure III-1

also reveals significantly different plasma levels for the two labels (on a percent dose basis) from 2 to 8 hours. The fact that the two labels are declining at different rates is shown clearly in Fig. III-2, in which the ratio in plasma of ^{3}H to ^{14}C in each rabbit is calculated and then averaged for all rabbits. There is a clear and significant change in the ratio, indicating an initially faster rate of removal of the cholesterol label and at later times a faster decline of the DPPC label. The data for all rabbits also exhibited a very early phase (Ø-.75 hours) in which the plasma levels oscillated. Fig. III-3 shows typical data from one rabbit during this time period (most of these very early (.Ø5-.15 hr.) points were left out of Fig. III-1 for clarity).

The effect of liposome surface charge on the plasma disappearance of the label is shown in Fig. III-4. After neutral liposome administration the inulin label is present at much higher levels than after negative liposomes. Both, in turn, are higher than levels after free inulin (average data from two rabbits). The liposome curves also exhibit a multiphasic nature on this semilog plot.

Fig. III-5 shows the effect of liposome size on the disappearance rates. The small (French press) liposomes show much higher plasma levels with time than do the large liposomes, and most of this difference can be attributed to the different falloff rates in the first hour. The curve for free ^{14}C-sucrose (not shown) was very similar to that

for free inulin and fell below the curve for the large liposomes.

Discussion.

The data presented here suggest that liposome properties such as size and surface charge are important determinants of their fate in vivo, and as such are in general agreement with the earlier findings of Juliano and Stamp (79). It is significant that these two factors have also been implicated, with the same qualitative findings, for other colloidal materials (16). However, following the in vivo fate of liposomes is much less straightforward than for the inert colloids due to some of the interactions of the liposomes with the biological environment.

The first step in monitoring the liposomes' fate is the choice of marker, specifically whether it should be a polar (in the entrapped aqueous spaces) or nonpolar (bilayer-associated) compound. The results of the dual lipid label studies imply that at least one of the labels was giving a false indication of the liposome plasma levels. This was expected, since lipid exchange processes are known to occur in the blood between the various lipid depots (40). This does not mean that all lipid labels are unreliable, but it does mean that the potential for lipid exchange should be kept in mind. The dual label studies also indicated that liposome disposition may depend on the animal's state of

health. This finding is reasonable since liposome fate is thought to depend in part on RES activity, which in turn is known to be affected by infection and general state of health (16). Finally, the data in Fig. III-2 suggest that simple mechanical interaction may be involved in the disposition - the oscillating levels between Ø-.75 hours are very similar to the gradually dampening blood level oscillations seen after administration of inert microspheres (2Ø), presumably resulting from the lodging, displacement and relocation of particles in "wave form" as they traverse the various capillary beds.

The alternative is the aqueous space marker. Ideally this should be a compound that is completely restricted to the aqueous spaces so that it doesn't confer any added properties on the liposome; this is important in identifying the properties of a given liposome type. This type of marker has the advantage that as long as the bilayer structure remains intact (regardless of lipid exchange processes) it will stay in the liposome. Its principal disadvantage is that the liposomes' blood-induced leakiness will result in label release. In such cases, however, the kinetics of the remaining intact liposomes may still be monitored if the clearance of the free (i.e. released) label is much greater than that for the entrapped label. ^{14}C-inulin was chosen as the aqueous space marker for these experiments because it fulfills these criteria--it's a large, polar molecule and so

shouldn't interact with the bilayer. As Fig. III-4 shows, the half-life for free inulin is short enough so that it can be used in comparing the plasma kinetics of the various liposome types. For cases in which the kinetics of the free and entrapped marker are similar, the liposomes' fate can be quantitated by separating the free and entrapped marker in the plasma sample using column chromatography (Chapter II).

The results of the surface charge study (Fig. III-4) provide a good example of how these stability (leakiness) aspects can affect the interpretation of liposome data. It has been shown (41) that negative liposomes are less stable in plasma (i.e. leak a greater fraction of their contents) than are neutral or positive ones. This effect probably causes, in part, the lower plasma levels for the negative liposomes, since the free inulin is excreted faster than liposome-entrapped inulin. However, the surface charge probably exerts an additional, non-stability effect because these results have been seen for the plasma clearances of other, non-degradable colloids (16).

It was also found here that larger liposomes were removed more rapidly from the blood than smaller ones. This size effect has been used (79) to explain why multiphasic curves are usually seen after administration of heterogeneous-sized liposome batches (i.e. different sizes are removed at different rates). However, multiphasic curves were seen here for the FP liposomes, which are very

homogeneous (61,62). These findings argue against too specific an interpretation of liposome plasma/blood curves (e.g. if single or multiexponential, or making designations analogous to distribution or elimination phases). Part of the complexity surely arises because of exchange (for lipid labels) or leakage (for aqueous); in the latter case the free and entrapped label each has its own distribution and elimination characteristics. Thus, although there are significant differences in the plasma curves of the various liposome types, they are the combined result of several processes that can affect apparent liposome disposition.

Several factors argued against continuing these studies in elucidating the role of the RES in liposome disposition. First, although plasma studies are informative, they aren't capable of showing what transpires at individual RES organs. In theory the sum total rate of organ uptake of intact liposomes can be measured with plasma data and might be valuable in indicating what is happening at the organ level. However, the liposomes are also undergoing breakdown (leakage), and distinguishing the two processes isn't technically easy, especially when the kinetics of the free and entrapped marker (inulin) are so different. Thus, the next series of in vivo studies (Chapters V, VI, and VII) was done in mice so that liposome fate in individual RES tissues could be followed.

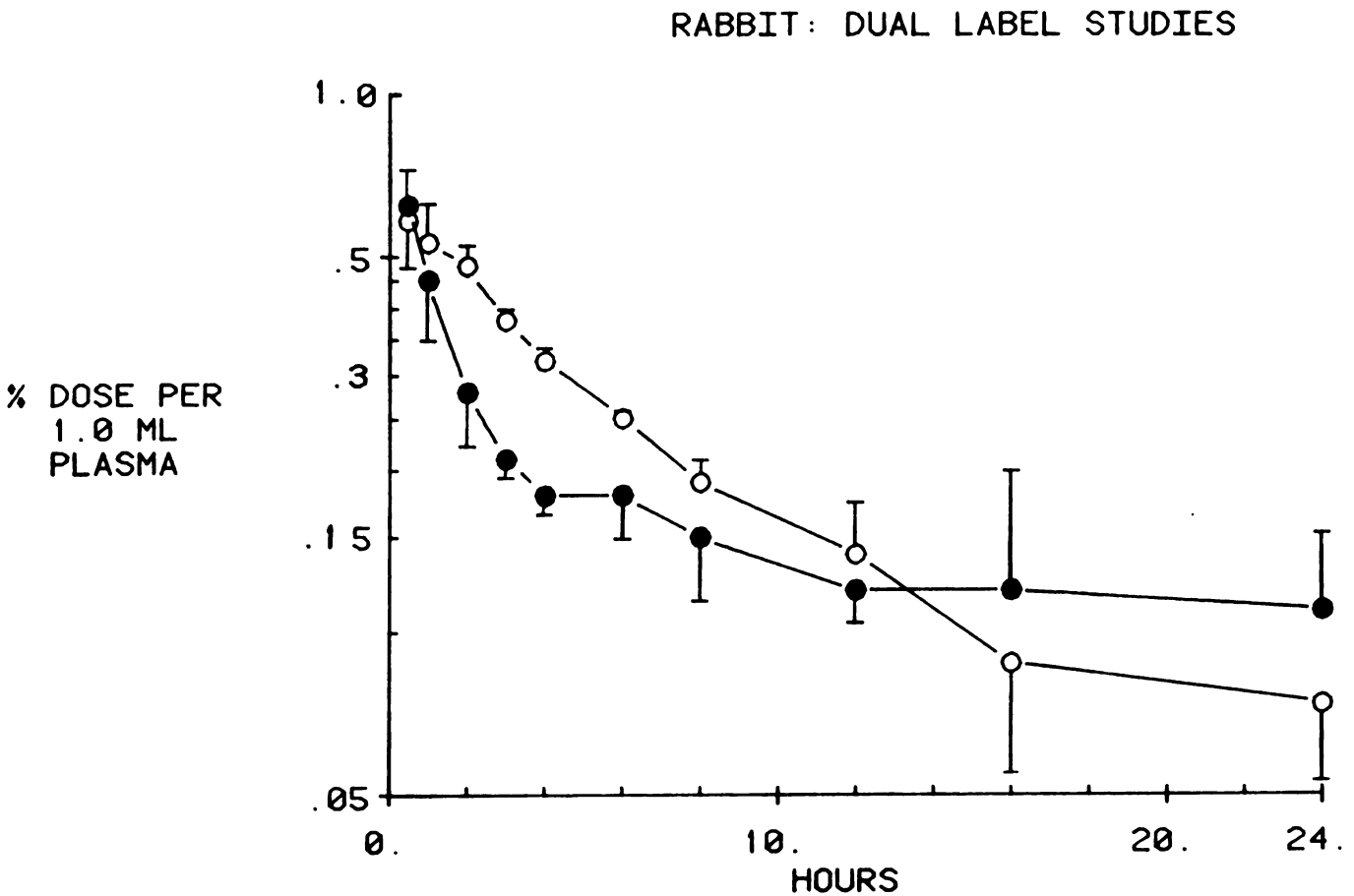


Figure III-1. Plasma levels after administration of 10 mg lipid/kg liposome doses to rabbits. Liposomes were of the PC/CH = 7/3 composition, labeled with 14C-cholesterol (closed circles) and 3H-DPPC (open circles). Values are given as % dose per 1.0 ml plasma. Mean plus or minus one S.D. (n=3).

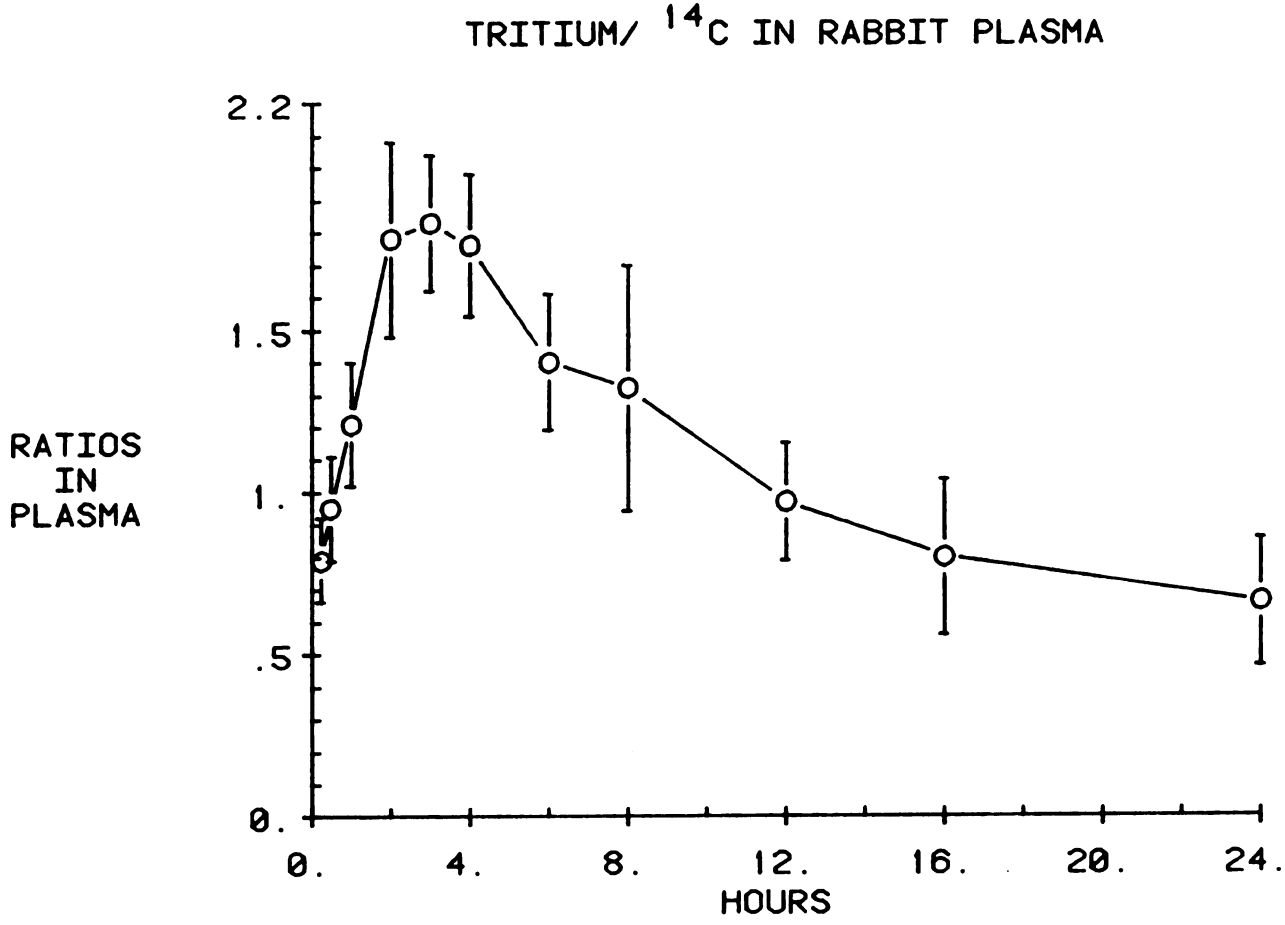


Figure III-2. Ratios of ^{3}H to ^{14}C in plasma for the dual lipid label study in rabbits. Details as in Fig. III-1.

Table III-1. Plasma level data parameters for the dual lipid label studies.

Rabbit No.	Sick?	^{3}H label alpha phase halflife (hours)	^{3}H label alpha phase time period (hours)	^{3}H label beta phase halflife (hours)
1	no	4.5	0.5-10	14
2	yes	12	1-24	a
3	no	5	1-8	16
4	no	5	0.5-5	b
5	no	5	1-14	14
6	yes	11	1-24	a

a: no beta phase evident
b: rabbit catheters failed at 5 hours

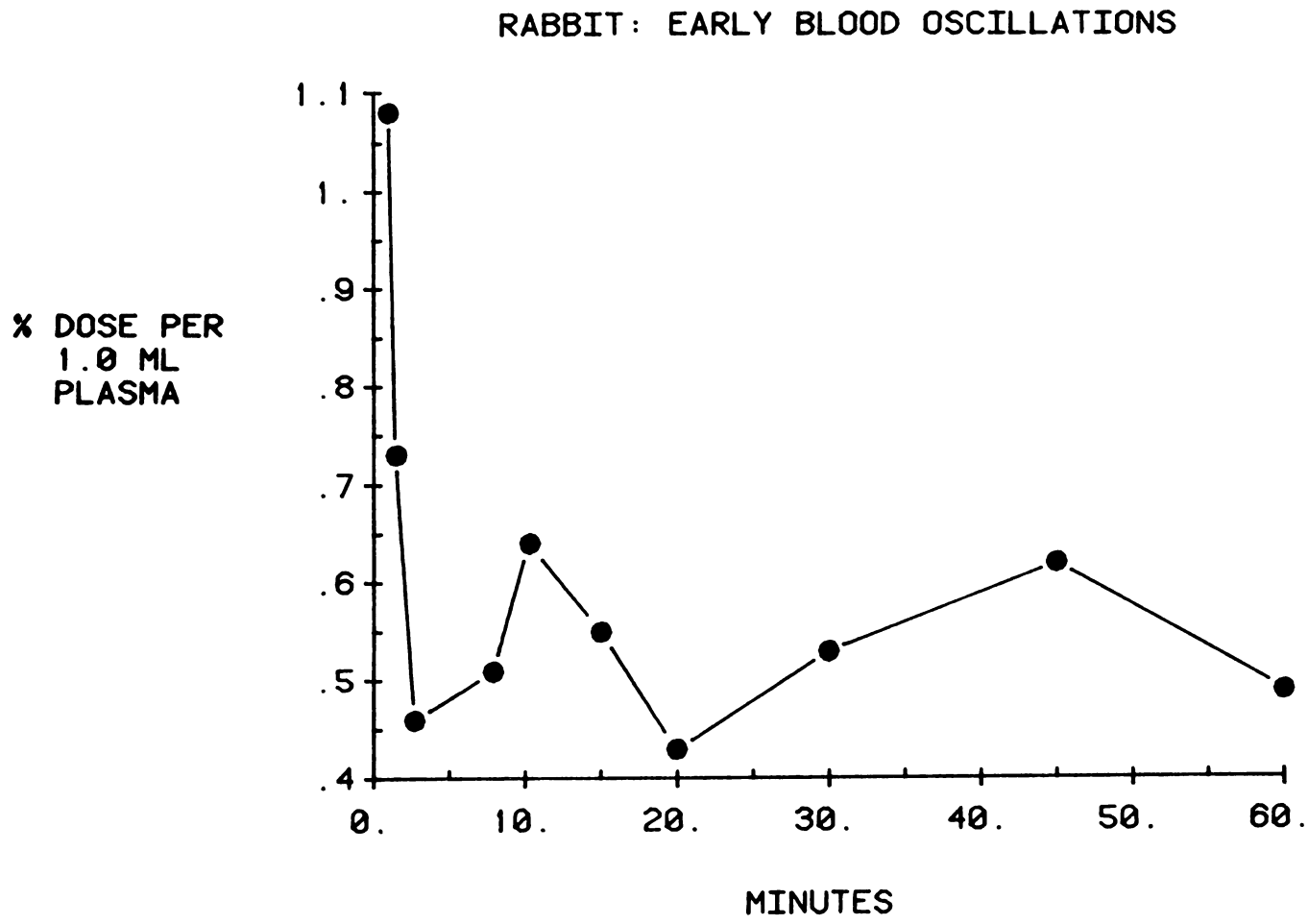


Figure III-3. Oscillating nature of the ^{3}H-DPPC levels at early times in the rabbit dual lipid label study. Data for one representative rabbit are shown. Other details are as in Fig. III-1.

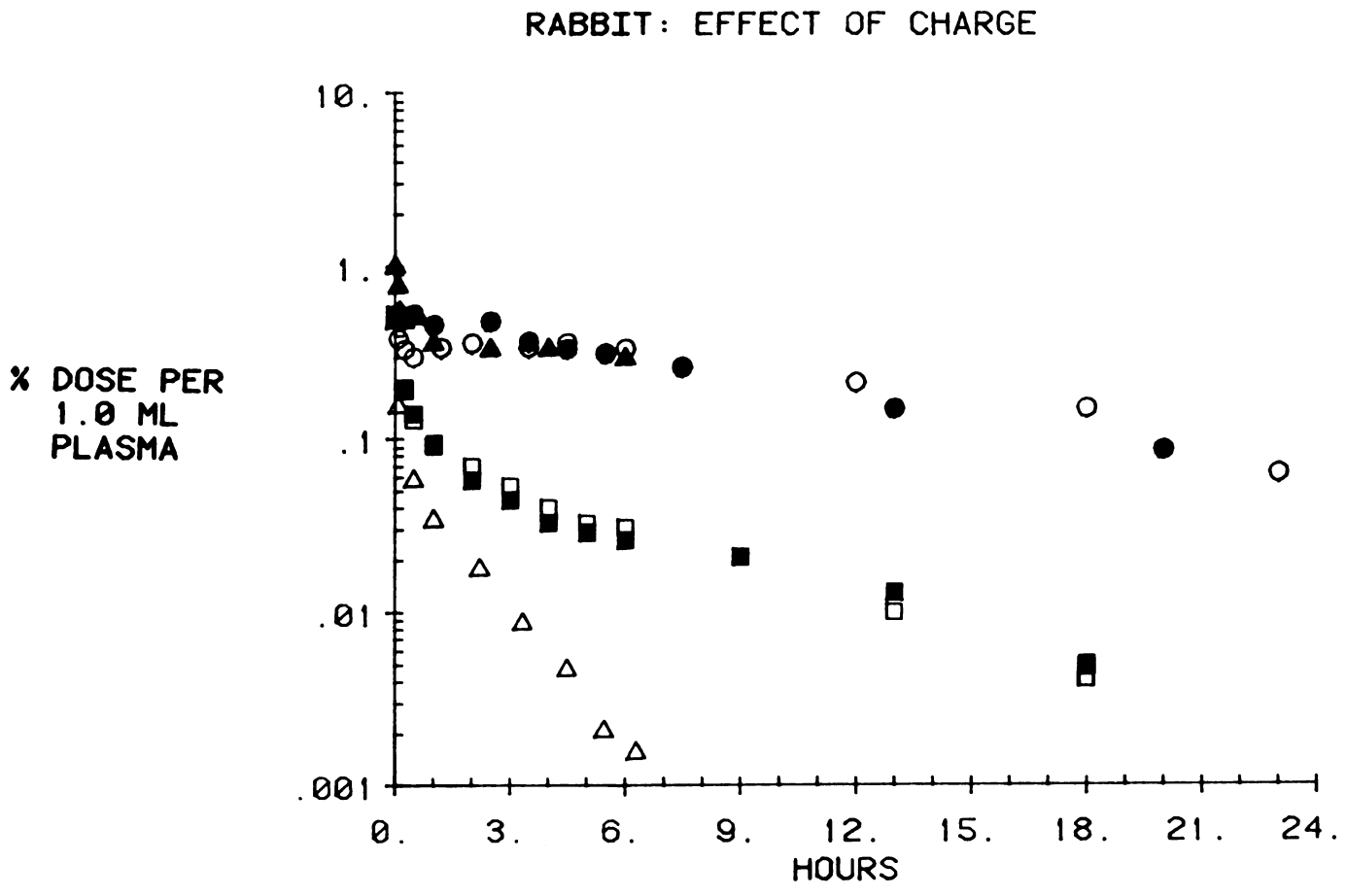


Figure III-4A. Effect of liposome surface charge on the plasma levels of 14C-inulin entrapped in small unilamellar (French press) liposomes administered at 10 mg lipid/kg. Circles and closed triangles are for neutral liposomes with the PC/CH = 7/3 composition (three rabbits), squares are for negative liposomes of the PC/PA/CH = 7/1/3 composition (two rabbits). Open triangles are for free inulin (average of two rabbits).

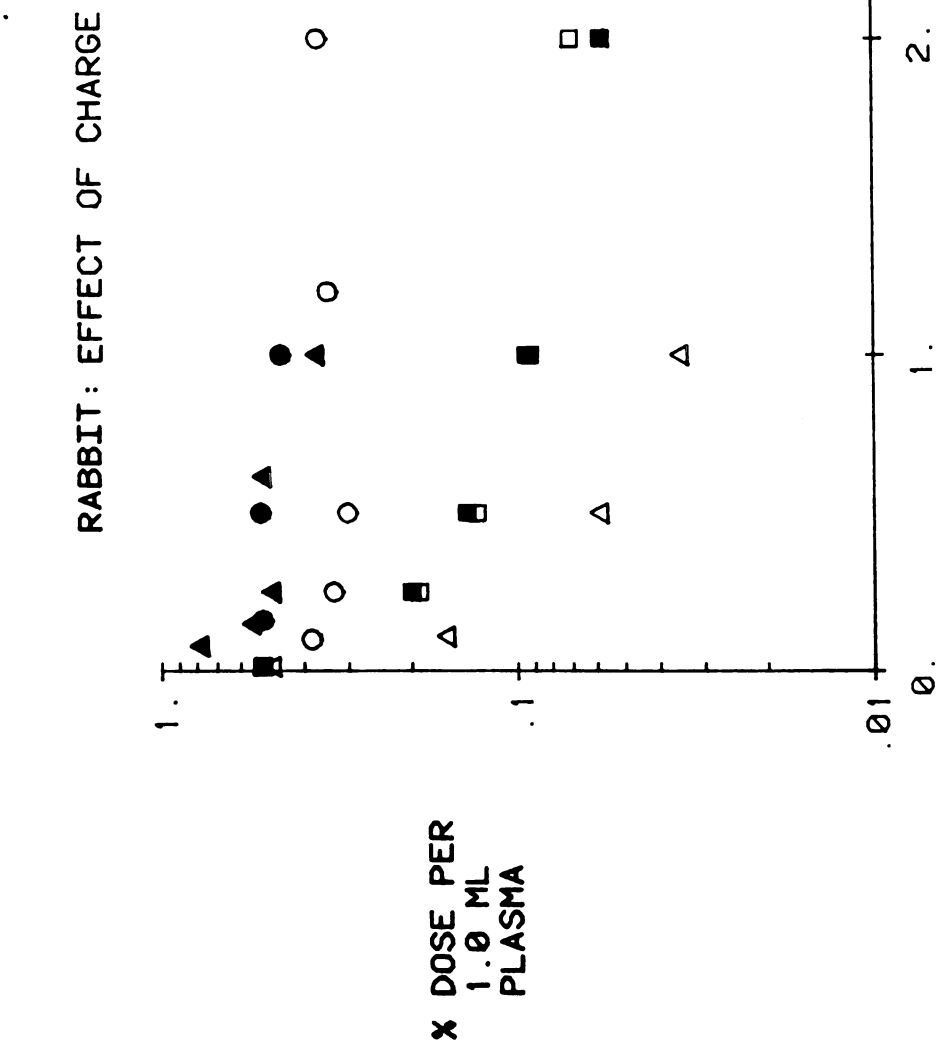


Figure III-4B. Expanded presentation of the early data in Fig. III-4A.

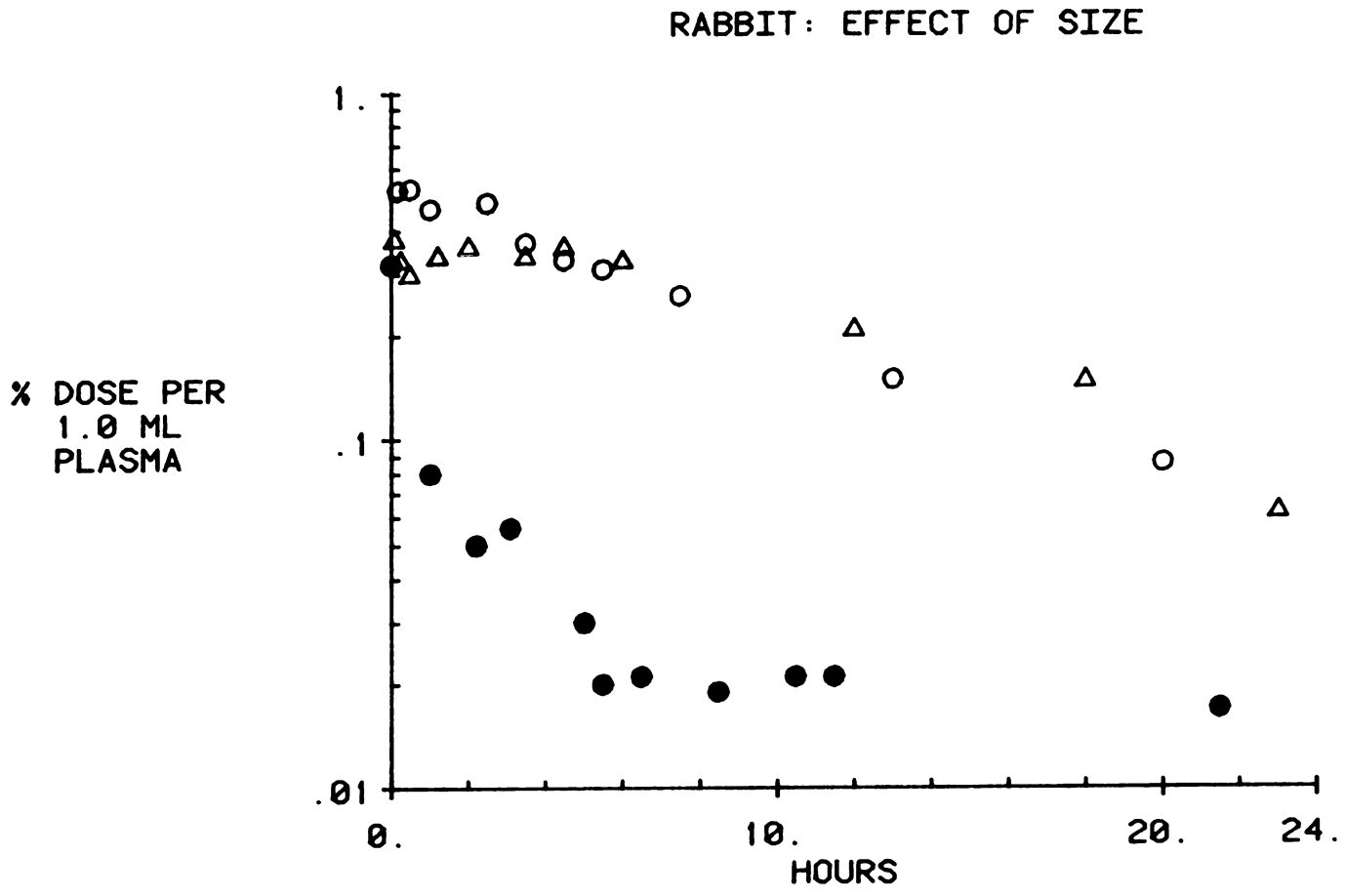


Figure III-5. Plasma levels after administration of ^{14}C-sucrose in French press or large multilamellar liposomes at 10 mg lipid/kg and of the PC/CH = 7/3 composition. Open figures are for French press (two rabbits). Closed circles are for large liposomes (one rabbit).

CHAPTER IV: LIPOSOME DIALYSIS FOR IMPROVED SIZE DISTRIBUTION

Introduction.

It is well recognized that liposome properties, both as model membrane and drug carrier systems, are highly dependent on their size (79). The differences in plasma time course and tissue distribution seen between large multilamellar and small unilamellar vesicles are now well established (106), and even different size classes of large multilamellar vesicles can have significantly different pharmacokinetics (75). Unfortunately, the size distribution of the original multilamellar preparation described and characterized by Bangham (24) is very heterogeneous and poorly reproducible. The use of this preparation for *in vivo* tissue distribution studies may thus mask some differential effects of size; indeed, complex blood level time courses have been attributed by some investigators to the heterogeneous size distribution of the administered liposomes (79). Several procedures have attempted to improve the size distribution or encapsulation efficiency of the liposome preparation: sonication and centrifugation (53,54), detergent dialysis (55-57), injection methods (58-60), French pressure cell (61,62), reverse phase evaporation (63), and others (73,74). All suffer from at least one of three faults: (i) the resulting size range is too wide or unpredictable (ii) the method produces liposomes in only one size range or (iii) there are restrictions on composition or

specific solutes, e.g. calcium or EDTA. However, a significant improvement in size distribution can be obtained by extrusion of a heterogeneous population through straight bore pore polycarbonate membranes of defined pore size (64,65); this defines the upper size limit for the population but does not affect the lower end of the size distribution (control of the lower end may be critical if liposome pharmacokinetics depend in part on the surface area or number of injected liposomes). In this chapter an improvement on the extrusion technique is made when the extruded liposomes are subsequently dialyzed against the same type of membrane with the object of removing the smallest liposomes, and thus narrowing the size distribution from its lower end. Such dialysis can also be used to characterize the size distribution.

Materials and Methods.

Chemicals. All chemicals and reagents were as in Chapter II.

Preparation of Liposomes. Mechanically dispersed (MD), extruded, or French press (FP) liposomes were prepared as in Chapter II. They were composed of PC/PA/CH/a-T in the molar ratios 4/1/1/.05 or 4/1/4.5/.1 and were made at 10 micromoles lipid/ml. In most cases ^{14}C-cholesterol was used as liposome marker; in several control studies ^{14}C-sucrose was used as the marker. Dialysis of liposomes across the Uni-

pore membranes was done at 5°C as described in Chapter II. Unless otherwise specified, the dialysate side was changed frequently to maintain sink conditions.

Electron Microscopy of Liposomes. For electron microscopy, aliquots of the liposome suspensions were diluted two-fold with a 25 percent glycerol solution and kept at room temperature for two hours afterward. The samples were then quickly frozen in liquid freon at approximately -155°C and then stored at -190°C in liquid nitrogen. Stored samples were transferred to the specimen stage of a freeze-fracture device (Balzers, Berkhamsted, U.K.), fractured at -115°C, and then platinum-carbon coated. The replicas thus formed were cleaned in dilute hypochlorite overnight, then rinsed in several successive distilled water baths for ten minutes each. A final 30 second acetone rinse removed any remaining lipid. Replicas were then picked up on flamed 200 mesh grids and viewed on a Siemens 1A electron microscope at 80 KV. Magnification was determined using a carbon calibration grid.

Results.

Dialysis of Liposomes. Figures IV-1A and 1B show the results of studies in which crude multilamellar (MD) and French press (FP) liposomes (both labeled with ^{14}C-cholesterol and of the PC/PA/CH/a-T = 4/1/4.5/.05 molar ratio composition) were dialyzed with membranes of various

pore sizes. In both cases there is a clear trend of more rapid and extensive dialysis as the membrane pore size increases. Fig. IV-2 shows the results of a study in which Ø.6, Ø.4, and Ø.2 micron-extruded and FP liposomes were dialyzed against Ø.8 micron membranes. Here there is a clear trend of more rapid and extensive dialysis as the liposome size decreases. Fig. IV-3 shows the combined results of three separate studies in which 1.Ø micron-extruded liposomes (again with the 4/1/4.5/.Ø5 composition) were made and treated as follows: half of the batch was predialyzed for 2Ø hours with Ø.8 micron membranes, the other half with .Ø5 micron membranes (without monitoring ^{14}C). These sub-batches were then dialyzed again, both with Ø.8 micron membranes, for 72 hours (with ^{14}C levels monitored, as shown in Fig. IV-3). There is a significant difference in dialysis patterns due to the predialysis. Samples of both sub-batches, taken after the predialysis step, were saved for electron microscopy.

Another study similar to that depicted in Fig. IV-3 (and with the same liposome composition and label) was done to show the effects of the dialysis on a very heterogeneous population. A liposome batch was made consisting of 1.Ø micron-extruded liposomes and FP liposomes (7Ø percent and 3Ø percent, respectively, of the total lipid present). A sample of this heterogeneous batch was saved and the rest was dialyzed for 21 hours with 1.Ø micron membranes. Elec-

tron microscopy was then done on the dialyzed and undialyzed samples (see below).

Control Studies. The effect of the dialysis system on liposome stability was assessed as follows: 0.8 micron-extruded liposomes of either the PC/PA/CH/a-T = 4/1/1/.05 or 4/1/4.5/.1 composition, containing ^{14}C-sucrose as label, were dialyzed against 0.05 micron pore size membranes; these two compositions (differing in cholesterol content) were chosen as medium and high stability liposomes (41). It was previously established that these same liposome types, when labeled with ^{14}C-cholesterol, would not dialyze across .05 micron membranes, and so the appearance of ^{14}C-sucrose on the dialysate side would be evidence of liposome instability. It was found that after 24 hours of dialysis 3 and 0 percent, respectively, of the entrapped sucrose had leaked from the less stable and more stable liposome types.

For the case where the label does dialyze across, it was important to know if the liposomes dialyzed across intact. To test this, 0.4 micron-extruded liposomes of the PC/PA/CH/a-T = 4/1/4.5/.05 composition containing ^{14}C-sucrose were dialyzed to equilibrium against 3.0 micron membranes (the size distribution of these liposomes is such (64) that all are small enough to dialyze across). Samples of the dialysate side were chromatographed on a Sephadex G-50-80 column, and all of the radioactivity appeared in the void volume, indicating that the liposomes had dialyzed

across intact.

Other control experiments further characterized the system. It was found that the dialyses were concentration-dependent; that is, with all other variables held constant (e.g., liposome size, membrane) samples with higher lipid concentration will dialyze more slowly, and to a lesser extent, than very dilute samples. The agitation rate and the cell fill volume were also studied. It was found that three different agitation rates (i.e. motionless, gentle test tube inverter, and the horizontal shaker method described in Chapter II) gave dialysis rates that covered a six-fold range. It was also found necessary to leave a head space of air when filling the cells, so that the dialyzing solution had the opportunity to agitate sufficiently. However, when these various conditions are held constant the dialyses are very reproducible.

Electron Microscopy and Liposome Sizing. Freeze fracture electron micrographs were done for the 1.Ø-extruded and for the mixed batch (1.Ø + FP). Examples of dialyzed and undialyzed samples from the latter batch are shown in Fig. IV-4. These show very clearly that the dialysis removed most of the very small liposomes from the preparation. Apparent liposome diameters were measured directly from micrographs in both experiments with a ruler or calipers and apparent size distributions were constructed by assigning each liposome to a size category of one mm width, e.g. 1-2 mm, 2-3

mm. Diameters of liposomes with oblong shape were calculated by averaging the values for their long and short axes. For each liposome type and treatment several micrographs were used. For the 1.Ø + FP mixture, measurements were made from 8Ø7ØX micrographs, except for the smallest liposomes, which were measured on 26,Ø9ØX blowups of regions on the same 8Ø7ØX micrographs. On the 26,Ø9ØX micrographs all liposomes with diameter Ø-3.3 mm were counted; on the 8Ø7ØX micrographs all liposomes greater than 1.Ø mm were counted. Thus, all liposomes less than 1.Ø mm on the 8Ø7ØX micrographs were determined by scaling up the results for the 26,Ø9ØX blowups. For the 1.Ø micron-extruded experiments, 6472X micrographs alone were used. The total number of liposome profiles measured and counted in each experiment is shown in Table IV-1. There are, however, certain inherent biases in the freeze fracture/cross sectional method of sizing. First, a given fracture plane may not make an equatorial cut through the liposome, making its apparent diameter smaller than its actual one. Second, it is more probable that a large liposome will be cut than a smaller one, for any given fracture plane; this probability is directly proportional to the liposome's diameter, and this factor has the effect of making the apparent average liposome size larger than the actual average size. The direction that the overall bias takes depends on factors such as the population size range and the shape of the actual distribution. These biases were corrected using the mathematical approach

developed by Wicksell (66). From these corrected distributions plots of cumulative surface area or cumulative total liposome volume versus diameter were constructed.

Table IV-1 shows the calculated diameters of the liposomes with the average surface area and volume for each category. The corrected and uncorrected frequency versus diameter plots for the dialyzed (i.e., those remaining after dialysis) and undialyzed 1.0 micron-extruded liposomes are shown in Figs. IV-5, A and B, respectively. There is a clear upward shift in average size for the dialyzed liposomes, with a doubling in average corrected diameter (from 0.156 to 0.3 microns). It is also seen that the correction procedure shifts the size distribution to smaller sizes. The corrected cumulative surface area and volume curves, not shown, also exhibited dramatic shifts toward larger diameters for the dialyzed liposomes, which is reflected in the changes in the diameters of the liposomes with the average surface area and volume (Table IV-1). Figures IV- 6A, 6B, and 6C show plots of the corrected frequency, cumulative surface area, and cumulative volume for the dialyzed and undialyzed mixture experiment (1.0 + FP). The size frequency plot (6A) shows a shift toward larger sizes after dialysis, although most of the liposomes were still in the smallest size category. The cumulative area and volume plots (6B,6C) show large shifts, as reflected in the average surface area and volume calculations (Table IV-1).

All differences noted between dialyzed and undialyzed preparations were highly significant; in the case where the fewest number of liposomes was measured (195, Table IV-1) the difference between mean diameters for the uncorrected dialyzed and undialyzed distributions was significant at the p less than 0.01 level (1-tailed t-test using frequency versus log diameter distributions (transformed from Fig. IV-5B)). For the corrected distributions (Fig. IV-5A) the percent difference between mean diameters was greater.

Discussion.

The initial hypothesis was that liposomes could be dialyzed across the same type of membrane used in the extrusion sizing method. The ensuing studies suggested that liposomes could indeed be dialyzed intact across these membranes, and in a very reproducible and predictable manner. That is, a given liposome population's size distribution can not only be narrowed with dialysis, but also characterized by its dialysis patterns against various membranes.

The dialysis data for the FP liposomes also suggested (Fig. IV-2, FP curve) that when the preparation is very homogeneous the dialysis should be first order, and thus that the dialysis rate of a heterogeneous population could be described by a sum of first order processes (or exponentials), one for each narrow size class. Rough estimates of the dialysis rate constants for various sizes were made from

these (Fig. IV-2) and other data, and applied to an undialyzed liposome distribution (Fig. IV-5A) to simulate the dialysis process based on the assumption of first order kinetics. Fig. IV-7 shows a plot of estimated dialysis rate constants against Ø.8 micron membranes, versus liposome diameter. Points on the graph were for ^{14}C-inulin, FP liposomes, and Ø.2 micron-extruded liposomes (it was also assumed that liposomes with diameter Ø.7 microns would not dialyze to any significant extent). Two rate constant estimates were made for the Ø.2-extruded liposomes, assuming diameters of Ø.1 or Ø.18 microns, because these liposomes are not as monodisperse as the FP, and their dialysis pattern is not really first order (Fig. IV-2). Two curves were thus constructed (Fig. IV-7) by hand (no simple mathematical transformations gave linear plots); two sets of estimated rate constants were then interpolated for the arbitrary size categories used in the micrograph counting. Fig. IV-8 shows three plots: the actual post-dialysis distribution for the 1.Ø-extruded liposomes (shown before in Fig. IV-5B), and two simulated distributions, obtained by applying the two sets of estimated dialysis rate constants to the pre-dialysis distribution. It is seen that the simulations did a reasonable job of estimating the dialyzed distribution given the system's limitations: 1) the Unipore pores themselves are not really monodisperse, as the manufacturer claims that maximum pore size is +Ø% to -2Ø% of rated pore size 2) the rate constant estimates were fairly crude and 3) the

potential exists for large liposomes to block pores, and thus impede the dialysis of small liposomes. The results do suggest, however, that the assumption of first order dialysis is reasonable even for heterogeneous preparations.

This dialysis technique offers several significant advantages over currently available methods for removing small liposomes from the distribution: (i) separation by centrifugation is complicated by the fact that liposomes of the same diameter may have different densities (i.e. number of lamellae) and vice versa, (ii) separating sizes by filtration is of little utility because: liposomes above their phase transition are extruded and made smaller (64), and so filtration of the commonly used egg lecithin liposomes, which have their Tc below $0^{\circ}C$, is unfeasible (some filtration can occur at high lipid concentration (64) but it is likely that it is not a size-discriminating process); although liposomes apparently may be retained to some extent by filters below their phase transition (67,68), the effect of their concentration at the filter interface (and caking effects) isn't known;, (iii) gel exclusion chromatography methods are currently limited by the size retention limit (approx. 0.1-0.2 microns for Biogel A-150M (55)) and for the same reason are of no value in characterizing the size distribution of liposomes larger than this, (iv) newer techniques such as field flow fractionation (70), though theoretically adequate, are not yet feasible on a practical

scale. Other methods that give very homogeneous distributions (i.e. sonication, detergent dialysis) are too limited as far as available sizes or are too dependent on very precise control of experimental conditions (4). Finally, the dialysis technique allows removal of nonentrapped solutes (e.g. for ^{14}C-inulin, half-life for dialysis = 1 hour) along with the smaller liposomes.

The advantages of using the dialysis method are thus clear, and relate mainly to the need to control the lower end of the liposome size distribution. A very good case in point is provided with the 1.0-extruded + French press batch, which is very heterogeneous. It was seen that, for the undialyzed case, the smallest size class accounted for 99 percent of the number, 58 percent of the surface area, but only 7 percent of the total liposome volume. Dialysis of these resulted in a tremendous reduction in the number of small liposomes (Fig. IV-4), although it apparently was only 95 percent complete (they still accounted for 94 percent of the total number of vesicles present after dialysis). The effect was that the cumulative surface area and volume curves were much closer together for the dialyzed as compared to undialyzed liposomes. This disparity is a very important consideration when liposomes are prepared for in vivo use, for several reasons. First, the liposomes' fate is usually followed with an entrapped volume marker, which is located predominantly in the larger liposomes. Second,

the liposomes' fate is thought to depend on their interaction with molecules in the biological fluids (6,43), which may be a function of liposome surface area (a significant fraction of which may come from the smallest liposomes). Also, it is very likely that liposomes of different size have significantly different in vivo dispositions. Thus, liposomes prepared for in vivo use must have narrow size distributions that are easily reproducible. As the data presented here demonstrate, this mainly involves defining and fixing the lower end of the size distribution.

In conclusion, the dialysis technique can be used to narrow the size distribution of liposome preparations, and can also be used to characterize the distribution. The chief advantages in using the technique are its reproducibility and its flexibility with respect to choice of extruding and dialyzing membrane pore sizes, allowing a wide range of average liposome sizes.

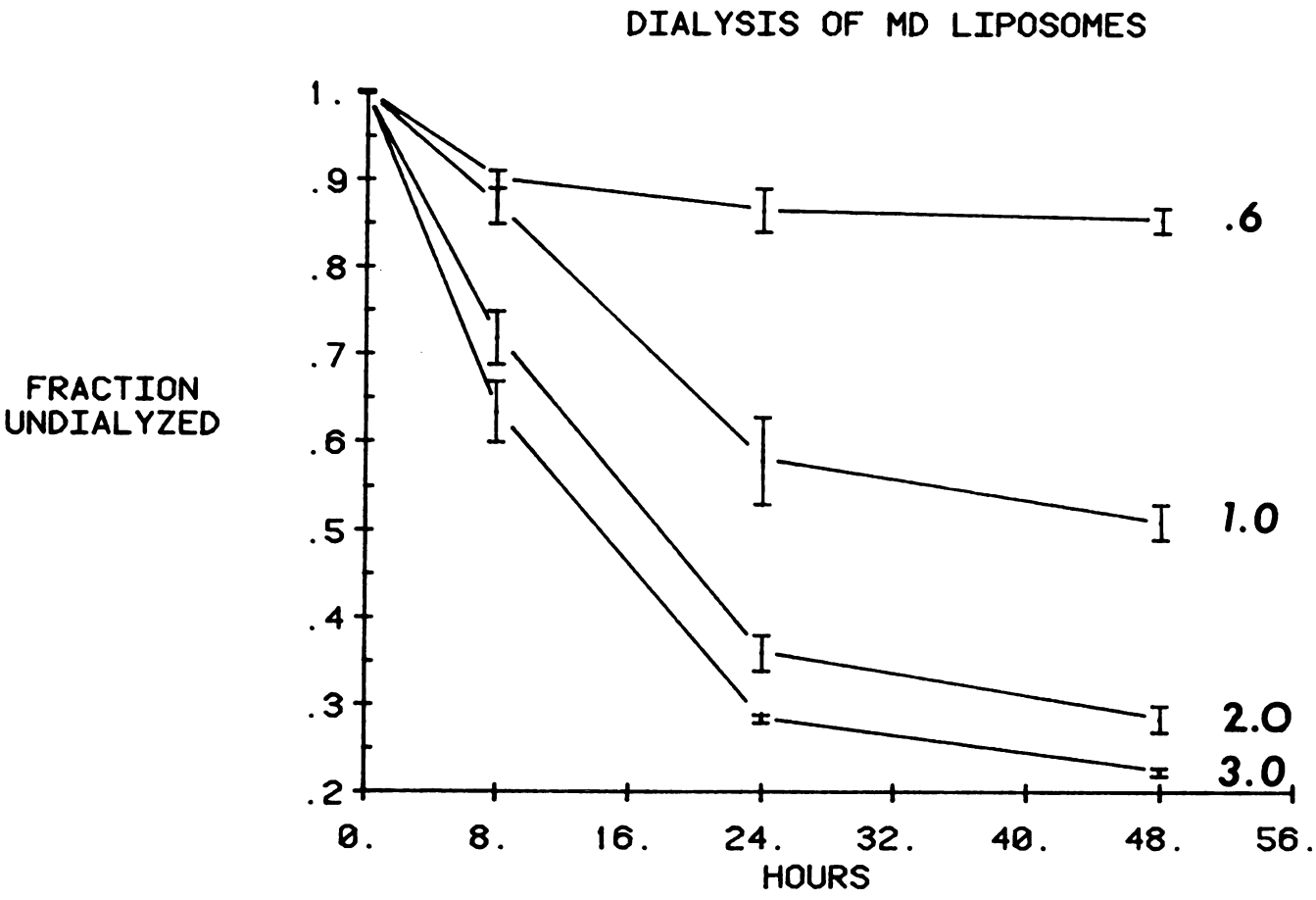


Figure IV-1A. Dialysis of large multilamellar (MD) liposomes with 0.6, 1.0, 2.0, or 3.0 micron pore size Unipore membranes. Liposomes were prepared at 10 micromoles lipid/ml and were labeled with 14C-cholesterol. Ranges (n=2).

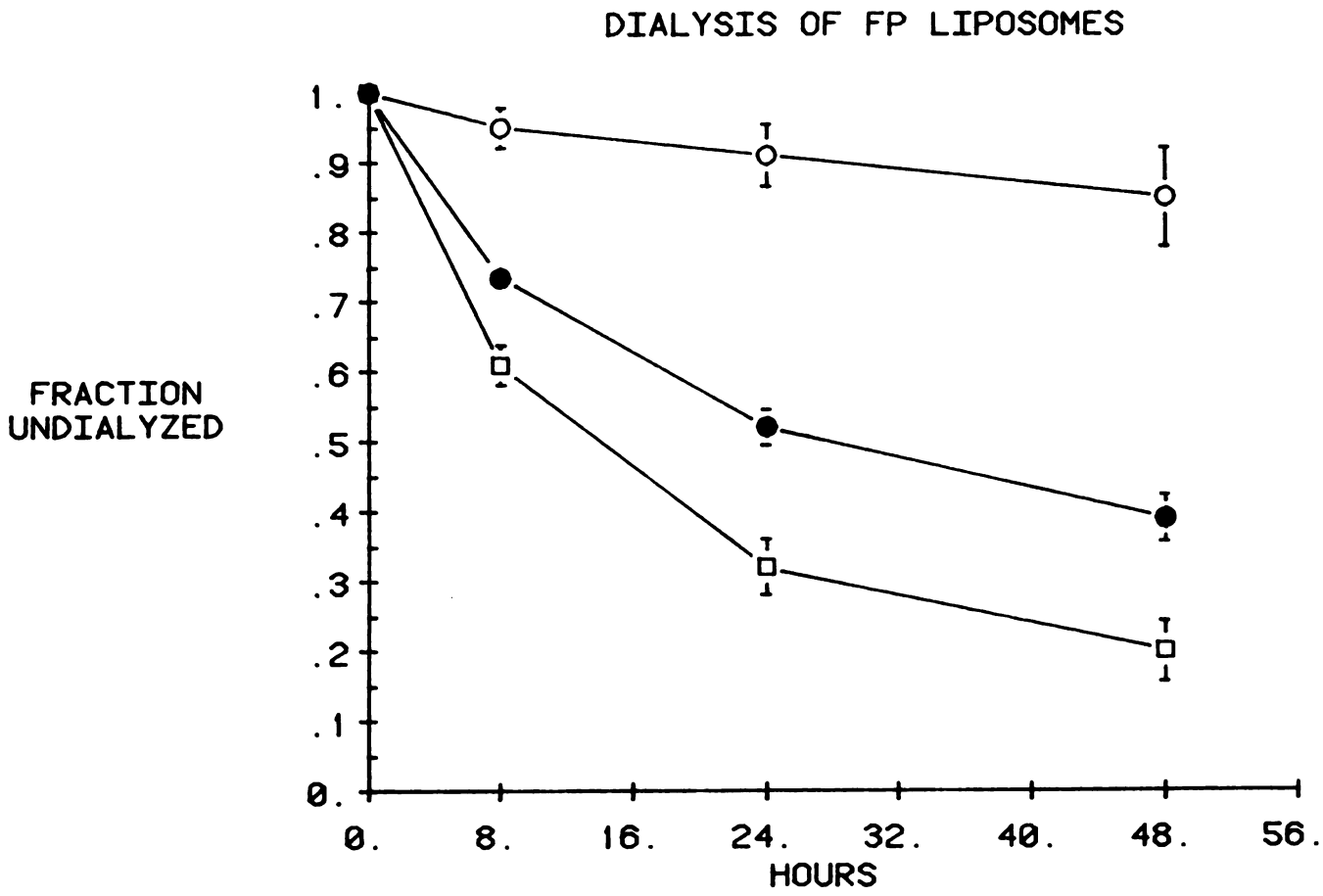


Figure IV-1B. Dialysis of small unilamellar (French press) liposomes with 0.05 (open circles), 0.1 (solid circles), or 0.2 micron pore size (squares) Unipore membranes. Other details as in Fig. IV-1A. Mean ± one S.D. (n=4).

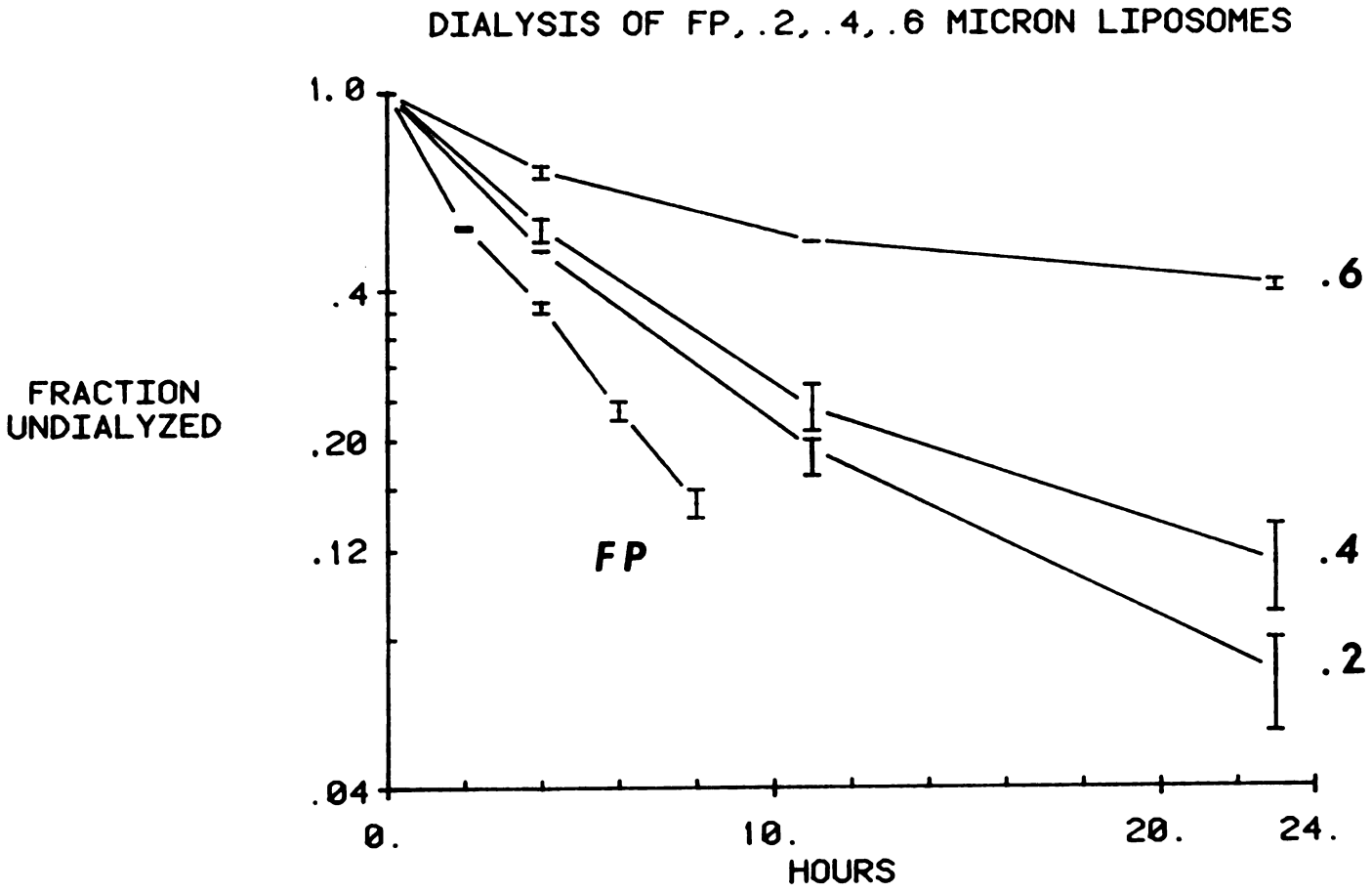


Figure IV-2. Dialysis of various liposome sizes with 0.8 micron Unipore membranes. Sizes dialyzed were 0.2 micron-extruded, 0.4-extruded, 0.6-extruded, or French press liposomes. Other details as in Fig. IV-1A. Ranges (n=2).

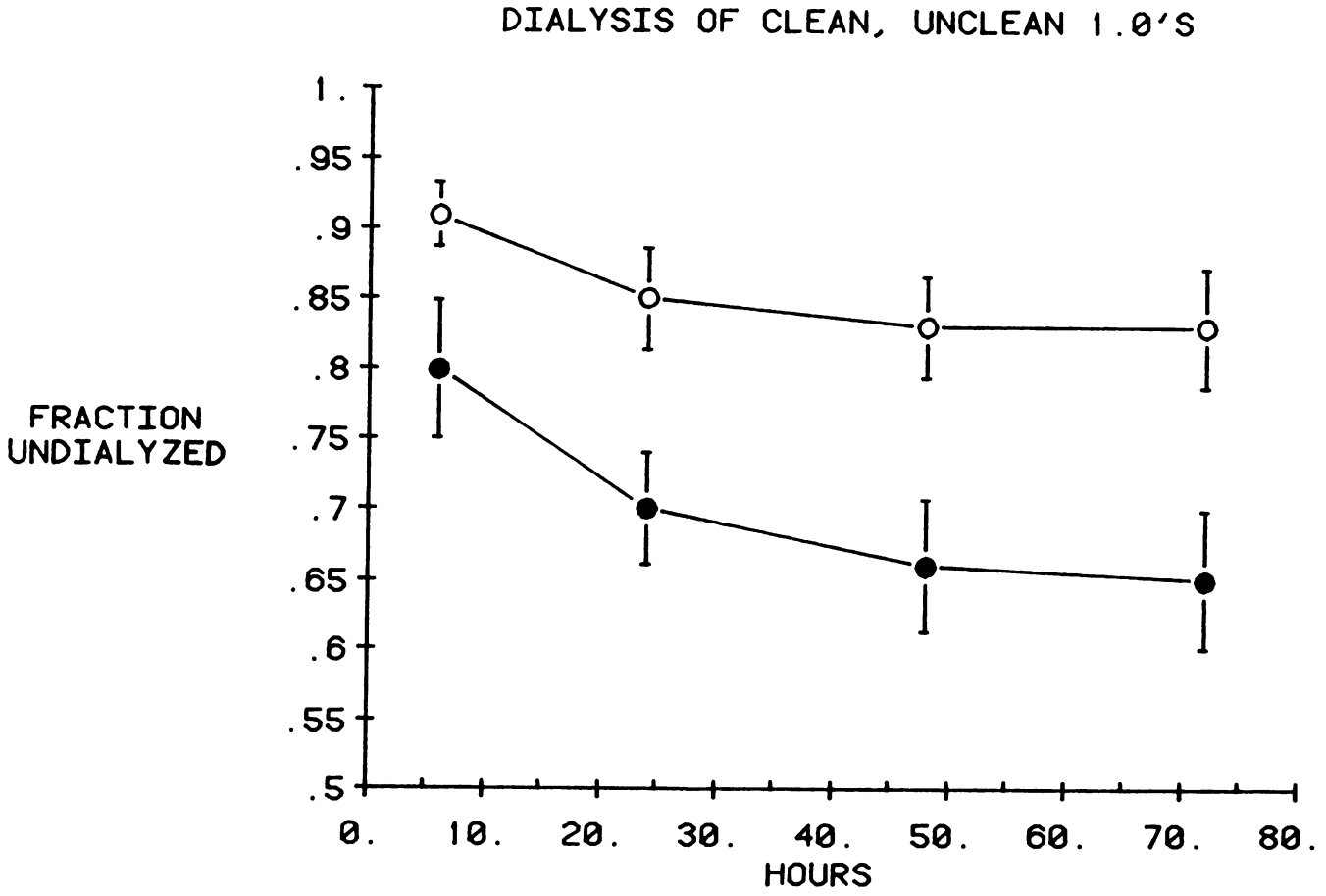


Figure IV-3. Dialysis of two types of 1.0 micron-extruded liposomes with 0.8 micron membranes. One group (closed circles) had been predialyzed with 0.05 micron membranes (i.e. essentially undialyzed). The other group had been predialyzed with 0.8 micron membranes for 20 hours. Other details as in Fig. IV-1A. Mean ± one S.D. (n=9).

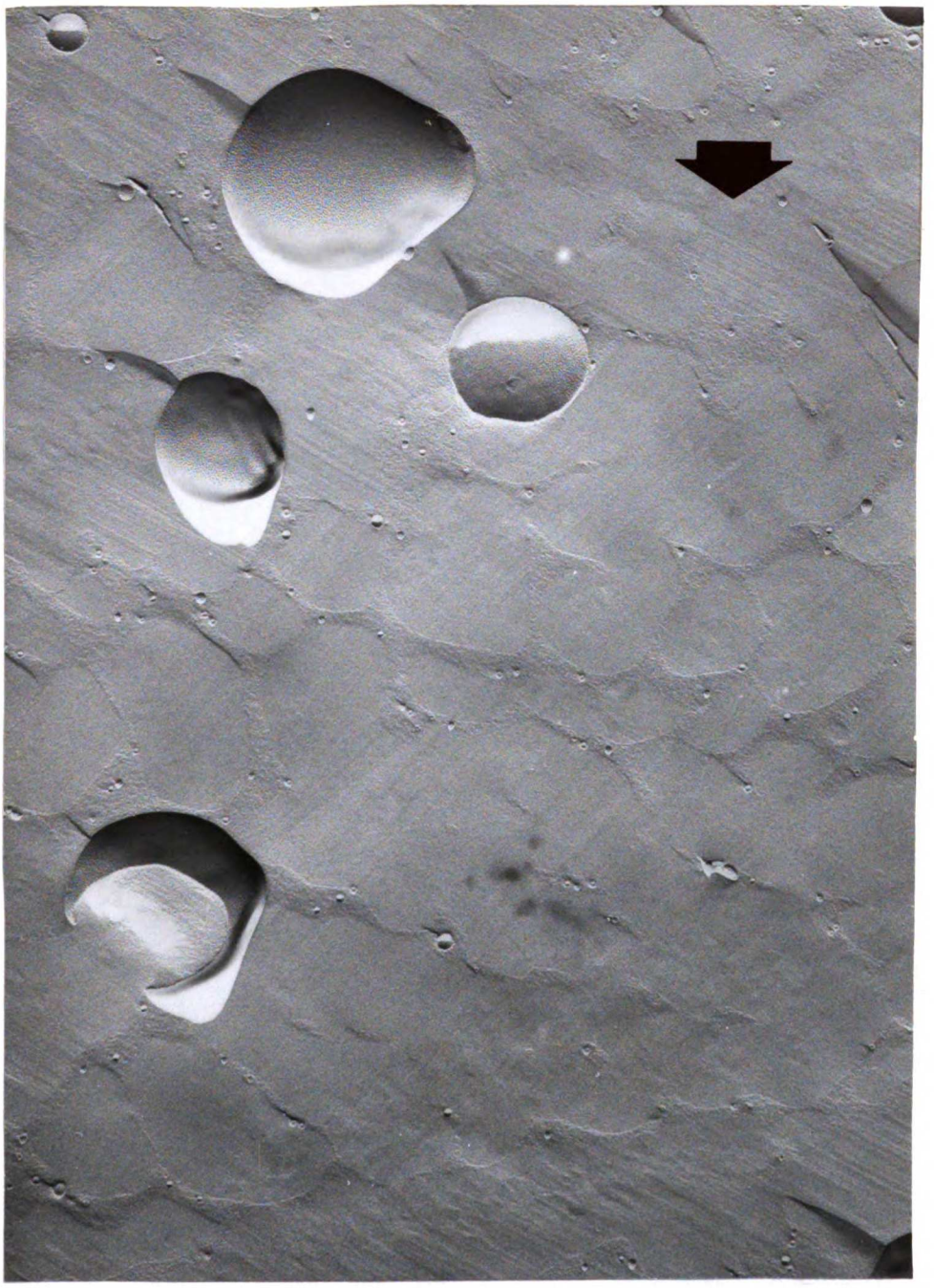


Figure IV-4A. 82,000X freeze fracture electron micrograph of the undialyzed 1.0 micron-extruded + French press mixed batch.

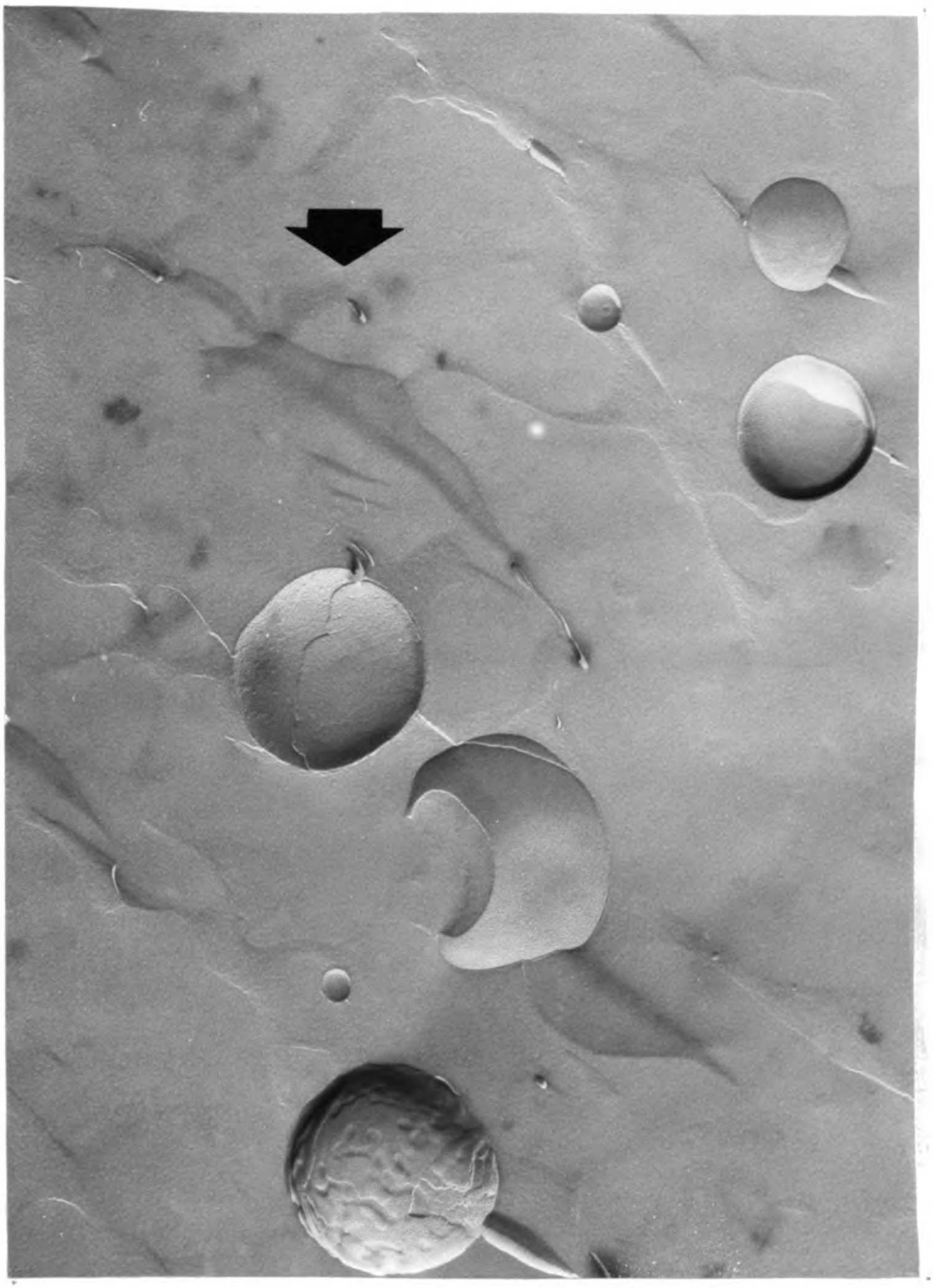


Figure IV-4B. 82,000X freeze fracture electron micrograph of the 1.0 micron-dialyzed 1.0 micron-extruded + French press mixed batch.

Table IV-1: Size data for the 1.0-extruded and 1.0 + FP batches taken from electron micrographs. Liposome diameters are expressed in microns.

Liposome Type	number of liposome profiles counted	corrected average liposome diameter	corrected diameter of the liposome with the average surface area	corrected diameter of the liposome with the average volume
1.0's undialyzed	979	.156	.227	.31
1.0's dialyzed	195	.30	.40	.494
1.0 + FP undialyzed	9923	.033	.040	.076
1.0 + FP dialyzed	1066	.047	.105	.213

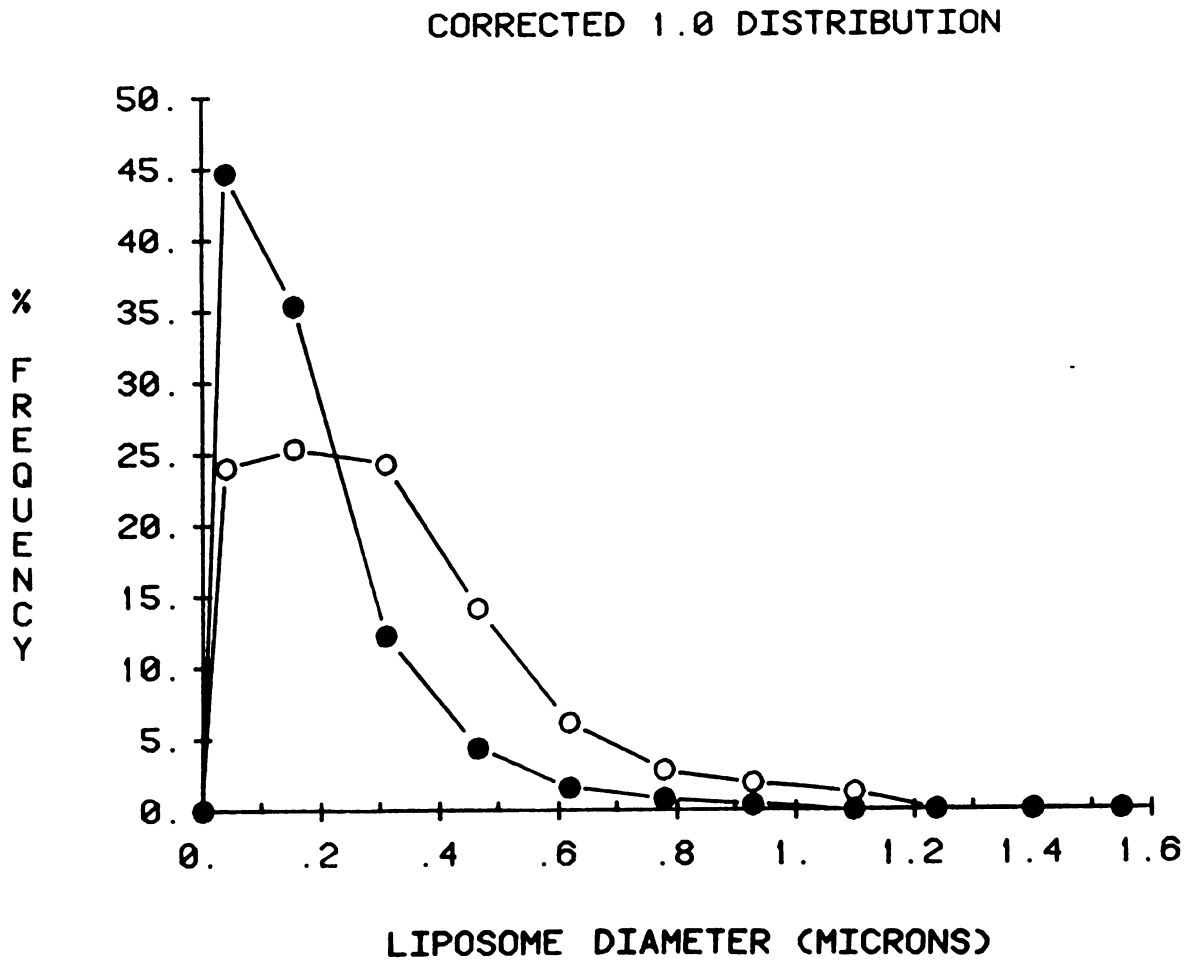


Figure IV-5A. Corrected size frequency distributions for 1.0 micron-extruded liposomes. Closed circles are for undialyzed liposomes. Open circles are for dialyzed liposomes (20 hours with 0.8 micron membranes).

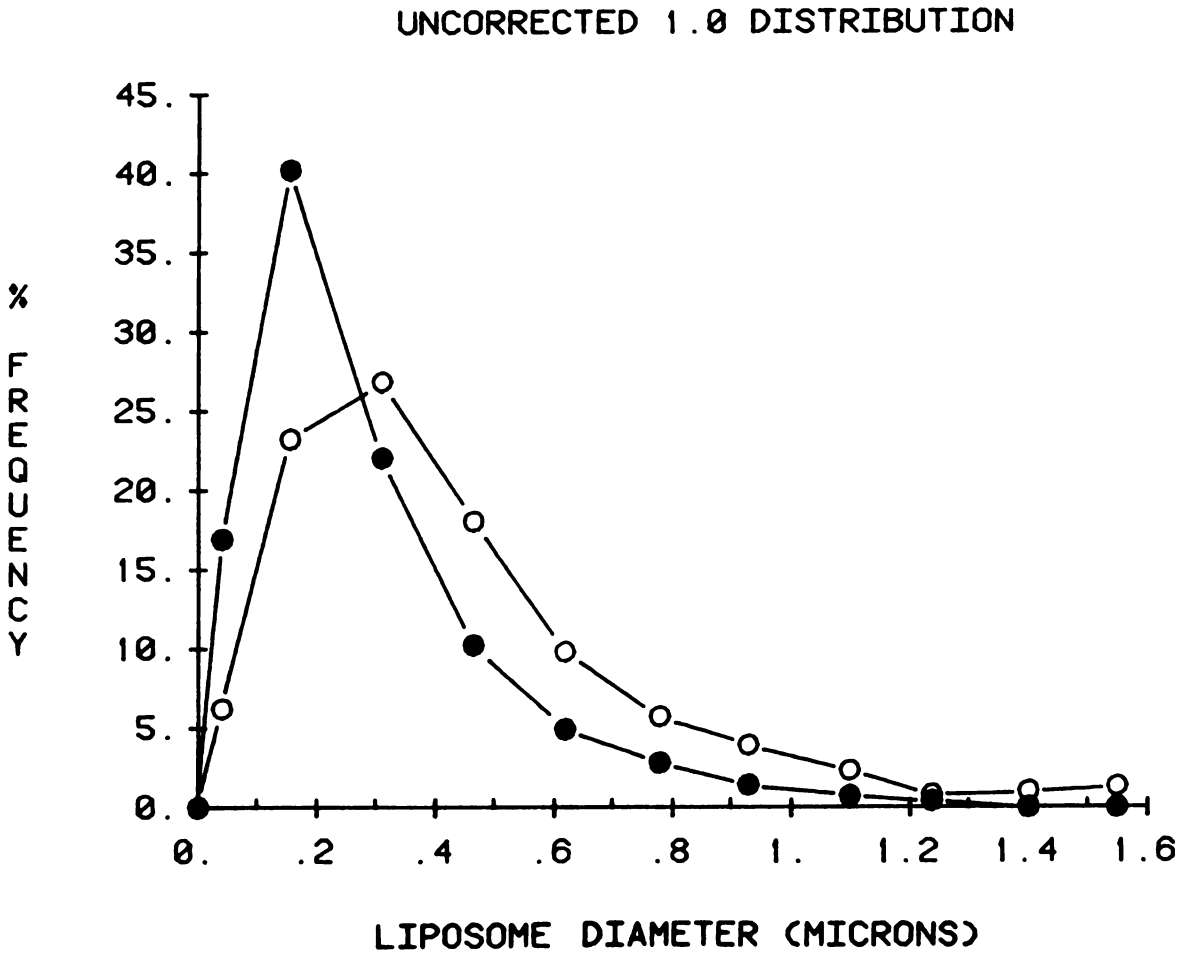


Figure IV-5B. Uncorrected size frequency distributions for 1.0 micron-extruded liposomes. Closed circles are for undialyzed liposomes. Open circles are for dialyzed liposomes (20 hours with 0.8 micron membranes).

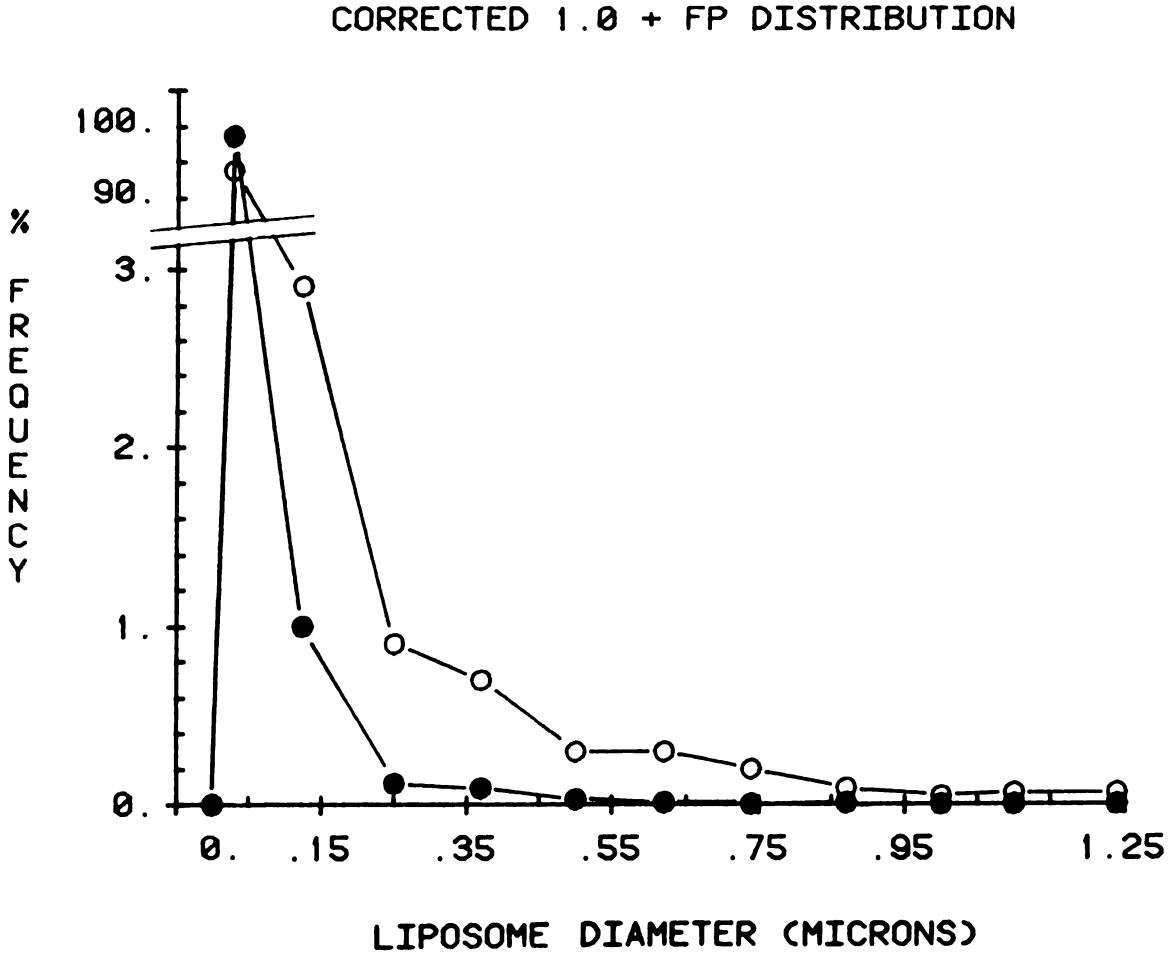


Figure IV-6A. Corrected size frequency distributions for the 1.0-extruded + French press mixed batch. Closed circles are for undialyzed liposomes. Open circles are for dialyzed liposomes (20 hours with 1.0 micron membranes).

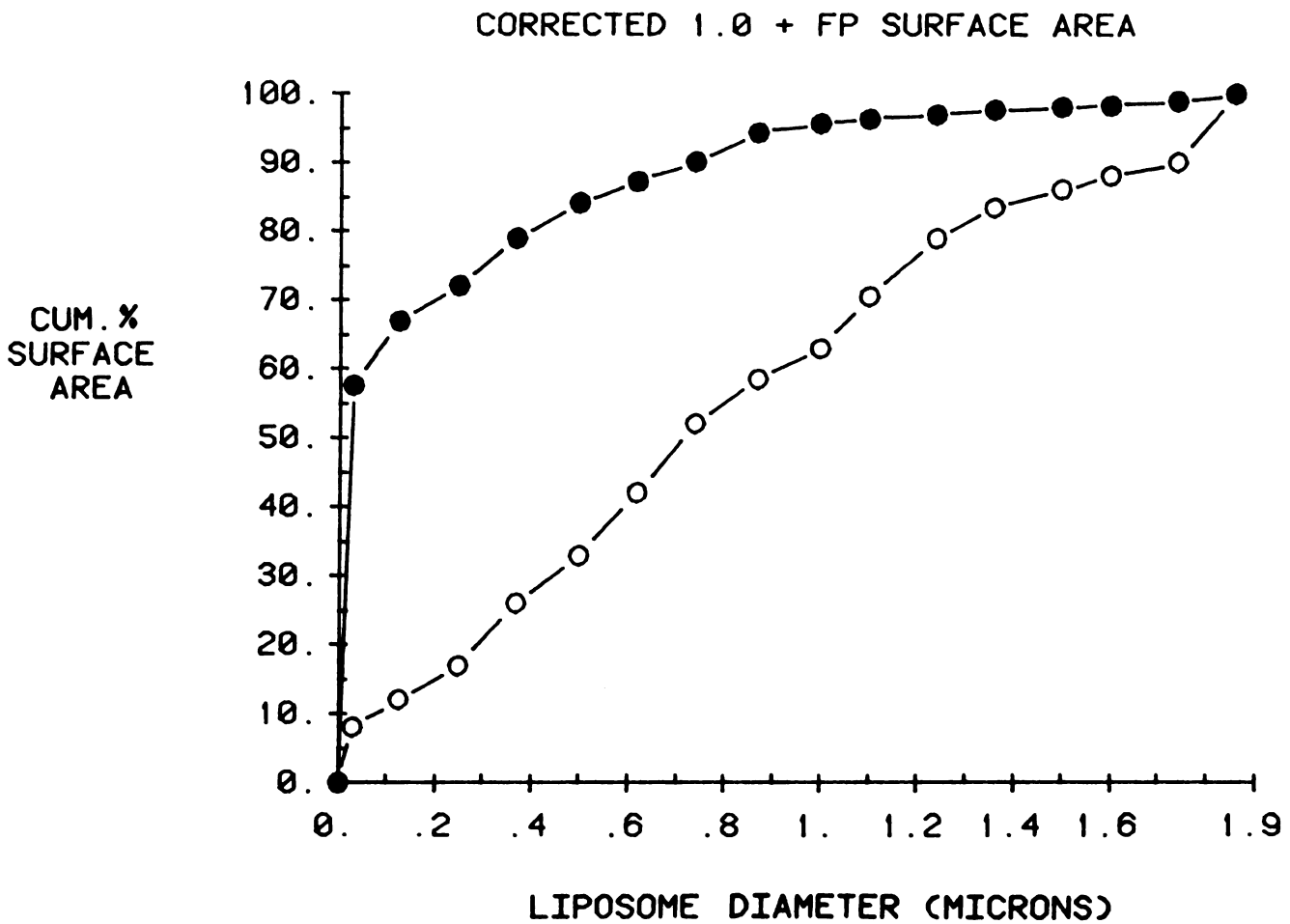


Figure IV-6B. Corrected cumulative liposome surface area plots for the 1.0 + FP batch (details as in Fig. IV-6A).

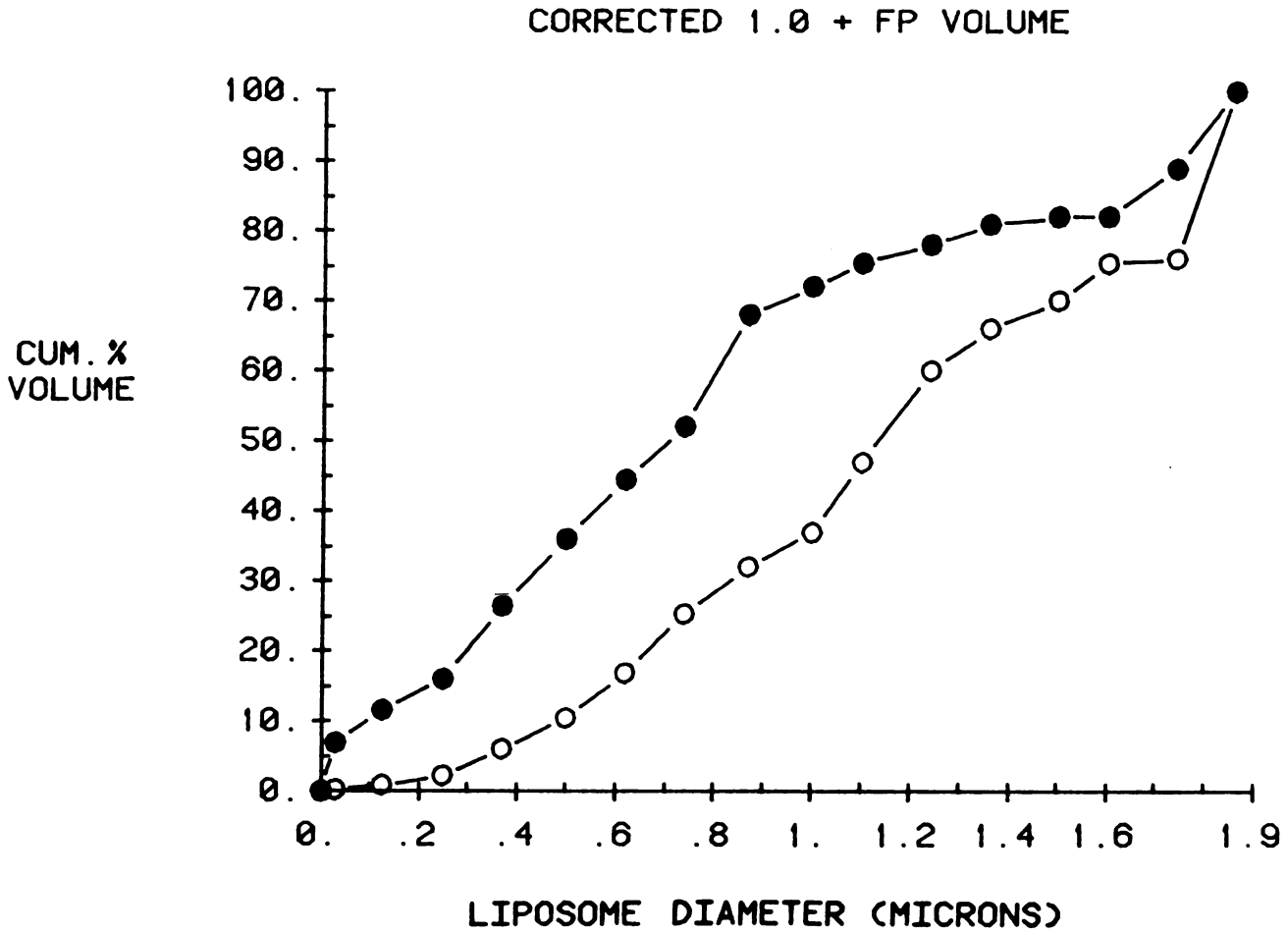


Figure IV-6C. Corrected cumulative total liposome volume plots for the 1.0 + FP batch (details as in Fig. IV-6A).

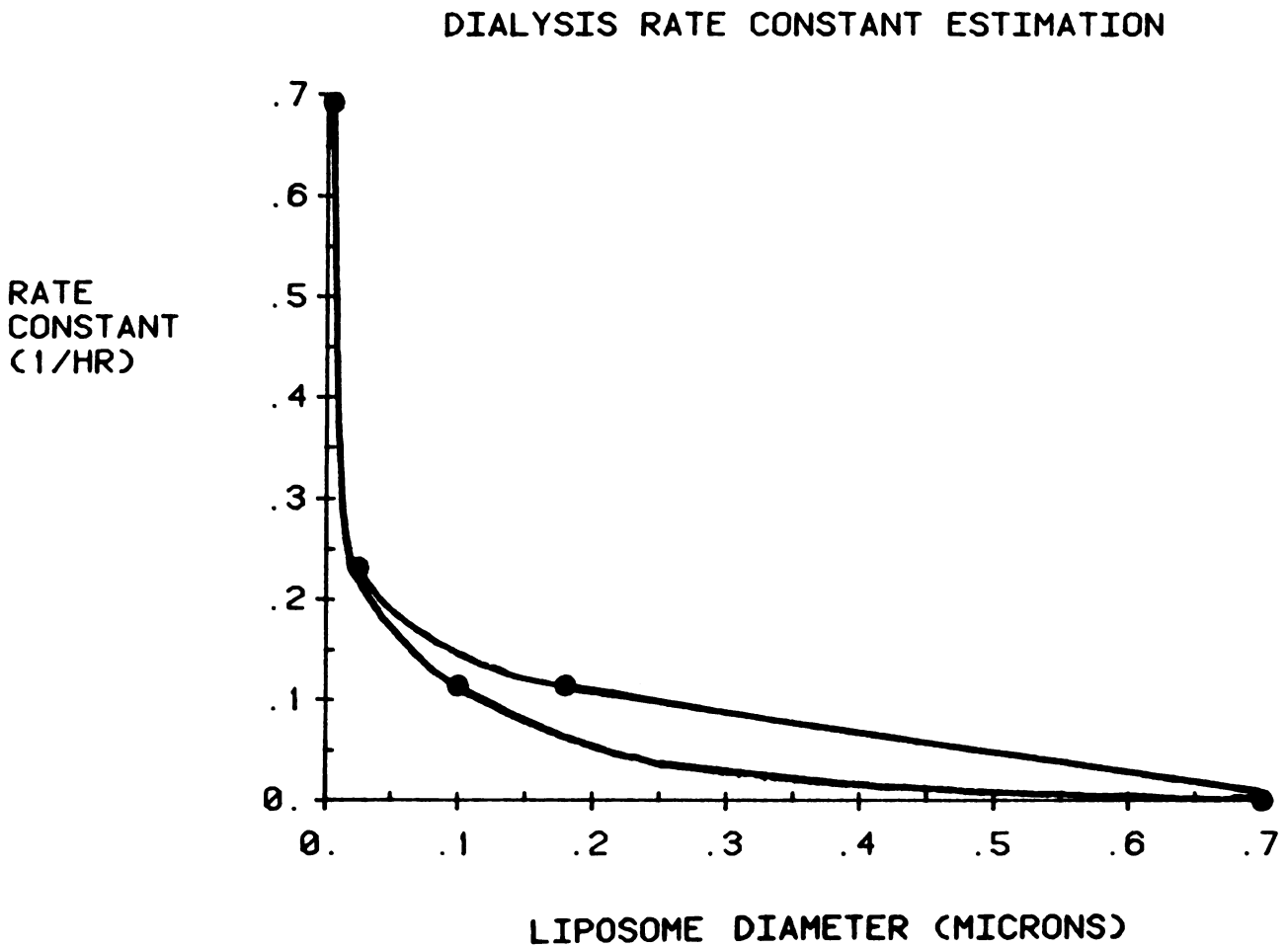


Figure IV-7. Plot of estimated first order dialysis rate constants for various liposome sizes: 14C-inulin rate constant (smallest diameter), French press (0.02 micron diameter), 0.2 micron-extruded (assuming 0.1 and 0.18 micron diameters because of size heterogeneity) and 0.7 micron liposomes (assuming negligible dialysis). Two curves were drawn through the two estimates for the 0.2-extruded size estimates.

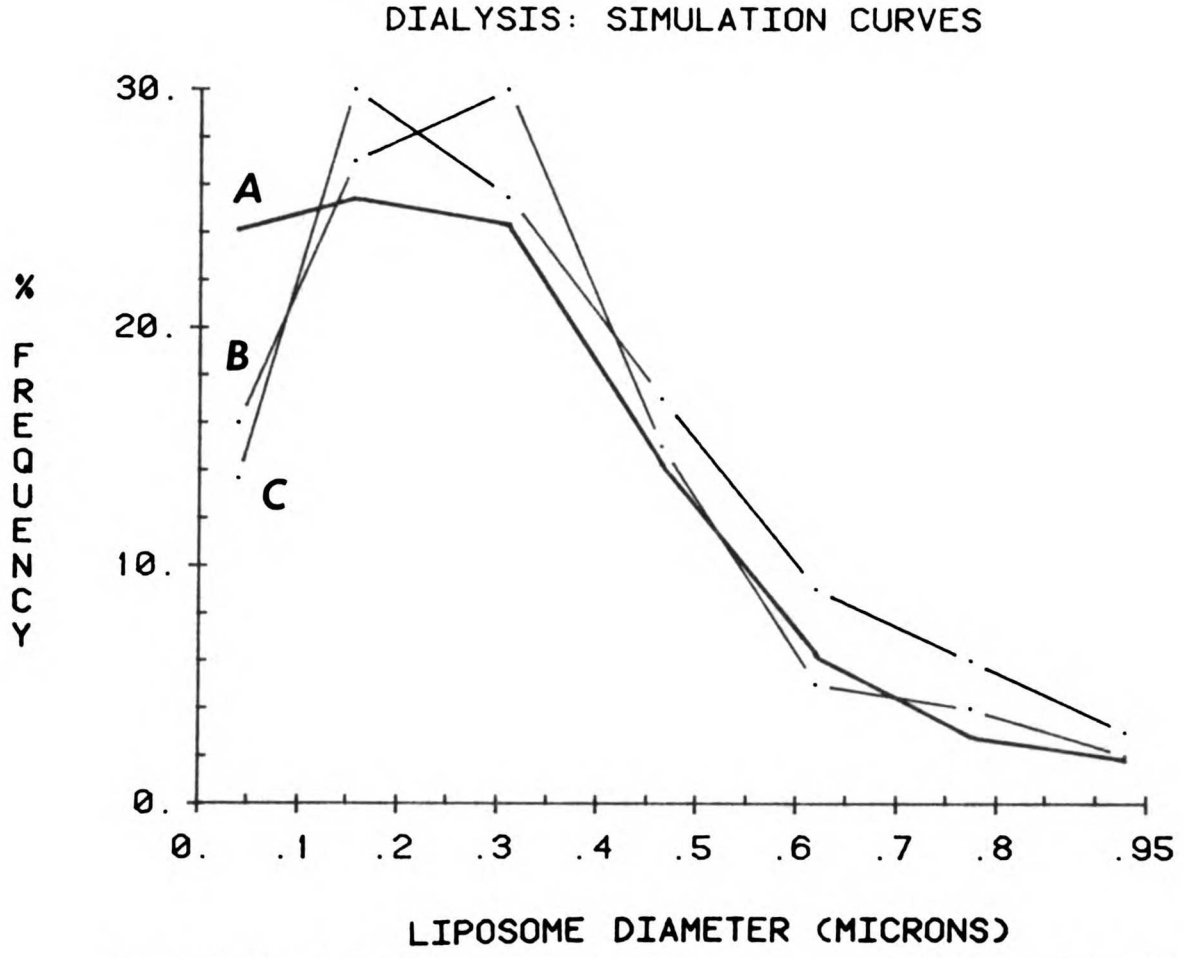


Figure IV-8. Dialysis simulation curves. The actual post-dialysis pattern for the 1.0-extruded liposomes is shown as a continuous line (A) (shown before in Fig. IV-5A). Lines B and C represent simulations of the dialysis obtained by applying rate constants estimated from Fig. IV-7 to the undialyzed distribution.

CHAPTER V: THE DOSE DEPENDENCY OF LIPOSOME DISPOSITION

Introduction.

When the fate of intravenously-administered liposomes is followed, e.g. via an entrapped aqueous space marker, the marker (and presumably the liposomes) accumulate to a significant extent in organs that contain cells of the reticuloendothelial system (RES). This occurs principally in the liver and spleen where, depending on the liposome type, animal species, and time after administration, a total of up to 90 percent of the injected dose of the marker may be found.

The significance of this RES localization was not lost on early investigators (5,104). The RES is a diffuse system with cells positioned along the vasculature in the liver, spleen, and bone marrow, in the lung alveoli, and in the tissue interstices generally (Fig. V-7). One of its functions is to phagocytize colloidal and particulate material such as effete red blood cells, denatured proteins, cellular debris, foreign organisms, and exogenously administered substances such as colloidal carbon or gold (the term "colloid" can arbitrarily encompass suspensions with particle sizes ranging from ten Angstroms to several microns).

An extensive literature exists on the *in vivo* fate of these so-called "inert" colloids (e.g. carbon and gold)(16). After intravenous administration they are taken up primarily

by RES cells in the liver and spleen, in a two-step process: first binding to the cell, then phagocytosis; the latter step is presumed to take place quickly after the binding (18). In fact, this assumption is used whenever the blood disappearance rate of one of these colloids is used as a general indicator of "RES function" (16). This RES uptake is a dose-dependent process that exhibits saturability in two respects. First, as the dose, i.e. number of particles, is increased the rate of colloid removal from the blood is decreased (21). Second, the tissue distribution changes with the dose (18). At very low, non-saturating doses, for which blood flow to the RES tissues is the rate limiting factor in the removal of the colloid from the blood, 95% or more of the colloid dose is taken up by the liver, the rest by the spleen. As the dose is increased the spleen takes up an increasingly larger fraction of the dose due to the saturation of the liver uptake. At extremely high doses the lung and bone marrow can take up significant fractions also, in what amounts to a system-wide "mobilization" in response to a saturating dose (19).

Because liposomes are colloidal, and because of their well-documented RES localization, it is reasonable to expect them to behave similarly to other colloids. The purpose of this study is to examine the possible dose dependency of liposome blood kinetics and tissue distribution. Other researchers report limited evidence that suggests that lipo-

some disposition may be dose-dependent (108,110), but none of these studies was a formal dose study per se; furthermore, the possibility of a time dependence in the dose effect was examined in the present study by following blood and tissue levels out to 72 hours. A final objective is to assess the extent to which the liposomes deliver their entrapped contents intracellularly in the various tissues studied by using an appropriate liposome aqueous space marker compound, ^{14}C-inulin.

Materials and Methods.

Chemicals. All chemicals and reagents were as in Chapter II.

Preparation of Liposomes. 1.0 micron-extruded, 0.8 micron-dialyzed liposomes with the composition PC/PA/CH/a-T = 4/1/1/.05 containing the ^{14}C-inulin marker were prepared at 37.5 micromoles lipid/ml as in Chapter II (inulin at 5mM in the buffer). Liposomes for the high dose were concentrated by adding 0.3 ml of 50% (w/v) sucrose to 1.1 ml of liposomes, centrifuging at 12,000 x g for 15 min., removing the upper concentrated liposome layer, and resuspending it in PBS to give a final concentration of 112.5 micromoles lipid/ml.

Tissue Distribution Studies. Liposomes were taken off dialysis and kept at 5^{o}C throughout the injection period.

Adult male ICR mice weighing 18-24 gm were used in the study. These mice were housed in the Animal Care Facility at U.C.S.F. and received a diet of Purina mouse chow #5Ø15 and water ad libitum, uninterrupted by the study. Mice were restrained for the injections in a plastic, cylindrical pill container with a hole cut in the cap for the tail. Syringes and solutions were as in Chapter III. Mice were injected with either 37.5 micromoles, 7.5 micromoles, or Ø.375 micromoles lipid per mouse (high, medium, and low doses) intravenously via tail vein, with Ø.25-Ø.35 ml injection volumes. Mice receiving imperfect injections were not used. To determine the percentage of free inulin in each dose a sample was applied to a 5 x 12Ø mm column of Sephadex G-2ØØ-12Ø equilibrated and subsequently eluted with PBS; the eluate fractions were analyzed by scintillation counting and the percent of inulin entrapped was calculated based on the ^{14}C recovered in the void volume and free inulin fractions.

Mice were anesthetized (with ether) at 1,24,48 or 72 hours after injection and approximately Ø.5-1.Ø ml of blood was quickly removed from the jugular vein with a heparinized syringe. The mice were then sacrificed and the livers, spleens, (and in some instances brain, kidney, and lung) were removed and frozen for later analysis. In some instances an aliquot of the blood was centrifuged and a plasma sample was applied to a Sephadex column as described previously to determine the percent entrapped.

Tissue Analysis for Total Radioactivity. Duplicate aliquots of liver (0.14 to 0.24 g), spleen (0.04 to 0.08 g), whole blood (0.2 ml) and the radiolabelled liposome dose (0.05 ml) were transferred to combustion cups. The remaining carcass was placed in a container with 75 ml of water and homogenized using a Williams Polytron wet milling device (Brunwell Scientific, Rochester, N.Y.). Duplicate samples of the resulting slurry (1.3-2.0 g) were weighed into combustion cups and allowed to air-dry for 24 hrs. Dried samples were analyzed for total ^{14}C by scintillation counting as in Chapter II. Results for duplicate samples were averaged and the total radioactivity in liver, spleen, carcass and 1.0 ml of blood were calculated and converted to percent dose.

In Vivo Clearance of Free Inulin. In a separate study similar doses of ^{14}C-inulin in identical volumes of PBS were administered to mice which were subsequently sacrificed and analyzed as described above to determine the in vivo clearance of free inulin.

Results.

Free inulin was cleared rapidly by the kidney. Its half-life in blood after iv injection was 10-15 min., with 0.3% of the dose remaining in blood, 0.25% in the liver, and 0.015% in the spleen at one hour (Figs. V-1, A and B). Carcass values at one hour averaged 6.9%, due, presumably, to residual inulin in the bladder; this value was minimized by

removal of urine from the bladder prior to freezing the carcass. The 24 hour value for carcass was less than 1%. Thus the presence of free inulin after the liposome doses may have contributed slightly to the 1 hour carcass data, but was negligible at later times and in all other tissues.

Table V-1 shows the 1-hour ^{14}C levels for various organs and blood, as well as the percent of the counts in blood that were entrapped in liposomes, following the liposome doses. Blood values are expressed as percent dose per 1.0 ml and exhibit a definite dose dependency; the values at 24, 48, and 72 hours are identical to background in all cases. Lung, brain, and kidney levels, done only in limited cases as controls, are negligible.

The other principal tissues of interest are liver, spleen, and carcass. Fig. V-2 shows the results for the liver: a definite dose effect, seen as higher relative levels for the lower doses, is seen at the 1 hour time point. This effect has completely diminished by 72 hours and a similar plateau is approached by all three doses. The spleen results, shown in Fig. V-3, also show a dose dependency, but one that differs from that in the liver: the effect is maintained over the 72 hours, and higher relative levels are seen as the dose is increased. The carcass results, shown in Fig. V-4, show evidence of a dose effect at later times which is significant at the 24 and 48 hour time points; the levels fall rapidly and reach a plateau.

Fig. V-5 shows the results for the totals in vivo ; here there is no evidence for a dose effect, and all values approach a similar plateau.

Discussion.

The results of this study can best be evaluated by comparison with the results one would expect for inert colloids, and to those expected (based on certain preconceived notions) for liposomes. Fig. V-6 depicts these "expected" dose study results. Curves A and B are plots of percent of injected dose found in all RES tissues vs. time for low and high doses, respectively, of a colloid such as gold. The delayed uptake for the higher dose reflects the saturation phenomenon mentioned in the introduction. In both cases, however, all of the dose is eventually found in RES tissues. The liposome case is different, for two important reasons. First, it is well known now that certain factors in blood can "destabilize" liposomes with respect to the leaking out of their entrapped contents (44-48). When this "leakiness" effect occurs a significant portion of entrapped marker can be released quickly into the blood, resulting in a less than 100% distribution of the marker to RES tissues. The second reason is more important as far as evaluating liposome tissue distribution, and concerns the nature of the entrapped marker. Most of the compounds that have been entrapped in liposomes to date have physical properties that preclude their use as accurate indicators of intracellular delivery

by liposomes. This is because the compounds used are often capable of entering and leaving the target cells on their own, or are metabolized to compounds that can. The situation which results is depicted in curve E of Fig. V-6, in which the tissue levels rise and fall with time due to the exit of the marker. This pattern has, in fact, been almost universally observed when tissue levels have been taken after administration of liposome-entrapped compounds. The principal disadvantage with such data is that it is impossible to ascertain what fraction of the dose has been delivered to the tissue in question. We felt, however, that this could be done by the proper choice of entrapped marker. We thus chose ^{14}C-inulin for the following reasons: its size and polarity prevent it from entering cells on its own (or leaving, if it is delivered there via liposomes); it has simple kinetics and is not metabolized. In short, it is a marker compound that should remain in the tissue to which it is delivered. Thus tissue levels that reach plateaus at later times but, because of the leakiness effect, approach values less than 100%, should be seen after the administration of liposome-entrapped ^{14}C-inulin. This situation is depicted, for two different doses, in curves C and D of Fig. V-6.

The results of this study lead to several general conclusions. First, in all of the principal tissues studied (liver, spleen, carcass, totals in vivo) and for all doses,

the levels approach plateaus at later times. This evidence supports our hypothesis that tissue level plateaus would be seen with a marker compound such as inulin; an argument will be presented later as to why these plateaus approximate the true level of intracellular delivery of the inulin by the liposomes. The second conclusion is that there is a pronounced dose effect at early times which is lost at the later times. This dose effect is consistent with that seen for other colloids such as carbon or gold. That is, the liposomes are subject to saturation kinetics, manifested by elevated relative blood and spleen levels and depressed relative liver levels as the dose is increased. As is the case for other colloids, the liver is the dominant organ in the liposomes' disposition; because of its large blood flow and high clearance capacity relative to the other vascular RES tissues (i.e. spleen and bone marrow), the extent to which the liver disposition of the liposomes is saturated determines the amount of colloid that will be taken up by the other tissues. In other words, a delayed liver uptake results in elevated blood levels over a longer time period, allowing the spleen (which is thought to have a higher cellular intrinsic activity for colloid uptake (16)) to sequester a larger fraction of the injected dose. The carcass values reported here (ie everything but removed blood, liver, and spleen) include the bone marrow, and maybe the dose effect seen in the carcass is due to uptake by the marrow.

These results also raise some intriguing questions. It is obvious that the data for the liver do not fit our predictions as depicted by curves C and D of Fig. V-6. Only the high dose data fit the expected pattern of a continuous rise of the tissue levels to the eventual plateau. The data for the low and medium doses show a very different pattern--an early decline in levels, followed by the plateau. A decline such as this can be accounted for in only two ways: first, a fraction of the marker that has been delivered to the cells at early times somehow partitions out of the cells at later times. However, the assumption was made earlier that the inulin marker, once delivered inside cells, wouldn't partition out. The only other explanation is that the marker levels in the liver at early times are not entirely constituted of intracellular inulin. Since the blood levels at one hour are very low for these two doses, and since the half-life for removal of free inulin from the blood is very fast, a significant fraction of the inulin in the liver at one hour must be located in extracellularly-bound liposomes.

It is our hypothesis that the following events occur in the liver disposition of the liposomes: first, if one postulates the presence of extracellularly-bound liposomes one must contend with the long-held belief that for colloids, removal from the blood can be considered the equivalent of intracellular uptake (that is, uptake occurs rapidly after

binding). Data is available that shows that these two phases of uptake are separable under specialized conditions in vitro (22), but only recently has evidence been reported that unambiguously shows that, in vivo , a colloid's binding to and uptake into Kuppfer cells can be very distinct events, separated by long periods of time (17,23). In fact, Kavet and Brain, in their recent review on the quantification of endocytosis, emphasize that "...ingestion is not a necessary consequence of attachment. Thus, particles may become cell-associated without being interiorized and unless detected as such may be falsely included with the intracellular compartment." (15). This is a very crucial fact in the case of liposomes, for it is very likely that extracellularly-bound liposomes are just as susceptible to the destabilizing influences of blood as are free-floating liposomes. What all of this thus suggests is that 1) tissue levels measured after administration of a liposome-entrapped marker may include substantial levels of extracellularly-bound, liposome-entrapped marker, even several hours after the dose is given and 2) these tissue levels may decline, because of the slow release of marker from extracellular liposomes due to the destabilizing effect of blood.

One final piece of evidence must be made use of, however, in order to explain completely the disposition of the three doses. Recent evidence suggests that the blood destabilizing effect may itself be saturable (41,44); that is, as

larger numbers of liposomes are incubated with a given volume of blood, a smaller fraction of the total liposome-entrapped marker is released. The liver data are thus explained as follows: in the low dose case, the liver quickly binds most of the liposome-entrapped dose of inulin (part of the inulin is released from the liposomes upon the initial mixing of the dose with the blood, so that the initial liver value is probably lower than 100% of the dose); however, because the intracellular uptake is slow, the bound liposomes continue to release inulin until they are taken up inside the cells. What is thus seen is a decline in liver levels over time until the inulin that remains is entirely intracellular, and the plateau is reached. In the high dose case, the initial liver binding is saturated and so the relative liver levels (that is, on a percent dose basis) are low and blood levels high. Thus the levels of extracellular liposomes (circulating and bound) remain elevated for longer periods of time, and are exposed to the blood's destabilizing factors longer. However, since this latter system is probably also saturated (we calculate that the ratio of mg lipid to ml blood is similar to that which causes saturation in <u>in vitro</u> systems (41,44)) the liposomes leak their contents at a relatively much slower rate. The net result is that the slowing-down of the two processes of uptake (a combination of binding and endocytosis) and blood-induced liposome leakiness are offsetting effects. Thus at later times the relative amount of inulin taken up inside cells is

similar for the two doses (the medium dose is apparently an intermediate effect).

As mentioned previously, the dose behavior of the spleen conforms to our expectations in that higher relative levels are seen as the dose increases. This effect, however, unlike that in the liver, is still evident at 72 hours, and may be due to two factors: 1) the amount of colloid available to the spleen initially is largely a function of the amount that has been bound by the liver and 2) the actual intracellular uptake may be faster in the spleen, so that most of what was initially bound there was also endocytosed. The ^{14}C levels in several other tissues (brain, lung, kidney) were also measured (see Table V-1). However, the initial experiments indicated that very little of the dose could be found in these tissues, and so they weren't analyzed routinely.

Several aspects of the experimental techniques used also deserve comment. The liposomes used were fairly large in size and had a relatively narrow size distribution. This was accomplished by the use of two techniques in the sizing procedure--extrusion through 1.0 micron pore size membranes (64) followed by dialysis against 0.8 micron membranes (Chapter IV). We feel that the excellent reproducibility seen in the tissue levels here (the high dose studies were done on two completely separate occasions, the medium dose study on three) was evidence of the liposome batch-to-batch

reproducibility that these sizing techniques afford us. The lipid composition used in the study was chosen based on *in vitro* liposome-plasma stability studies (41). The object was to choose a composition such that the liposomes were stable enough to retain most of their entrapped contents *in vivo*, yet "unstable" enough so that if significant longterm extracellular liposome tissue binding did take place, the marker would be released from them in a reasonable period of time (less than 72 hours) and would thus not be included as part of a "plateau" tissue level value. The decline in liver levels for the low and medium doses takes place over the first 24-36 hours, which was fast enough to allow us to see the plateaus by 72 hours.

Conclusions.

The results of this study have several general implications. First, it is obvious that liposomal dose (or liposome number) is an extremely important determinant of their *in vivo* disposition. It is unfortunate that *in vivo* work is still appearing in the literature in which the liposome dose isn't given and is difficult or impossible to determine from the other details given (the dose in terms of micromoles lipid per kg animal is currently acceptable, but the ideal would be to additionally specify liposome size and number (71)). Second, our data suggest that, after administration of a liposome-entrapped marker, a significant fraction of the marker may be located in extracellularly-bound liposomes

for long periods of time. This means, of course, that there will have to be a general reinterpretation of the significance of tissue levels for most compounds administered in liposomes. Specifically, tissue levels cannot be taken as an indicator of the intracellular delivery of the entrapped compound by the liposomes. Our data do suggest, however, that tissue level plateaus after administration of a liposome-entrapped compound such as inulin may be a valid indicator of intracellular delivery. This would be of importance for several reasons. First, it is necessary information for potential entrapped compounds so large or polar that their cellular uptake in the free form is zero. Second, for compounds that can penetrate cells to a limited extent and whose efficacy (and intracellular delivery) can benefit from a localized liposomal extracellular slow release effect, it is still important to know what fraction is delivered to cells via liposomes; this is because the drug's intracellular destination may be different by different entry routes.

Finally, it is apparent that the in vivo system is very complex, with at least two saturable processes affecting liposome disposition: RES organ uptake (which includes binding and endocytosis) and the destabilizing factors in blood. These processes compete and it is their interplay that determines the total organ binding and intracellular delivery of a liposome-entrapped compound. In this study the

complexity of this interplay was evidenced by the time dependence of the dose effect such that at later times levels in the tissues approached similar relative values. This finding, however, may not be true generally since the relative magnitudes of these saturable processes probably depend on (and vary at different rates with) liposome physical properties and the animal's physiological properties. These reasons make it all the more important that the dose behavior of a given liposome type be well studied so that its therapeutic efficacy can be optimized.

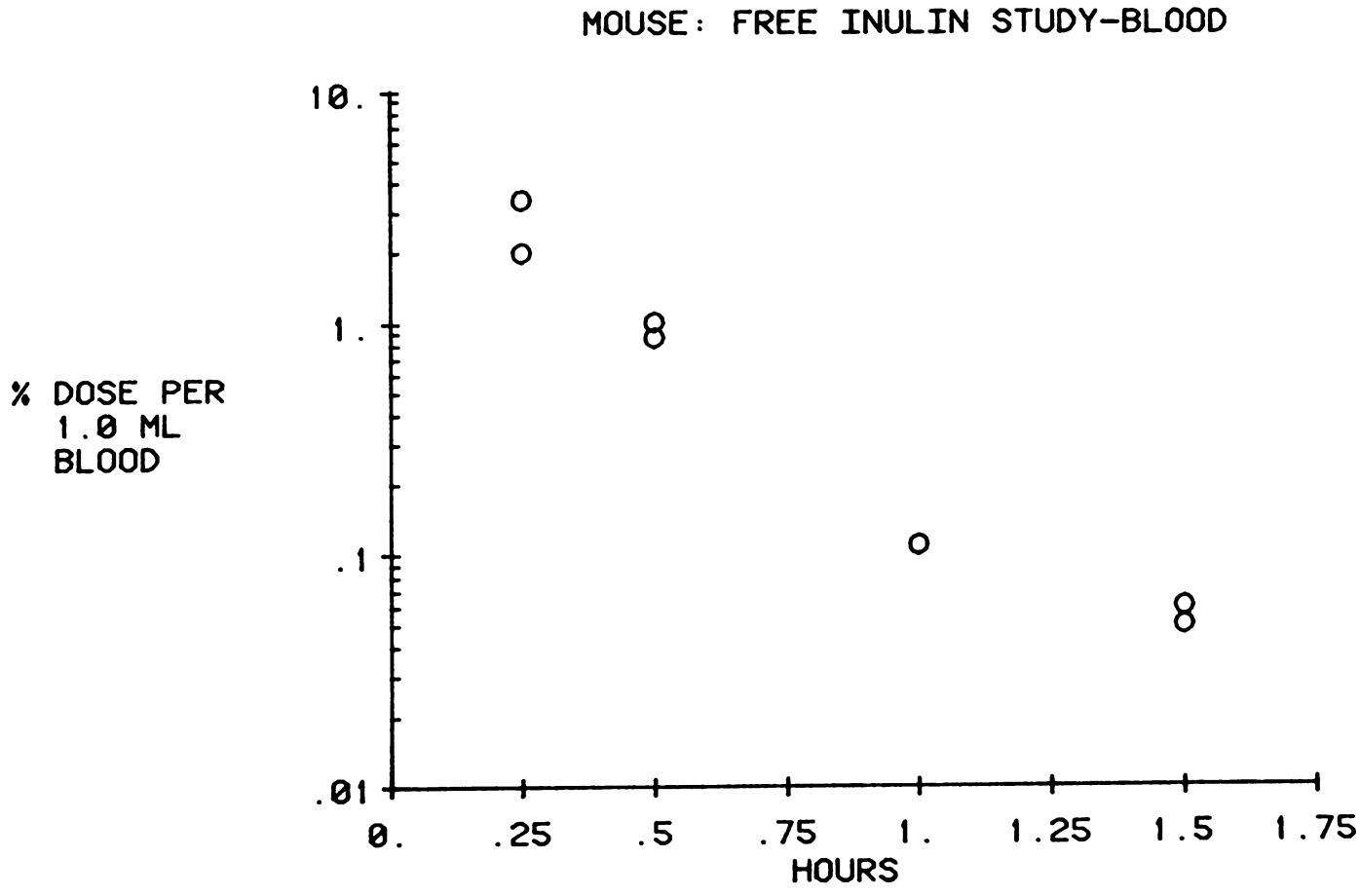


Figure V-1A. Blood levels after administration of free ^{14}C-inulin to mice. Levels expressed as % dose per 1.0 ml blood. Each data point represents one mouse.

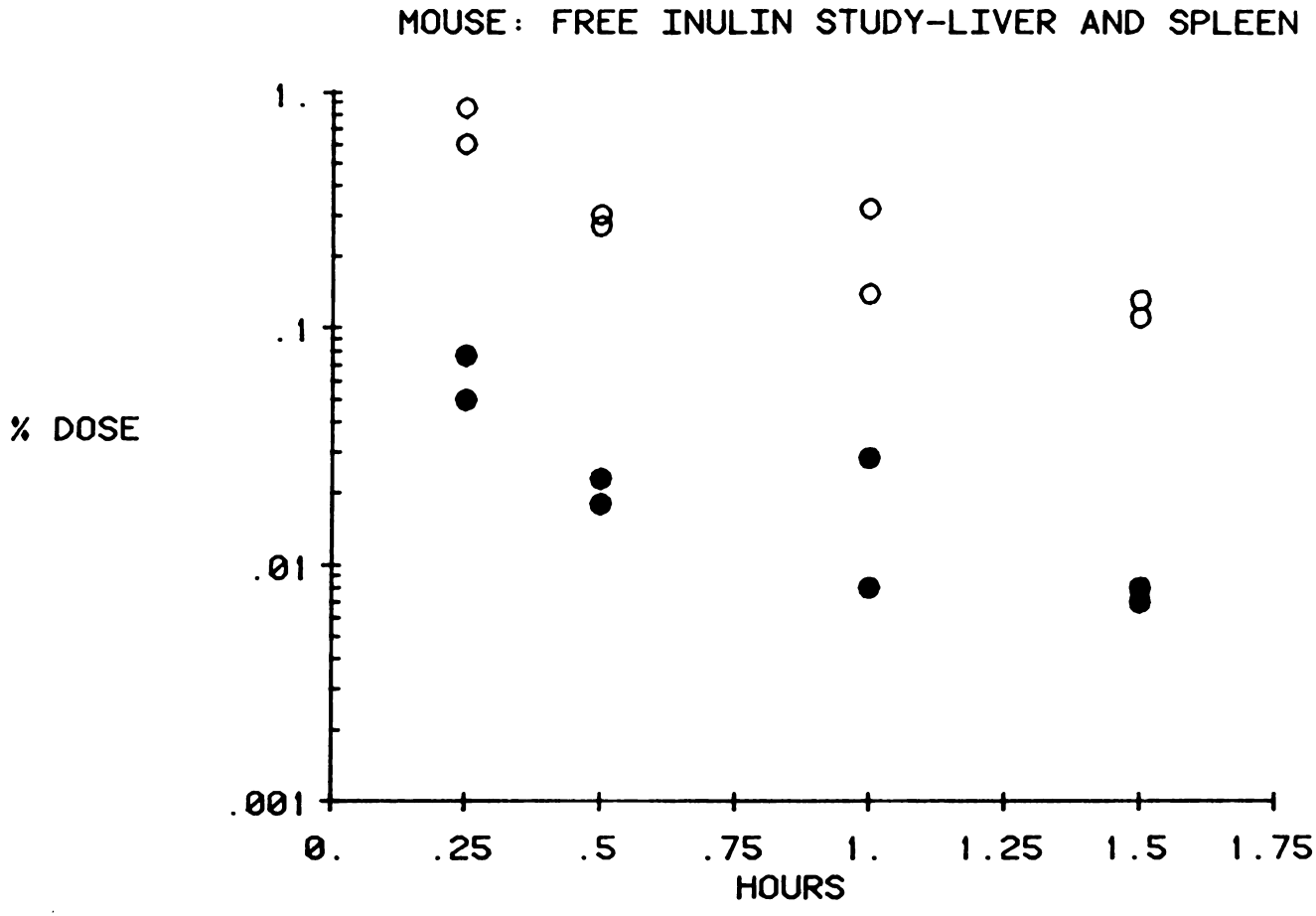


Figure V-1B. Liver (open circles) and spleen (closed circles) levels after administration of free ^{14}C-inulin to mice. Details as in Fig. V-1A.

Table V-1: Data[a] for various organs at one hour after liposome doses.

Dose	Blood Levels % dose/1.0 ml	% Entrapped in blood	Lungs	Kidneys	Brain
Low	0.39 ± 0.1 (3)	b	0.09	----	----
Medium	1.1 ± 0.71 (9)	10.1 ± 2.0 (3)	0.23 ± 0.04 (4)	0.825 ± 0.28 (4)	0.095, 0.18
High	10.4 ± 1.1 (4)	93, 95	0.75, 0.97	----	----

a % dose: mean ± one S.D., (n); or individual values where n < 2
b Blood values too low to do the measurement.

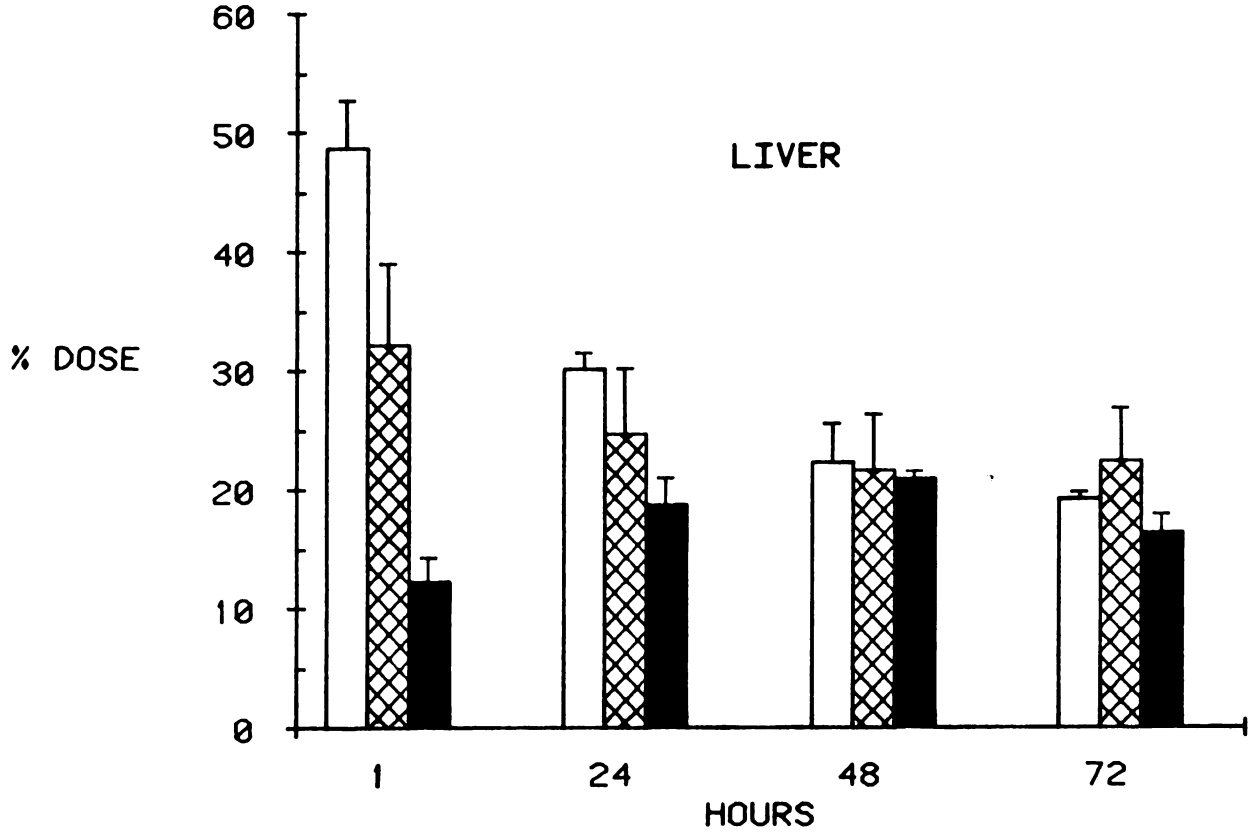


Figure V-2. Dose study (details on page 88). Liver ^{14}C levels after administration of liposome-entrapped ^{14}C-inulin for low (open figures), medium (crosshatched) and high (solid) doses. Mean + one S.D. (n = 3,10,4 for low, medium, high doses, resp.). Differences between low and high doses were significant at 1 and 24 hours but not at 48 and 72 hours.

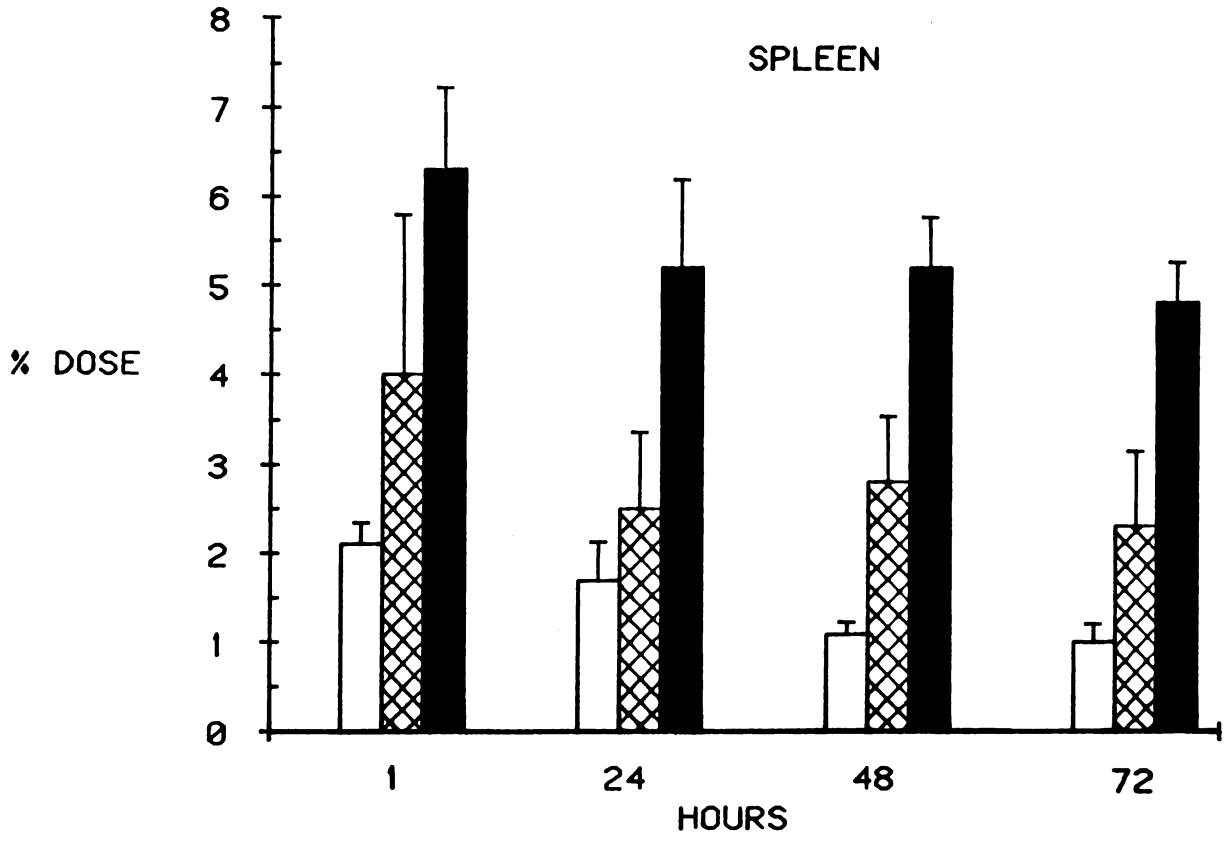


Figure V-3. Dose study: spleen results. Differences between low and high doses were significant at all time points (symbols and other details as in Fig. V-2).

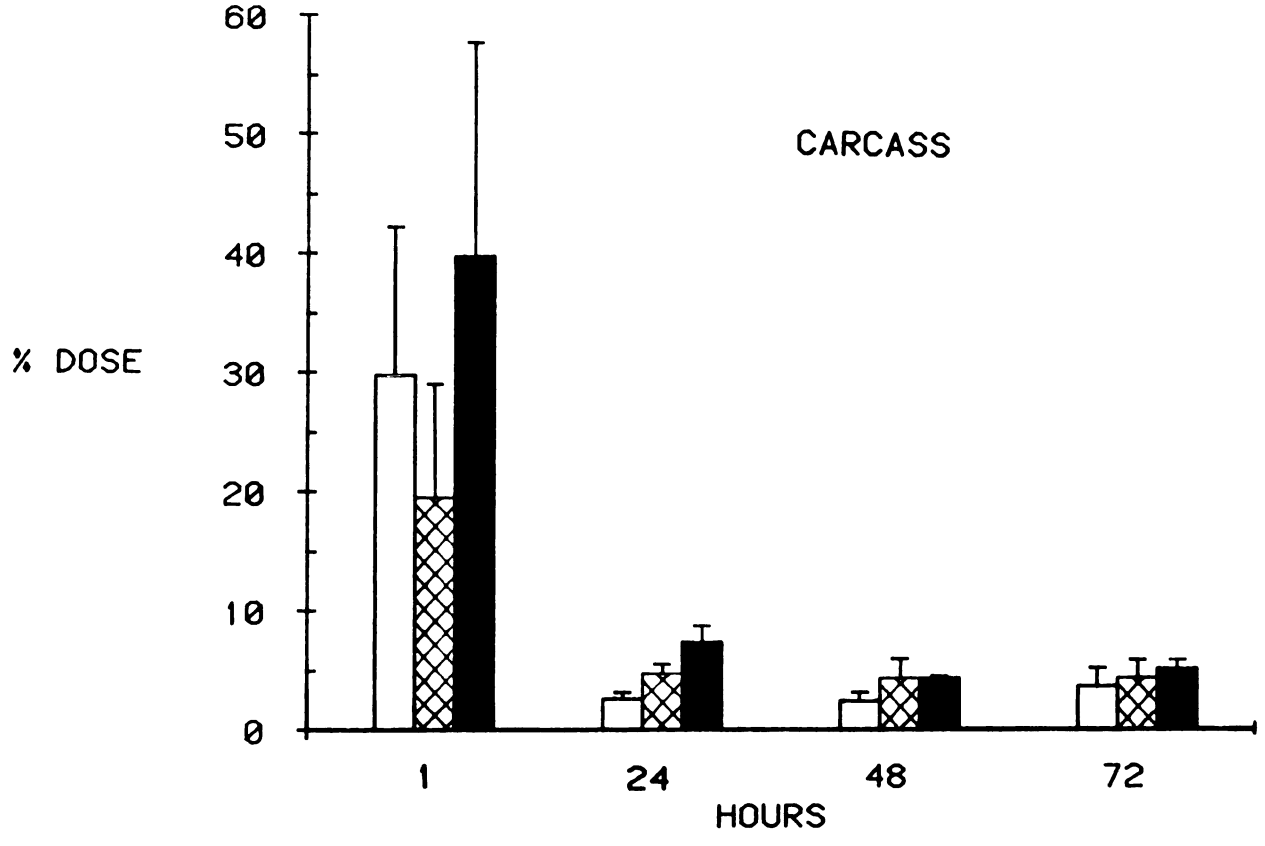


Figure V-4. Dose study: carcass results. Differences between low and high doses were significant at 24 and 48 hours but not at 1 and 72 hours (symbols and other details as in Fig. V-2).

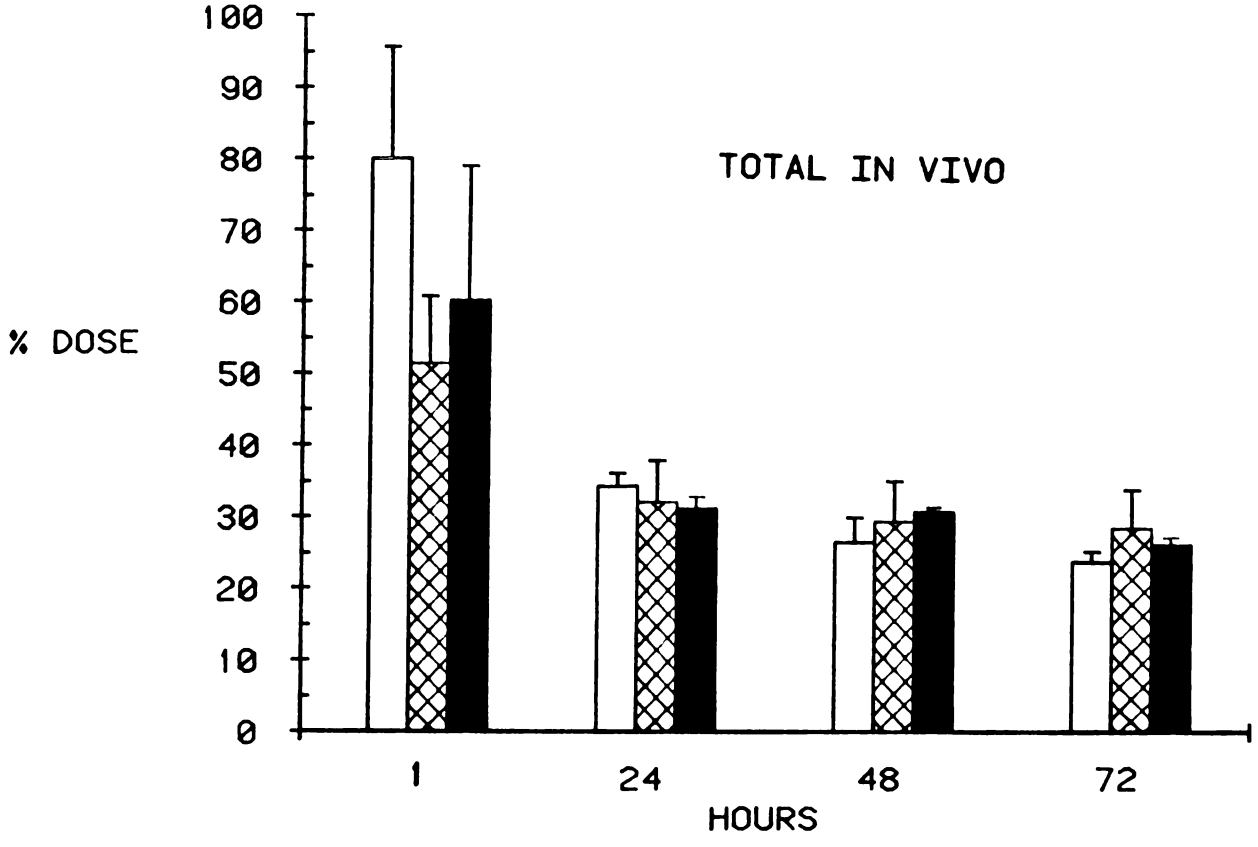


Figure V-5. Dose study: totals in vivo. No evidence for a dose effect was seen here (symbols and other details as in Fig. V-2).

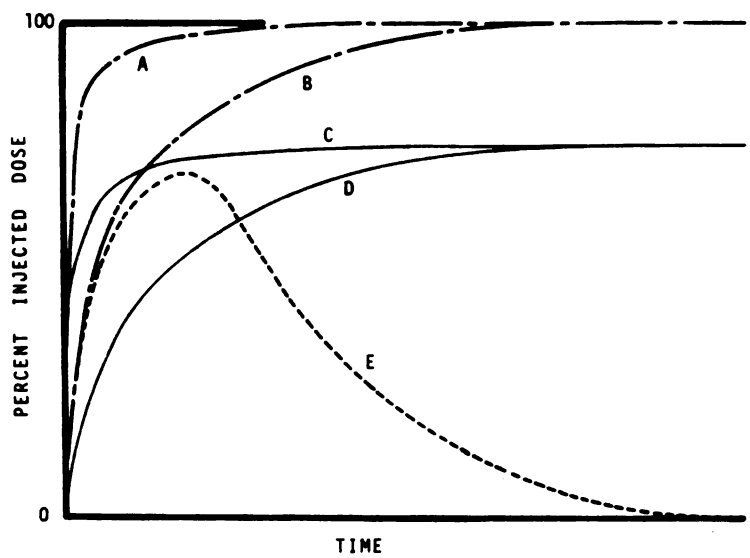


Figure V-6. Dose study. Expected results for a plot of percent dose in RES tissues vs. time after intravenous administration of: low and high doses of colloidal gold (curves A and B, resp.), low and high doses of liposome-entrapped 14C-inulin (curves C and D, resp.), or a liposome-entrapped marker that can partition out of cells (curve E).

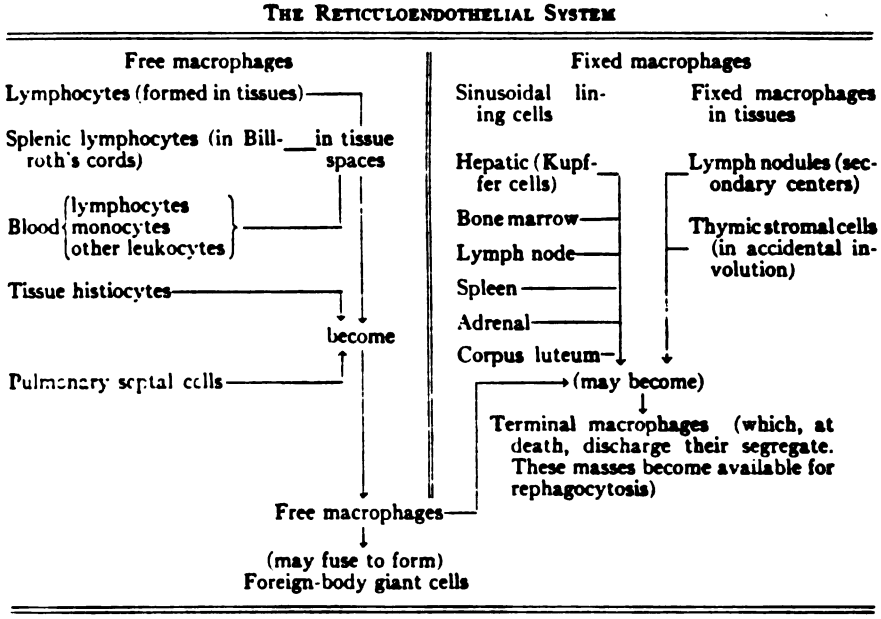


Figure V-7. Diagrammatic representation of the reticuloendothelial system (taken from R.N. Bailliff, ref. 19).

CHAPTER VI: PREDOSING TO ACHIEVE RES SATURATION.

Introduction.

One of the principal problems in developing liposomes as a versatile drug carrier has been their tendency to localize in RES tissues. Attempts have been made to direct liposomes to other tissues by attaching to them "homing" molecules such as antibodies (103,110) or glycolipids (96) that would impart a specific affinity to the liposome for the target tissue. These attempts, however, have only managed to produce slight shifts in tissue distribution, and a way must be found to avoid the RES uptake if liposomes are to become a truly target-specific vehicle.

The results of the dose study (Chapter V) showed that liposomes' *in vivo* disposition is dose dependent. The dose effect is manifested in a saturation effect in the liver and increased spleen and blood levels as the dose increases. It suggested that a possible way to avoid RES uptake would be to give a large predose of nonradioactive liposomes (to saturate the RES) followed by a small labeled dose. This predose strategy might also have the advantage (over the single high dose strategy) that the spleen uptake of the labelled liposomes could be avoided. The purpose of this study was to do such an experiment. In addition, the time dependence of the RES saturation was characterized by spacing the two doses apart by either 1, 5, or 24 hours.

Materials and Methods.

All chemicals and reagents were as in Chapter II. Mouse experimental details were as in Chapter V.

Preparation of Liposomes. 1.0 micron-extruded, 0.8 micron-dialyzed liposomes with the composition PC/PA/CH/a-T = 4/1/1/.05 were prepared at 20 micromoles lipid/ml with entrapped inulin (either nonradioactive or ^{14}C-labeled) at 5mM as in Chapter II. High dose liposomes were concentrated as in Chapter V.

Liposome Studies. A control group of mice received a 76 micromoles lipid/kg dose of liposome entrapped ^{14}C-inulin. Experimental groups received this dose also, but it was preceded by a 1500 micromoles lipid/kg dose of entrapped nonradioactive inulin. The time interval between doses was either 1, 5, or 24 hours. All groups of mice were sacrificed one hour after the ^{14}C-labeled dose, and ^{14}C tissue levels were determined as in Chapter V.

Results.

The ^{14}C levels in blood, liver, spleen, and carcass for all study groups are shown in Fig. VI-1. Control group levels are shown as horizontal shaded bars. Totals in vivo are shown in Table VI-1. Administration of the cold predose had a dramatic effect on the disposition of the ^{14}C dose when the two were spaced apart by one hour. The blood and spleen

levels were elevated and liver levels depressed compared to the control. The predose effect was almost entirely diminished, however, when the two doses were spaced apart by 24 hours. The results for the carcass and totals show the opposite trend: that is, the control and 1-hour predose values were similar, while the 5 and 24 hour predoses resulted in levels lower than the control.

Discussion.

The results of this study show that administration of a drug-free liposome predose can be used to delay the RES binding/uptake of a subsequently given liposome-entrapped drug, thus increasing its residence time in blood. This allows more time for the direction of the liposomes to other tissues, or for a systemic slow release of the drug from the liposomes in the blood.

These results were very similar to those of the dose study (Chapter V), especially with regard to the spleen behavior (increased levels after the 1 and 5 hour predoses). Similarly, the apparent recovery time for the RES "function", 24 hours, is in close agreement with the "recovery" seen in the high dose 24 hour liver results of Chapter V. These similarities were not necessarily expected, since the dose and predose studies are different experiments: that is, in the predose study the molecular "environment" (availability of opsonins or destabilizing factors ?) should be dif-

ferent for the hot dose as compared to the predose (the latter having adsorbed them); also, in the predose study, unlike the dose study, the spleen encounters high levels of circulating liposomes for one hour before the hot dose is given, and so its ability to take up the hot dose should be reduced. These differences presumably might have made the predose a more effective strategy than the single large hot dose. The fact that the spleen results were similar for the two types of studies, and that the saturation effects were of the same duration, suggest that these differences had no effect. The results, not unexpectedly, parallel those for other colloids: ample evidence shows that a large colloid predose can "blockade" the RES and alter the disposition of a succeeding smaller colloid dose (16).

The results for the carcass and totals, however, can not be explained by simple mechanisms involving saturable hepatic binding/uptake, and must await clarification in future studies.

In conclusion, predosing with cold liposomes, or giving a large hot dose of liposomes, can significantly delay the RES localization of the hot dose (the predose strategy does have the advantage that the hot dose can be very small). In this study, and the dose study, however, the doses given produced a saturation effect that lasted less than 24 hours. If this effect depends on liposome particle number, though, it should be possible to get the effect more efficiently

(and perhaps for greater duration) using smaller liposomes, because of their increased particle number per gm of lipid. Thus, in the next series of studies (Chapter VII) the use of small (FP) liposomes in achieving the saturation effect will be investigated.

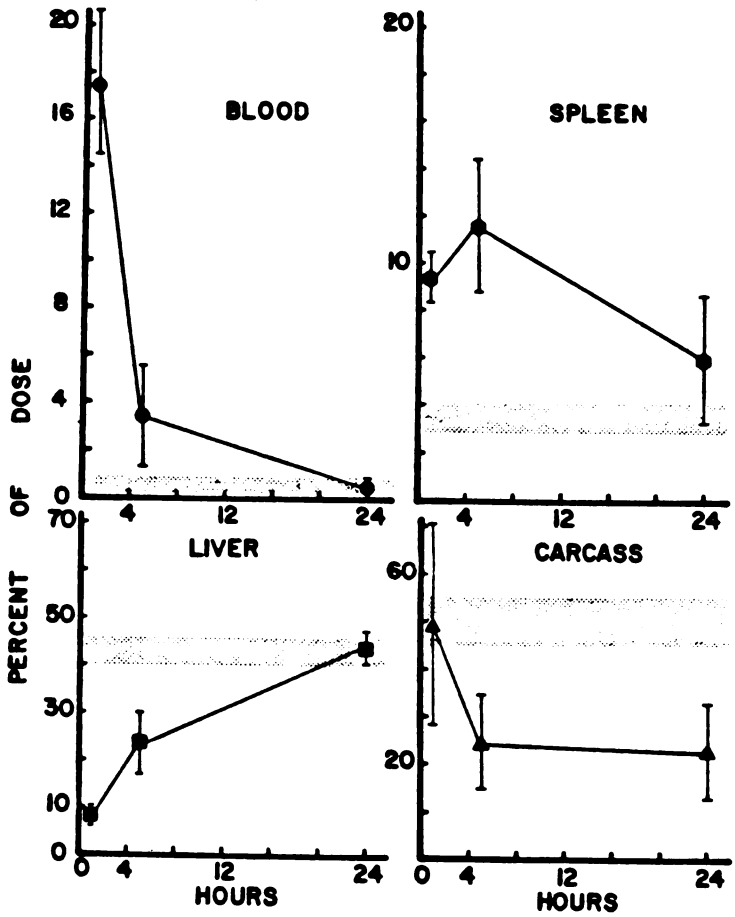


Figure VI-1. Predose study (details on page 112). Tissue levels at one hour after administration of the labelled dose plotted vs. time interval between the unlabelled and labelled doses (blood as percent dose per 1.0 ml). Shaded areas represent mean ± one S.D. for the control group.

Table VI-1: EFFECT OF A LARGE, UNLABELLED LIPOSOME PRE-DOSE ON THE PERCENT OF DOSE REMAINING *IN VIVO* AT ONE HR AFTER INTRAVENOUS ADMINISTRATION OF ^{14}C-INULIN ENCAPSULATED IN 1 μm DIA. LIPOSOMES.

Time separating the two intravenous liposome doses	Total ^{14}C recovered *in vivo* as percent of encapsulated ^{14}C dose* mean ± S.D.
1 hr.	84.8 ± 20.9
5 hr.	61.0 ± 12.8
24 hr.	72.4 ± 8.2
control	96.4 ± 7.0

*Calculated as the sum of values for carcass, liver, spleen, and total blood volume.

CHAPTER VII: EFFECT OF PARTICLE NUMBER, SIZE, AND STABILITY ON LIPOSOME DISPOSITION.

Introduction.

The results of the dose study (Chapter V) raised some intriguing questions concerning the nature of the RES saturation effect. The principal one was: was the saturation effect simply a function of the number of particles (liposomes) that the system encountered, or did the liposome size matter? This was an important question to answer, because if particle number is the dominant factor in the dose effect, then predosing to achieve the saturation (Chapter VI) can be done much more efficiently by using smaller liposomes (i.e. there are more liposomes per unit amount of lipid); on the other hand, there is evidence, for other colloids, that particles of different size have different blood clearances and organ distribution patterns (16), implying that their ability to cause RES saturation may be qualitatively or quantitatively different; this has been found even when an effort was made to administer the same total number of particles for the different sizes (18).

Studies were done to answer this question and it was found that increased particle number alone did not cause a saturation effect; this result led to an examination of the disposition properties of the French press liposomes, as well as an examination of the effect of liposome composition on disposition.

Materials and Methods.

Chemicals and solutions were as in Chapter II.

Liposome preparation and analysis. Preparation of liposome batches, animal procedures, and analysis of tissue levels were as in Chapters II and V. ^{14}C-inulin was used as the entrapped marker.

Liposome Studies. All mice received doses of 300 micromoles lipid /kg intravenously. This dose is equal to the "medium" lipid dose level of Chapter V. In one set of studies the doses were composed of 1.0 micron-extruded, 0.8 micron-dialyzed (for 16-20 hours) liposomes (Chapter IV) + French press liposomes, each type comprising 50% of the total lipid amount in the dose and with the 4/1/1/05 = PC/PA/CH/a-T molar ratio composition. Two types of studies were done with these liposomes: one in which the ^{14}C label was present only in the 1.0-extruded liposomes (henceforth designated 1.0* + FP) and one in which the label was present only in the French press (1.0 + FP*). A second set of studies was done, similar to the first except that the molar ratio composition was 4/1/4.5/.1 = PC/PA/CH/a-T. A third set of studies was done in which FP liposomes alone, of either the 4/1/1/.05 ("low stability") composition or 4/1/4.5/.1 ("high stability") composition were administered. In each case, however, all liposomes contained non-radioactive inulin at 5 mM in the buffer. The dose type abbreviations are:

1.0* + FP	4/1/1/.05
1.0* + FP	4/ 1/4.5/.1
1.0 + FP*	4/1/1/.05
1.0 + FP*	4/1/4.5/.1
FP	4/1/1/.05
FP	4/1/4.5/.1

Results and Discussion.

Particle Number Effect. In order to assess the importance of particle number in the dose effect (Chapter V) studies using the 1.0* + FP, 4/1/1/.05 liposome type were carried out, with this rationale: this mixed dose, although it contained the same total lipid amount as the "medium" dose (Chapter V) and was of the same lipid composition, had a much greater total particle number than the "high" dose (Chapter V) (size distribution measurements done on similar sized liposome doses (Chapter IV) confirmed this). Thus, if particle number is the dominant factor in the liposome disposition, an organ distribution pattern similar to that for the high dose should have been seen (Figs. V-2 to V-5). That is, depressed liver levels and elevated blood and spleen levels at early times, relative to those for the medium dose. 1.0-extruded liposomes were included in the dose (and their fate followed with a ^{14}C label) so that

proper comparison with the dose study results (in which 1.Ø's were used) could be made. Figures VII-1 to VII-4 and Table VII-1 show the results of the 1.Ø* + FP study. It was apparent that the expected high dose (saturation) effect was absent -- in fact the levels are higher in the liver and lower in the blood than for the medium dose 1.Ø's. The conclusion was that simple particle number was not the dominant factor in the liposomes' disposition, and the principal implication was that liposome size may play the most important role.

Effect of Location of Label. As a first step in examining the influence of liposome size on disposition, the 1.Ø + FP*, 4/1/1/.Ø5 study was carried out. By putting the label in the FP liposomes alone, and by comparing these levels with those found in the 1.Ø* + FP study, the fate of two liposome sizes in the same dose could, in essence, be studied. The 1.Ø + FP* results are also shown in Figures VII-1 to VII-4, and Table VII-1. Clear and significant differences in the tissue ^{14}C levels were seen for the two label locations (1.Ø or FP). These differences were significant at all time points in the liver and spleen, at 48 and 96 hours in the carcass, and at all times except 1 hour for the totals. The pattern was one of higher levels with the label in the 1.Ø's, except for the carcass, where the order was reversed.

These results suggested two plausible interpretations.

First, different liposome sizes may "interact" with the body differently. This may include different volumes of distribution (it may be that smaller liposomes have more access to extravascular spaces (6)), the existence of separate recognition sites on RES cells for different sizes (no known evidence), or different affinities for blood substances such as opsonins (6,43): for other colloids, in some cases one colloid has no effect on the disposition of a subsequently or concomitantly-administered dissimilar colloid (16). This lack of interaction is thought to depend on a difference in the colloid surface properties, which can be mediated through opsonins (16.). The absence of a saturation effect in the 1.Ø* + FP study may be a similar case.

An alternative explanation may be in the fact that FP liposomes are known to be less stable than 1.Ø's in <u>in vitro</u> plasma stability tests (41), primarily at low cholesterol composition. Thus, the lower tissue levels seen in the 1.Ø +FP* case may result from a lower <u>in vivo</u> stability (the difference in the totals supports this also). The carcass data, which exhibited the opposite trend, argue against but don't negate this hypothesis. An added implication here is that because the 1.Ø's and FP liposomes were co-administered, the FP may have enhanced the stability of the 1.Ø's by preferentially adsorbing the blood components that are thought to cause liposome destabilization (43). The result would be elevated 1.Ø levels as compared to the dose

study (1.0's alone), which was seen.

Elimination of the Stability Effect. In order to minimize the effect of liposome size differences on stability, the 1.0* + FP, 4/1/4.5/.1 and 1.0 + FP*, 4/1/4.5/.1 studies were done. At this more stable composition the size effect on stability is minimal, and so differences in tissue levels should principally indicate differences in tissue affinity for the two sizes. Figures VII-5 to VII-8 and Table VII-1 show the results for these studies. Again, different tissue levels were seen for the two different label locations. The facts that at one hour 100 percent of the dose is present in vivo for both dose types, and that (not shown) 100% of the counts in blood at one hour were entrapped in liposomes (measured by column chrom.), suggest that the liposomes were still intact. This means that the tissue level differences were due to different affinity or physical distribution properties for the two sizes. The carcass reversal effect (i.e. higher levels for the FP) seen for the 1.0 + FP, 4/1/1/.05 cases was seen here also (Fig. VII-7), further supporting the notion that the two sizes have different disposition properties.

Evidence for Interaction of the Two Sizes. The FP, 4/1/1/.05 study (Figures VII-9 to VII-12, Table VII-1) was done so that results for the two sizes administered separately could be compared (results for the medium dose

1.Ø's (Chapter V) are shown as dotted lines). In all cases except spleen the levels for the two sizes follow a pattern, i.e., different values at one hour and very similar values at all other times. This finding is in contrast to the mixed size studies, in which significant differences in tissue levels due to label location were seen at all times, and this suggests some sort of interaction between the two sizes. Interpretation is made difficult, however, by the fact that the total number of FP, or 1.Ø, liposomes given was twice as great in the single size doses as compared to the mixed doses (this is because the mixed doses were designed to have the same total lipid content as the single-size "medium" dose). The fact that the one hour FP, 4/1/1/.Ø5 levels are lower than the 24 hour level implies a saturation situation similar to that for the "high" dose (Chapter V), and could have been due to the increased FP particle number as compared to the 1.Ø + FP*, 4/1/1/.Ø5 dose. The FP, 4/1/4.5/.1 study was done to see if this dose effect was also present for the high stability composition (Figures VII-9 to VII-12, Table VII-1). A definite "dose-like" effect (i.e. depressed one hour liver levels) is seen for the liver curve, indicating that a dose-like effect occurred. Thus, evidence for some sort of synergistic effect (e.g. one size affecting the other's <u>in vivo</u> stability) caused by the presence of the two sizes together isn't conclusively shown by these data. However, they do show that different size liposomes, coadministered and

interacting with the same "pool" of biological molecules, have different disposition properties; this fact argues for using homogeneous-sized liposome populations.

Other Effects of Changing Composition/Stability. One conclusion that arises quickly from the studies using the two liposome compositions is that the higher stability liposomes (4/1/4.5/.1) give higher tissue levels, for both liposome types (1.Ø + FP and FP). Thus, in vitro plasma stability tests (41,44-48) predict in vivo stability well, and the concept (Chapter V) of in vivo disposition as a competing process (extracellular breakdown of liposomes versus cellular uptake) is reinforced.

There are other trends in the data that are associated with composition-- the rates at which tissue levels decline over time. The terminal slopes for each curve of the organ data, for all the mouse studies done at the "medium" dose level (all of this chapter and the medium dose, Chapter V) were fitted to a straight line equation and the slopes computed, as shown in Table VII-2. The straight-line fit was chosen as a simple indicator; it should not be inferred that this model is the best empirical or mechanistic attempt to describe the data. The data were parsed and summarized by organ and composition, with two striking features evident: first, the curves for the liver and totals are steeper for the higher stability liposome type. An explanation may be that the initial rapid falloff in liver levels over the

first 24-36 hours seen in the dose study (Chapter V), which were attributed to the release of inulin from extracellularly-bound liposomes, may be lasting much longer for the higher stability liposomes due to their decreased leakage rates. Second, the curves for the spleen have very small slopes. It is reasonable to assume that the Chapter V explanation for the "level" spleen curves (the spleen quickly takes up everything that is bound so that no extracellular release effect is seen) remains valid; it is further supported because the slopes are the same for both liposome compositions.

Further Considerations: Number Effect vs. Size Effect. The 1.0* + FP, 4/1/1/.05 experiment was undertaken with the expectation that the very large particle number would cause a saturation (high dose-like) effect. The negative results ruled out a simple particle number effect, but did not exclude a surface area effect. The size distribution data on a similar 1.0 + FP preparation (Chapter IV) revealed that the FP liposomes (30 percent of the total lipid) accounted for only 58 percent of the total surface area. This result would not be expected for a simple solid colloid. Liposomes are a special case in that the FP have a much lower ratio of entrapped water volume to lipid volume. Thus, in the 1.0* + FP case the total surface area was not as great as for the high dose that gave the saturation effect (Chapter V). As mentioned before, it is reasonable to expect that surface

area may affect liposome interaction with biological molecules, and thus their disposition. The role that surface area does play, however, must be clarified in future studies.

Conclusions. In conclusion, it was found that the dose effect (Chapter V) is not simply a function of particle number, but may depend on size or surface area. When a heterogeneous liposome sample is administered, different liposome sizes can have significantly different dispositions. This may result partly from differences in stability of the two sizes, but also involves intrinsically different affinity or physical distribution properties. Also, a synergism or interaction between sizes (e.g. one affecting the other's stability) cannot be ruled out. Finally, the liposomes' stability, as measured in vitro, is a good predictor of in vivo stability, as measured by tissue levels over time.

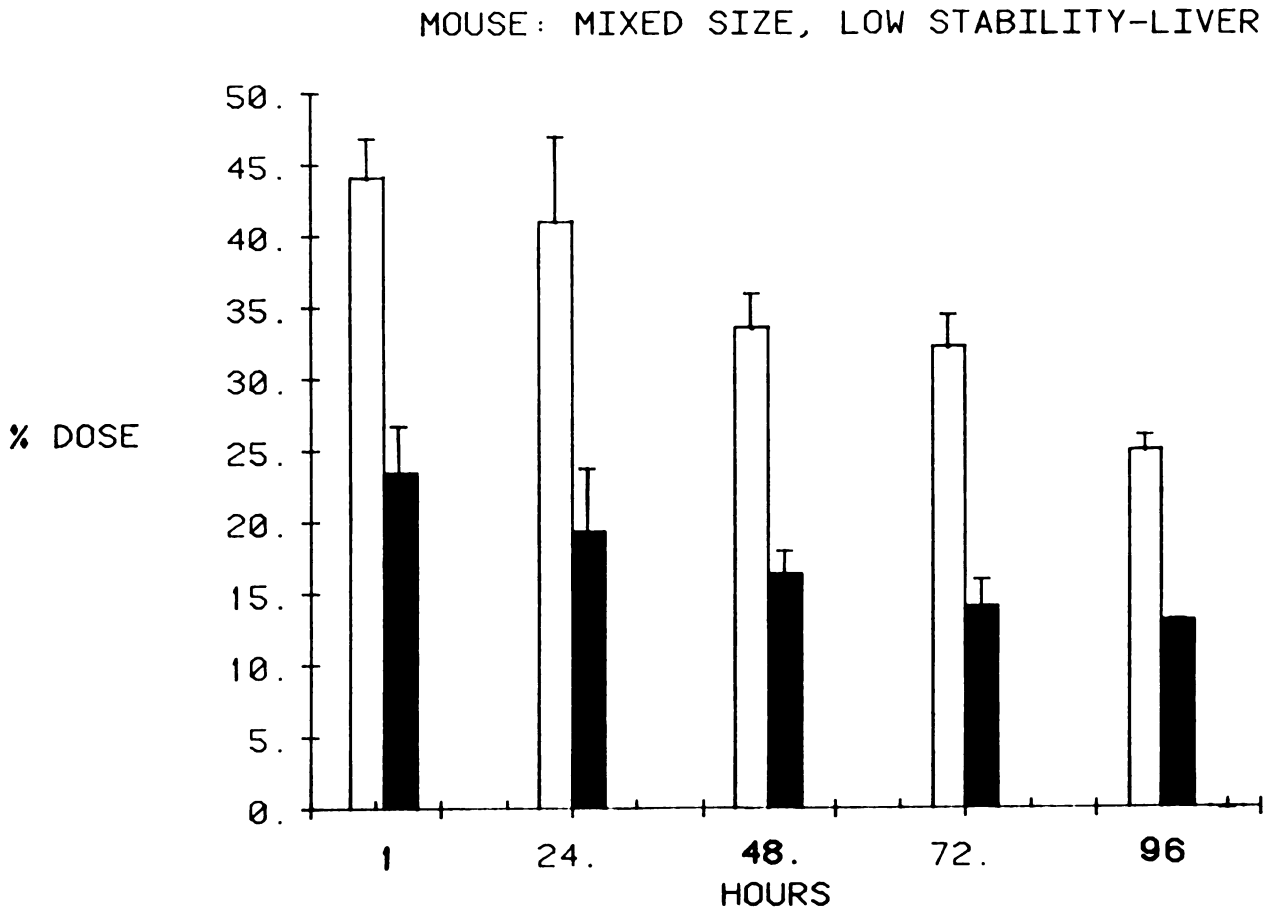


Figure VII-1. Liver results for the 1.0 + FP, 4/1/1/0.05 studies (details on page 119). Results for the 1.0* + FP study are shown as open figures. Results for the 1.0 + FP* study are shown as solid figures. Differences were significant at all time points. Mean + one S.D. (n = 4 at 1,24,72 hours and 2 at 48 and 96 hours).

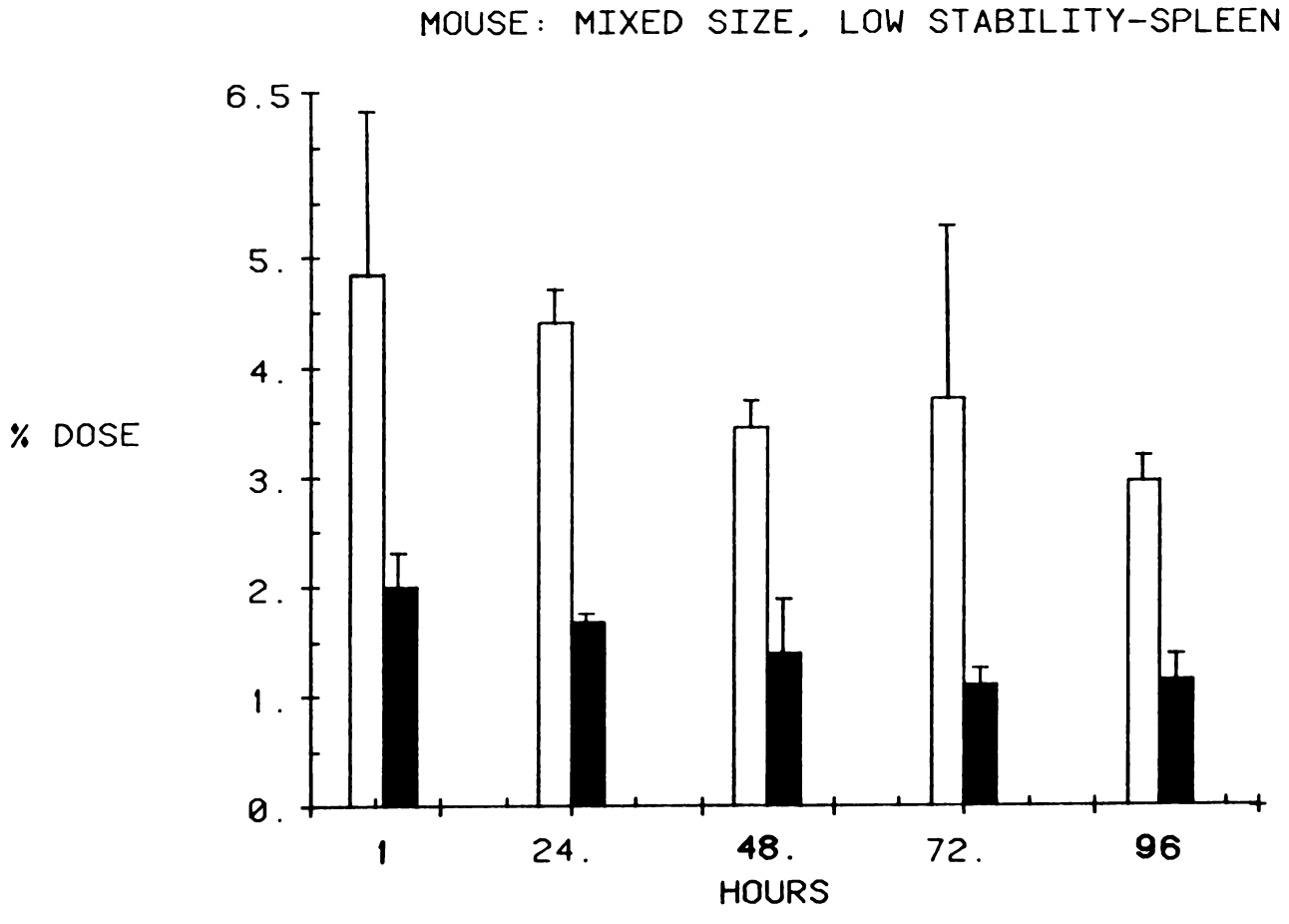


Figure VII-2. Spleen results for the 1.0 + FP, 4/1/1/0.05 studies. Differences were significant at all time points (other details as in Fig. VII-1).

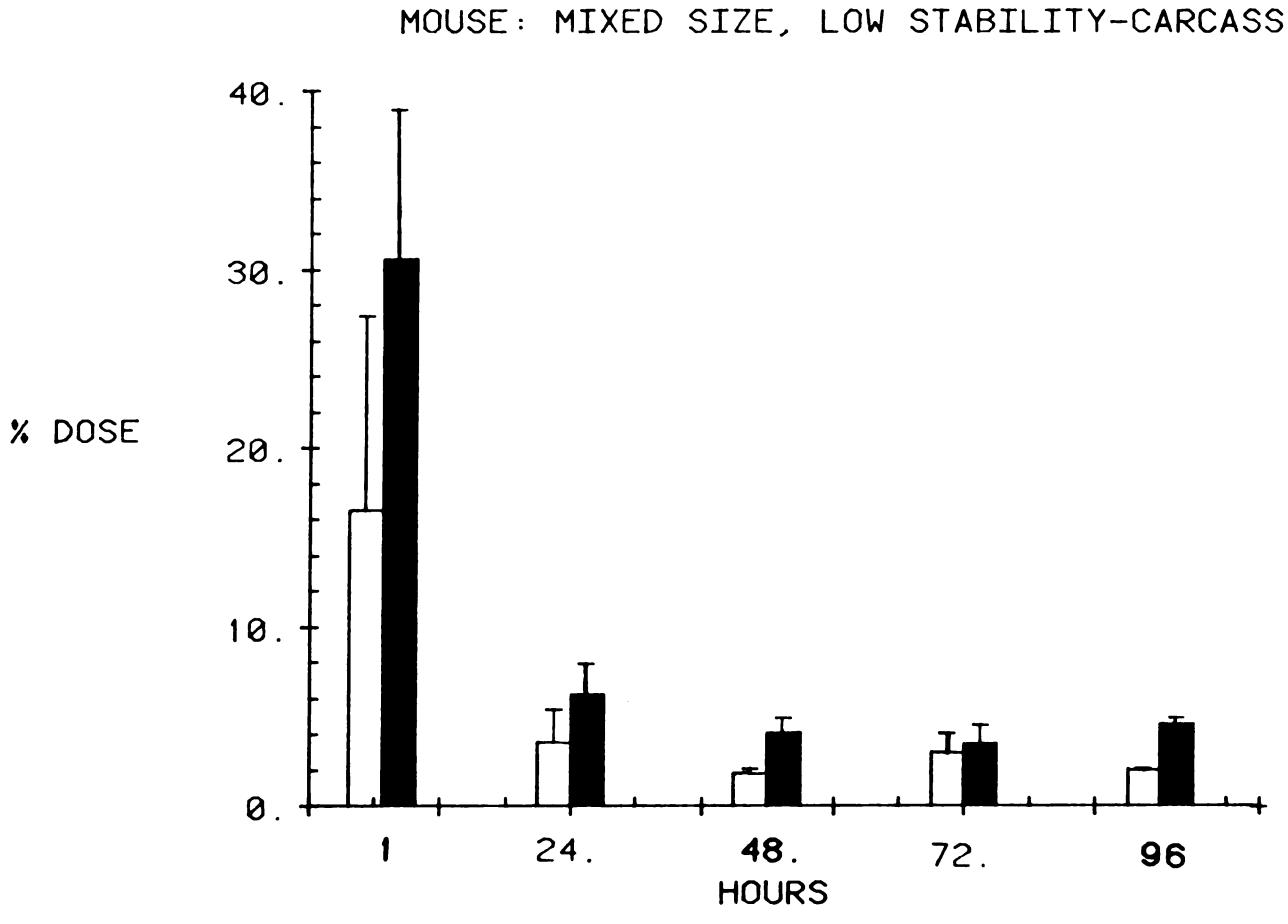


Figure VII-3. Carcass results for the 1.0 + FP, 4/1/1/0.05 studies. Differences were significant at 48 and 96 hours only. Other details as in Fig. VII-1.

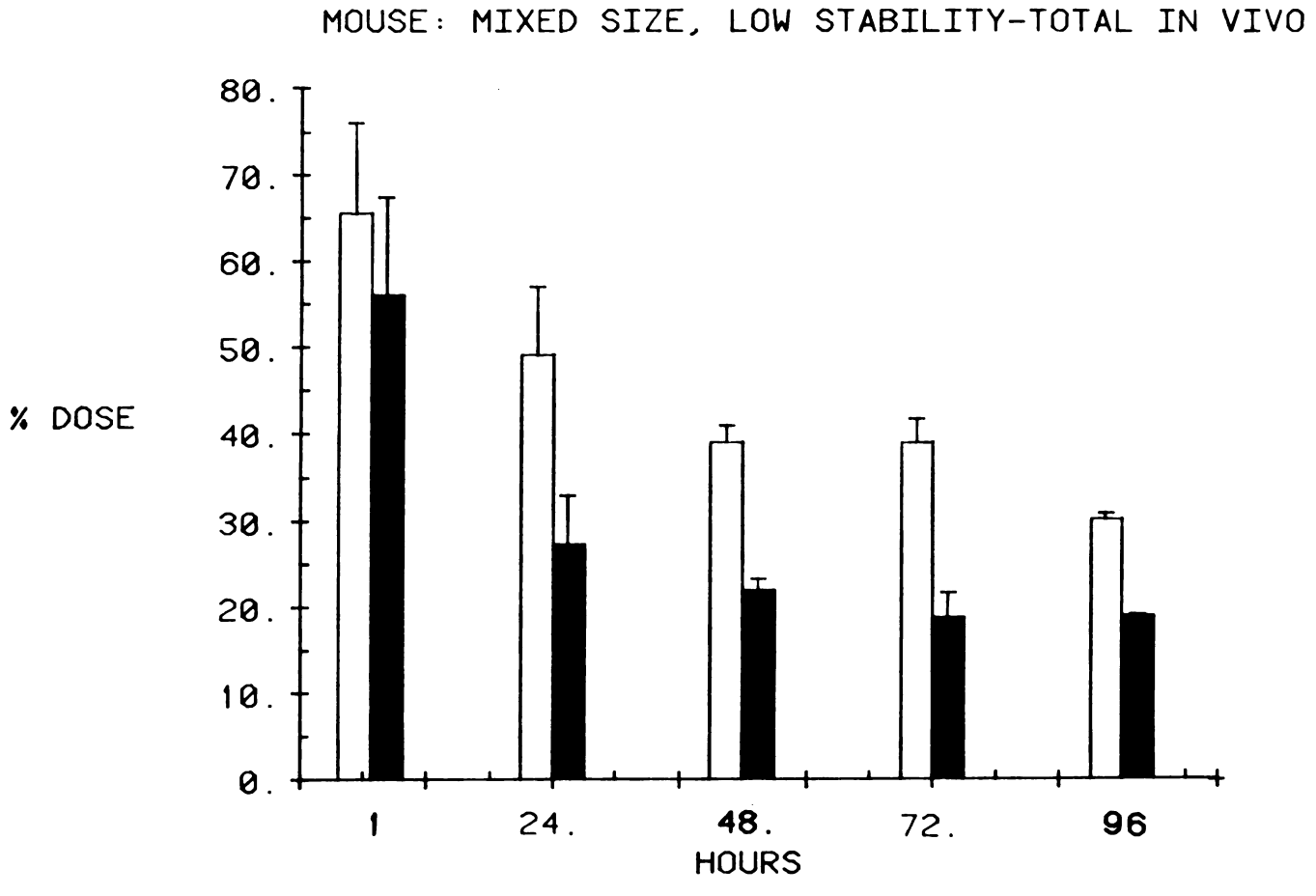


Figure VII-4. Totals in vivo for the 1.0 + FP, 4/1/1/0.05 studies. Differences were significant at all times except 1 hour (other details as in Fig. VII-1).

Table VII-1: One-hour blood levels for all studies in Chapter VII. Values expressed as percent dose per 1.0 ml blood. Values are reported as mean ± one S.D. or individually when n = 2. Values reported as zero were equal to background. Blood levels at all times later than one hour were equal to background.

Dose Type		% dose per 1.0 ml blood
1.0* + FP	4/1/1/.05	0.325 ± 0.23 (n = 4)
1.0 + FP*	4/1/1/.05	2.48 ± 0.86 (n = 4)
1.0* + FP	4/1/4.5/.1	.60, 0
1.0 + FP*	4/1/4.5/.1	6.2, 5.9
FP	4/1/1/.05	7.9, 6.2
FP	4/1/4.5/.1	17.8, 15.8

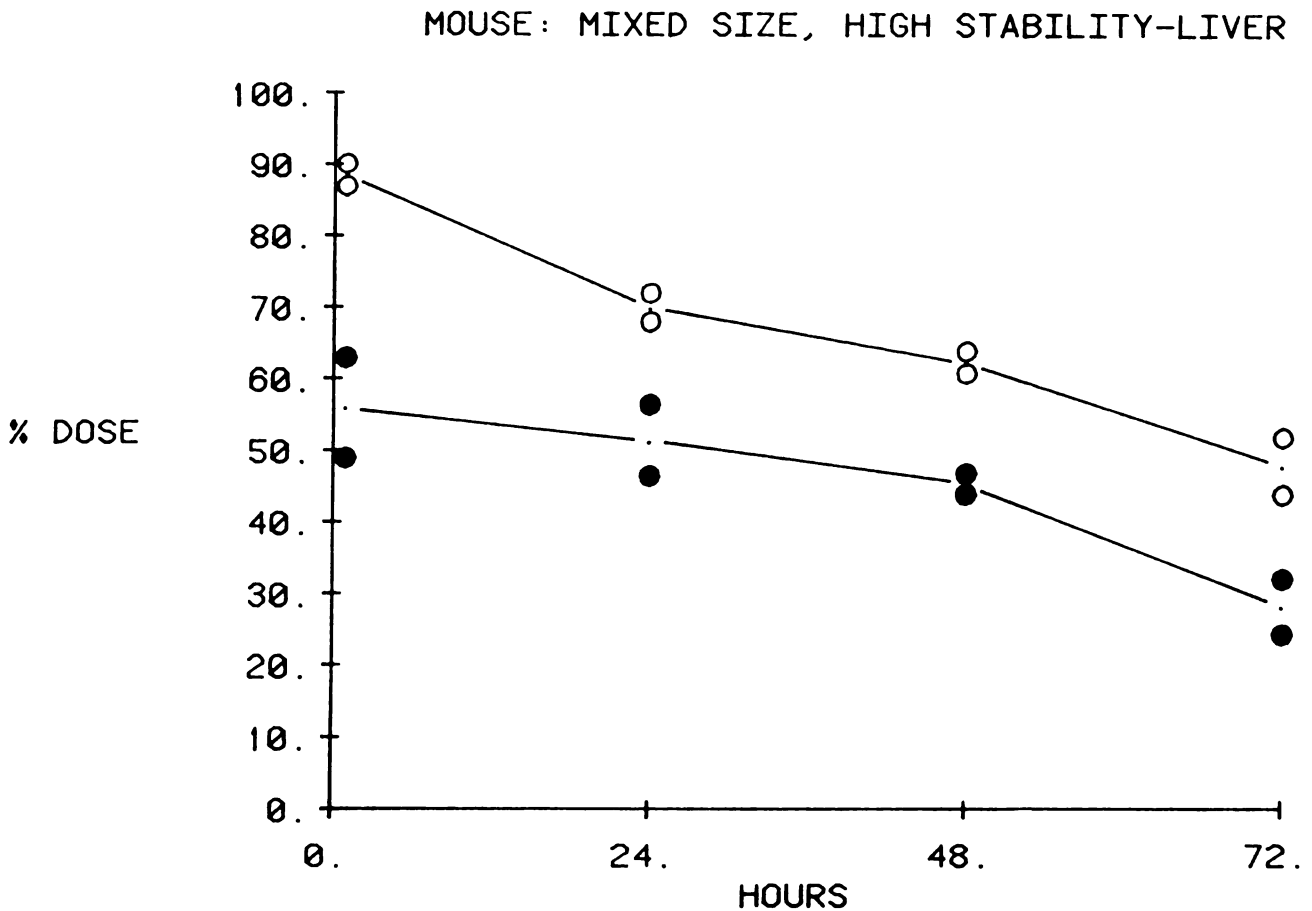


Figure VII-5. Liver results for the 1.0 + FP, 4/1/4.5/0.1 studies (details on page 119). Results for the 1.0* + FP study are shown as open circles. Results for the 1.0 + FP* study are shown as solid circles. Each data point represents one mouse.

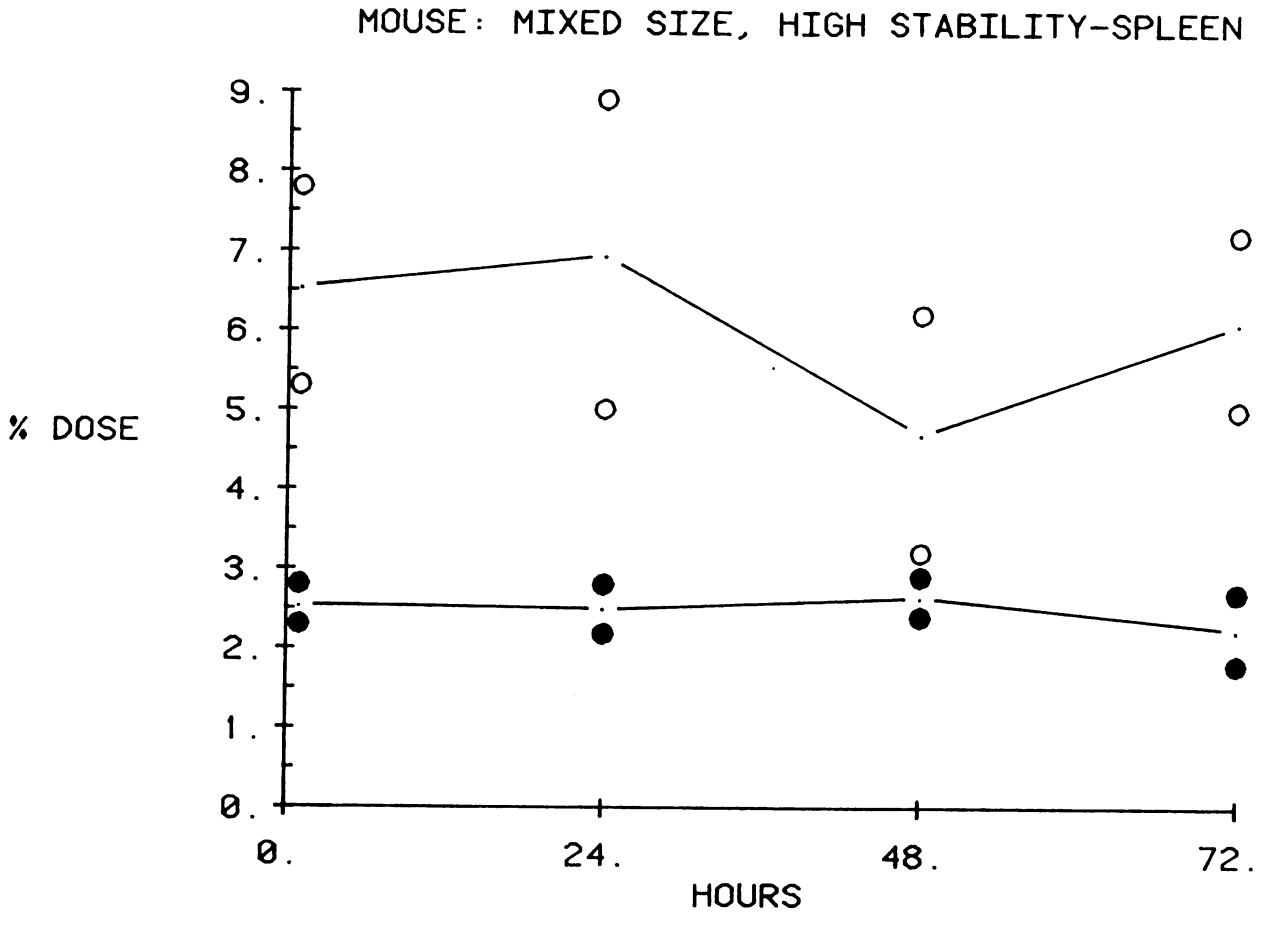


Figure VII-6. Spleen results for the 1.0 + FP, 4/1/4.5/0.1 studies. Details as in Fig. VII-5.

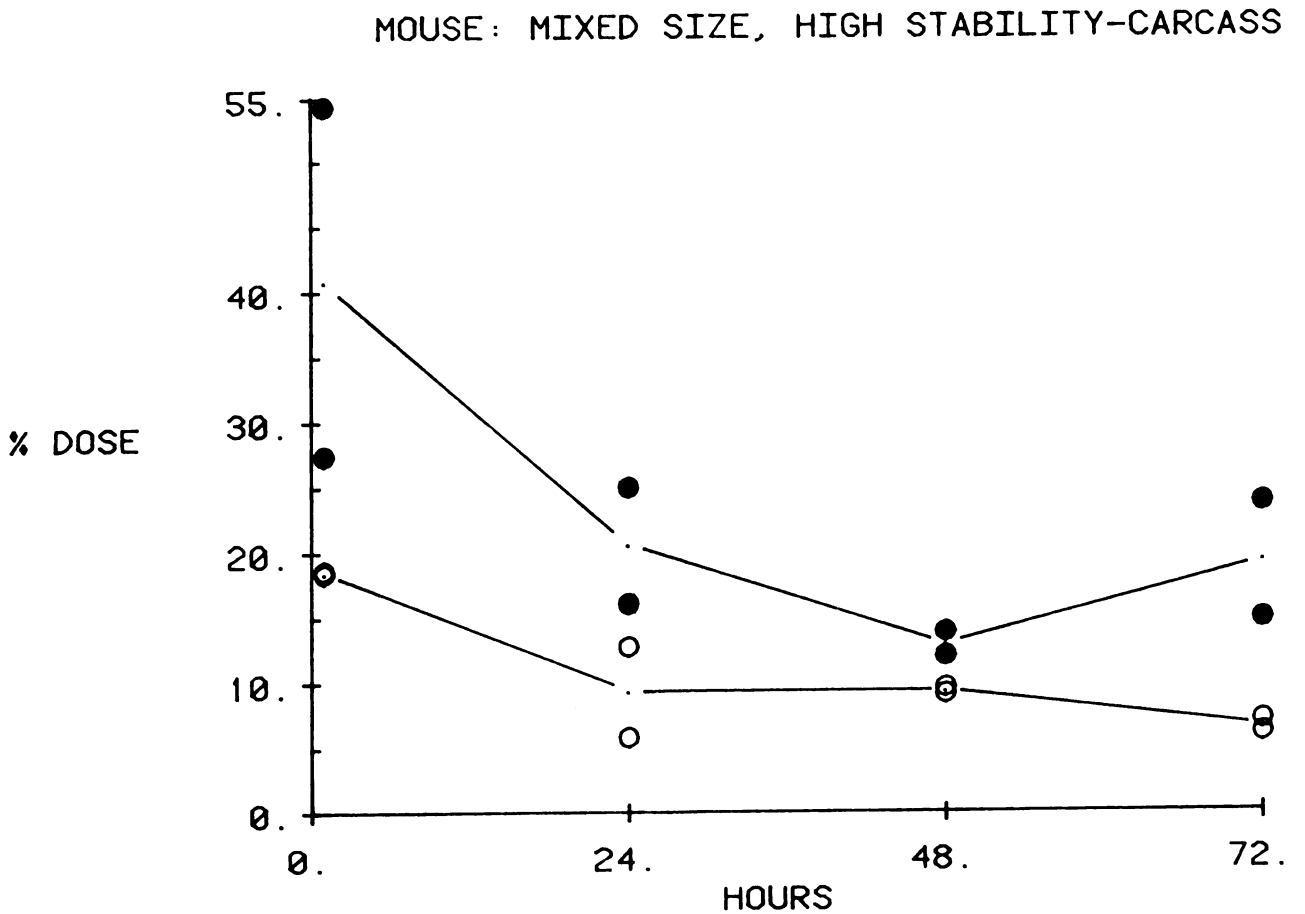


Figure VII-7. Carcass results for the 1.0 + FP, 4/1/4.5/0.1 studies. Details as in Fig. VII-5.

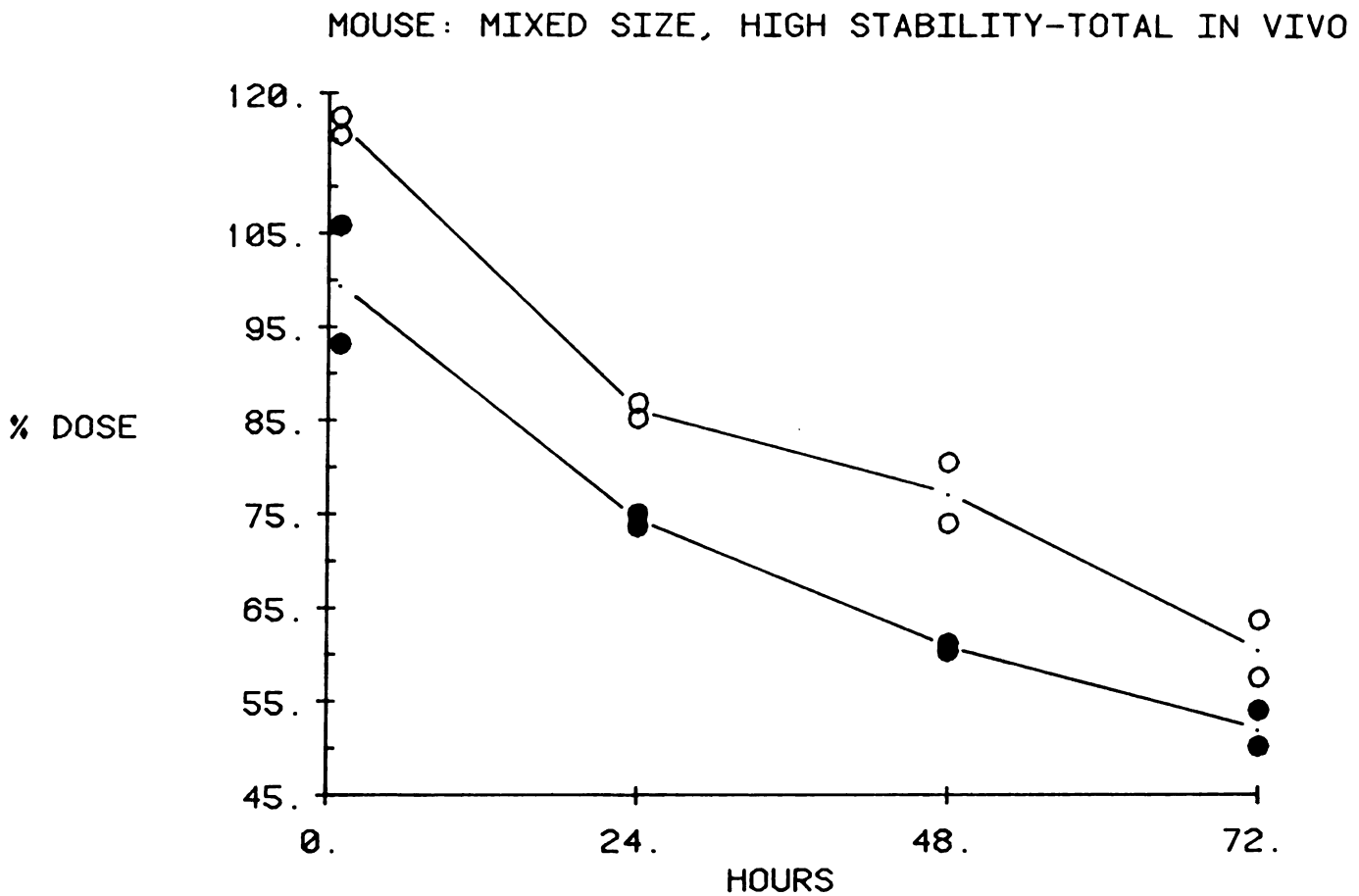


Figure VII-8. Totals in vivo for the 1.0 + FP, 4/1/4.5/0.1 studies. Details as in Fig. VII-5.

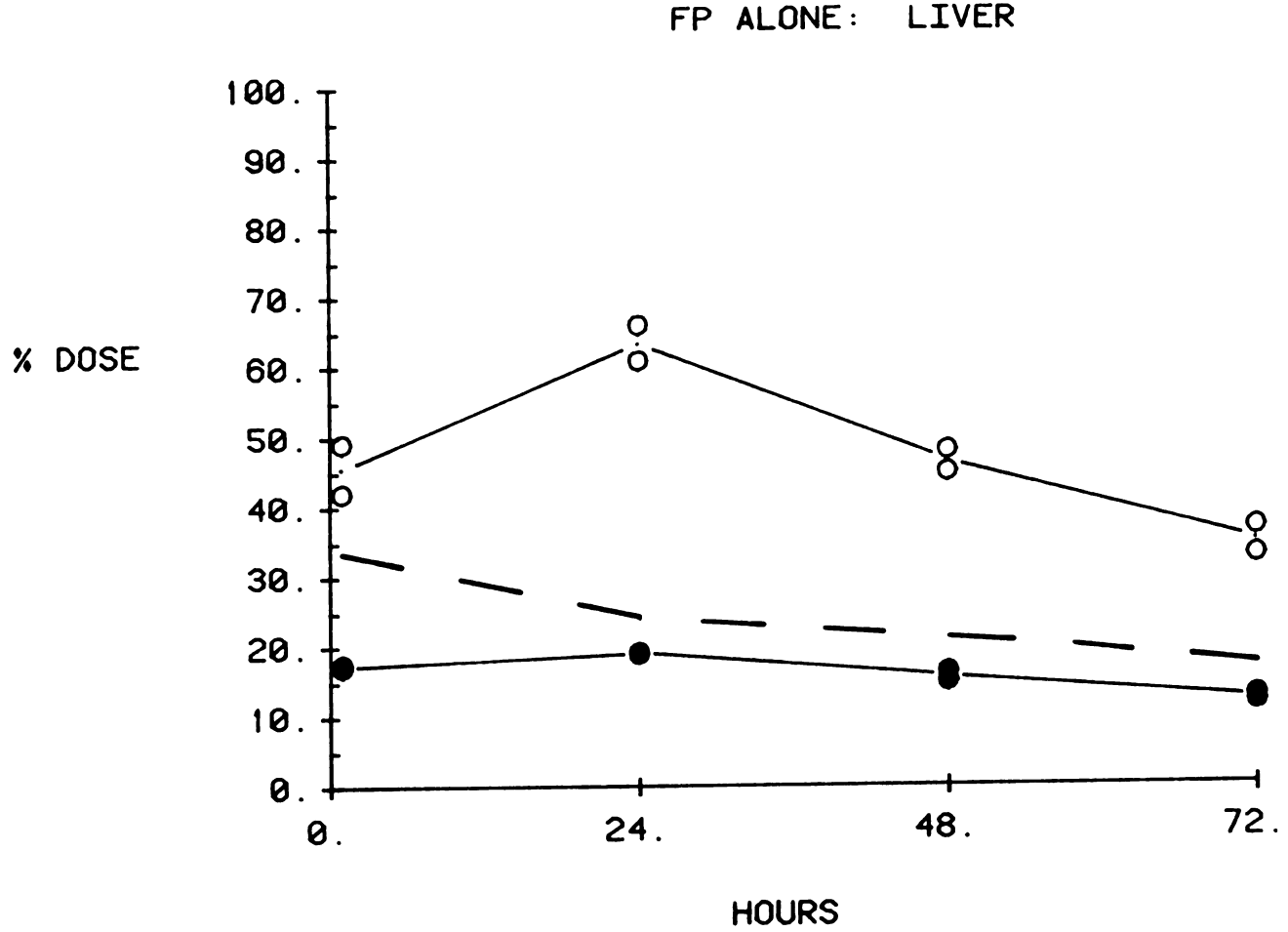


Figure VII-9. Liver results for the studies in which French press liposomes were administered alone (details on page 119). Results for the high stability liposomes are shown as open circles. Results for the low stability liposomes are shown as solid circles. Levels for the medium dose 1.0's (dose study, Chapter V) are drawn as dotted lines for comparison.

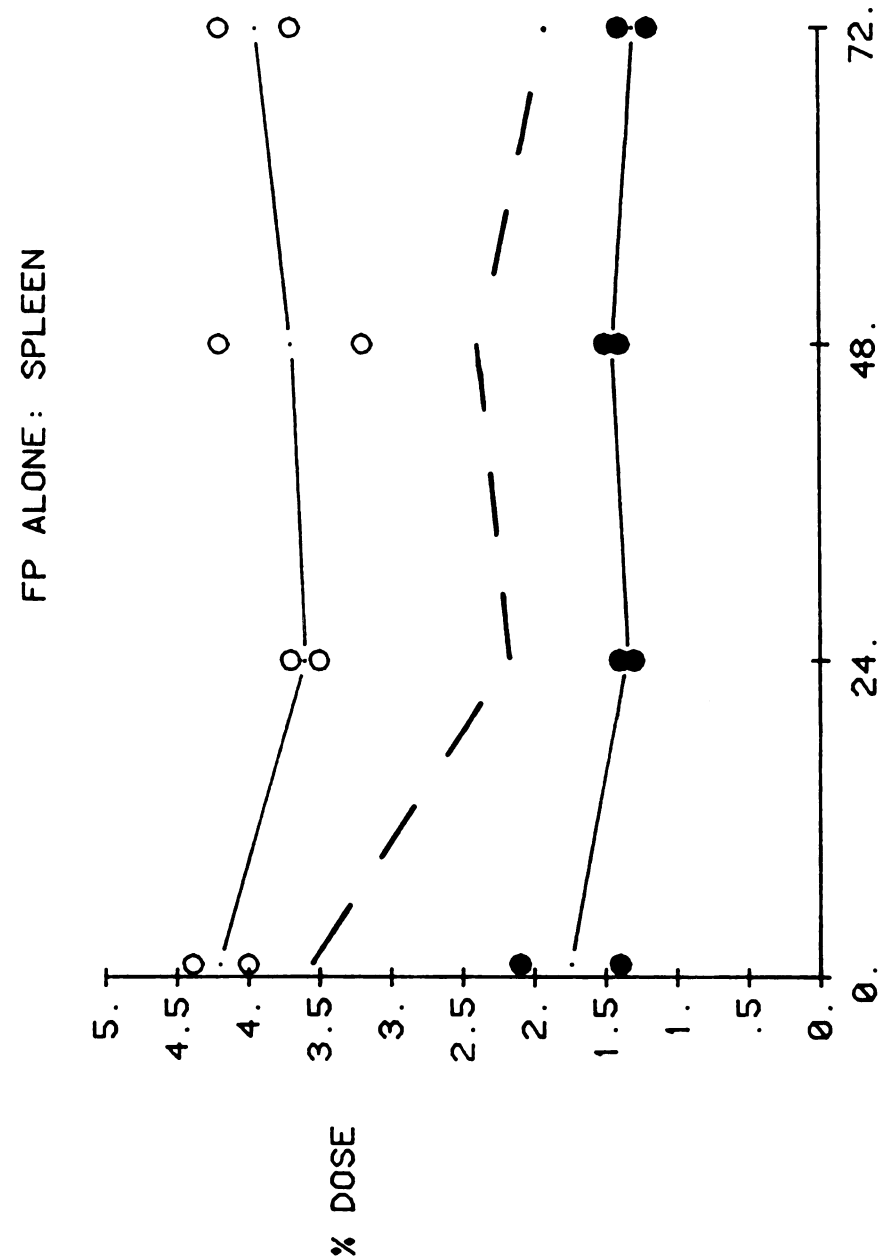


Figure VII-10. Spleen results for the FP alone studies. Details as in Fig. VII-9.

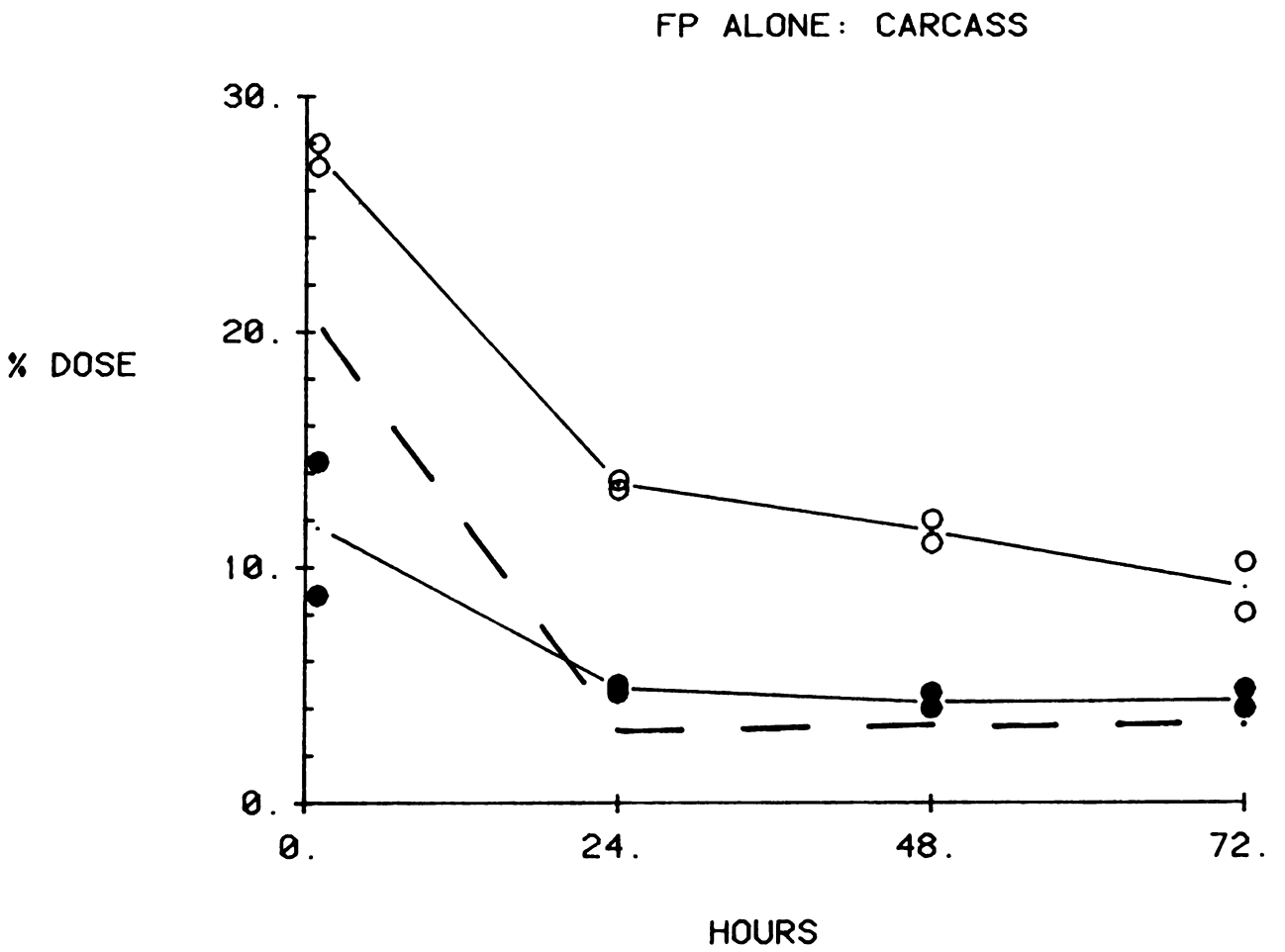


Figure VII-11. Carcass results for the FP alone studies. Details as in Fig. VII-9.

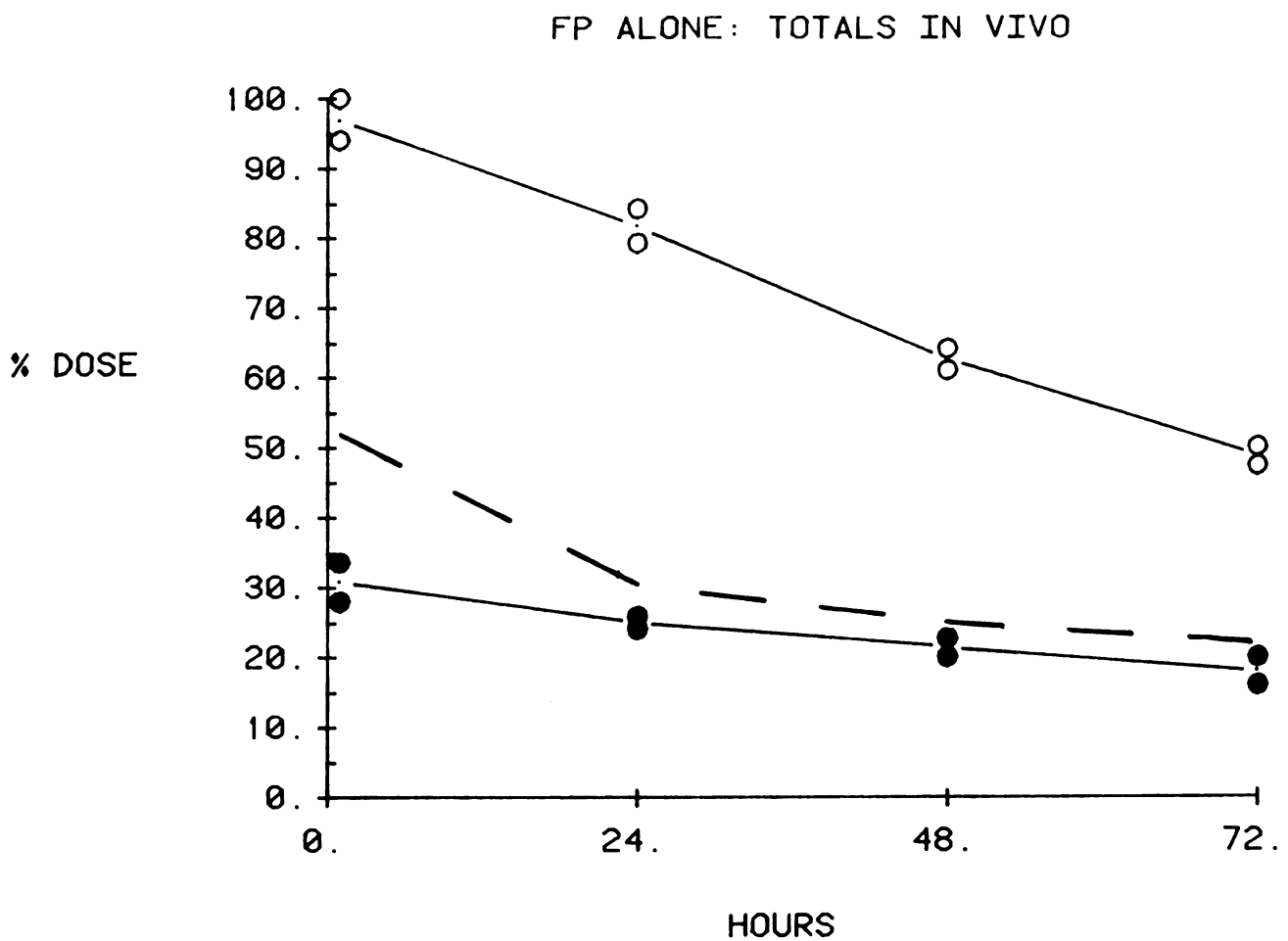


Figure VII-12. Totals <u>in vivo</u> for the FP alone studies. Details as in Fig. VII-9.

Table VII-2: Linear regression slopes for tissue data done at the "medium" dose level. Slopes given in % dose/hour:
Slope
S.D.
(n)

Liposome Composition	Liver	Spleen	Carcass	Totals
PC/PA/CH/a-T = 4/1/1/.05	−.11 ± .072 (4)	−.012 ± .007 (4)	−.0145 ± .012 (4)	−.15 ± .062 (4)
PC/PA/CH/a-T = 4/1/4.5/.1	−.50 ± .12 (3)	−.007 ± .008 (3)	−.058 ± .03 (3)	−.63 ± .085 (3)

CHAPTER VIII: GENERAL CONCLUSIONS.

The results of these studies have led to several general conclusions. First, the liposomes' in vivo disposition is very dependent on several physical parameters: size, surface charge, stability and dose. Ascribing observed liposome behavior to any one of them, however, is far from straightforward, since they are interdependent. For example, liposome size and surface charge affect tissue uptake, but they also affect stability, which in turn affects tissue uptake. Liposome dose can also affect stability (by saturation of the destabilizing factors in blood as the dose increases). Size and dose are important in determining tissue distribution, and may be exerting their effect through the common denominator of surface area. Thus, one must be very careful in attributing altered liposome disposition to a single factor.

Second, the RES disposition of liposomes cannot be treated in the same manner as that for other, inert colloids. Specifically, tissue levels of a liposome-entrapped label cannot automatically be interpreted as the level of intracellular delivery of the label by the liposomes. The data presented here suggest that there can be a significant amount of extracellular release of label from tissue-bound liposomes. In fact, all of the in vivo liposome efficacy data to date can be explained by a localized slow release effect (this is because all of the drugs used have some

activity in their free form, and thus some minimal ability to enter cells). Only in cases such as the liposome-mediated insertion of RNA into cells (77,87) can the intracellular delivery route be invoked exclusively.

It must be noted that in this thesis there were very few attempts to compare specific experimental results with those of other investigators. This is primarily because most of the studies reported here have not been done previously. In the case of the rabbit studies (Chapter III), even though one major objective was to document some previously reported results in a general way, there were enough important differences in experimental design to make highly specific comparison unfeasible. In fact, attempts to duplicate exactly another investigator's work may be futile because of the unreproducible nature of the techniques currently used in liposome studies.

It will also be noted that there were no attempts to fit mechanistic models to the data presented here. This is because the in vivo disposition of liposomes is very complex. Fig. VIII-1 shows what can happen to a liposome and its entrapped label (L) in blood: the liposome in blood can bind to cells (a) or become destabilized and release its label (b), which can then be excreted (c); it is also assumed that bound liposomes can release label (d); leakage of the label can potentially result in empty liposomes (e), which might exert a tissue effect (f)(since the liposome no

longer has its label, this would be difficult to model kinetically); bound liposomes can be intracellularized (g) and can release the label inside the cell (h); the label may then exit or reenter the cell (i) or be excreted (e.g. in the bile (j)); intact liposomes could presumably be exocytosed (k), but there is no evidence for this. The use of a marker such as ^{14}C-inulin simplifies the scheme somewhat (Chapter V), but it is still complex since at least two of the processes (a and b) are known to be saturable. Attempts have been made to fit models to *in vivo* data (notably Hwang's attempts (76)) but they have not resulted in any clarification of the underlying processes.

In the future, liposome research will probably center in these areas: 1) further improvements in formulation aspects (e.g. sizing, entrapment efficiency), 2) continued pharmacological testing of liposomes in animal model disease systems 3) continued improvements in specialized delivery, such as lung uptake or localized hyperthermia and 4) hopefully, attempts to further elucidate the basic *in vivo* disposition processes. The last will be very important if liposomes are ever to become the "magic bullet" of therapeutics.

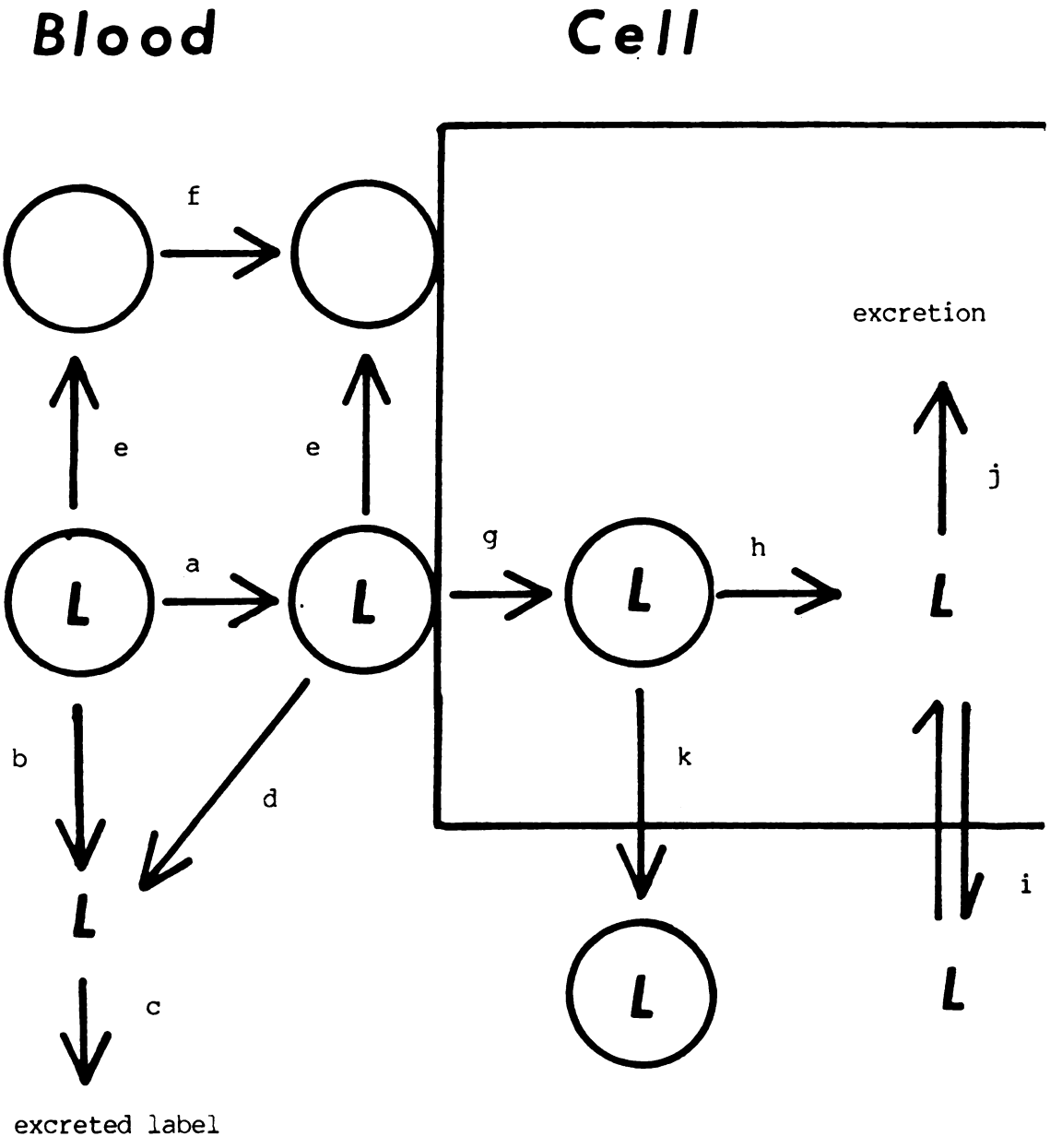


Figure VIII-1. Schematic diagram of the various things that can occur with liposomes and their entrapped markers in vivo.

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