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LIPOSOMES AS A DRUG DELIVERY SYSTEM

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INTRODUCTION

Liposomes have shown great potential as a drug delivery system. An assortment of molecules, including peptides and proteins, have been incorporated in liposomes, which can then be administered by different routes. Various amphiphathic molecules have been used to form the liposomes, and the method of preparation can be tailored to control their size and morphology. Drug molecules can either be encapsulated in the aqueous space or intercalated into the lipid bilayer; the exact location of a drug in the liposome will depend upon its physicochemical characteristics and the composition of the lipids.

Due to their high degree of biocompatibility, liposomes were initially conceived of as delivery systems for intravenous delivery. It has since become apparent that liposomes can also be useful for delivery of drugs by other routes of administration. The formulator can use strategies to design liposomes for specific purposes, thereby improving the therapeutic index of a drug by increasing the percent of drug molecules that reach the target tissue, or alternatively, decreasing the percent of drug molecules that reach sites of toxicity. Clinical trials now underway utilize liposomes to achieve a variety of therapeutic objectives including enhancing the activity and reducing toxicity of a widely used antineoplastic drug (doxorubicin) and an antifungal drug (amphotericin B) delivered intravenously. Other clinical trials are evaluating the ability of liposomes to deliver intravenously immunomodulators (MTP-PE) to macrophages and imaging agents (^{111}Indium) to tumors. Recent studies in animals have reported the delivery of water-insoluble drugs into the eye, and the prolonged release of an immunomodulator (interferon) and a peptide hormone (calcitonin) from an intramuscular depot. These trials and animals studies provide evidence of the versatility of liposomes.

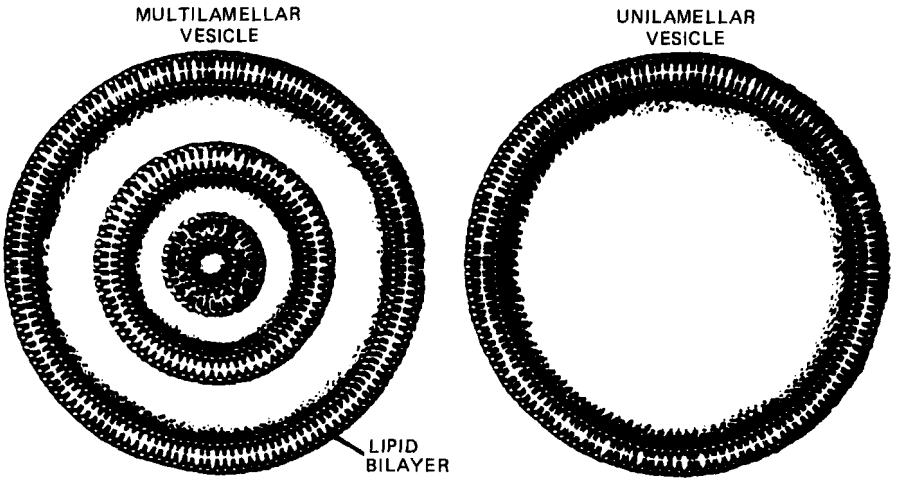

FIGURE 1
Diagramatic representation of multilamellar and unilamellar vesicles. Source: Ostro, M.J. (1987) Scientific American, 102-111.

A liposome is defined as a structure consisting of one or more concentric spheres of lipid bilayers separated by water or aqueous buffer compartments (Fig. 1). These spherical structures can be prepared with diameters ranging from 80 nm to 100 μm. When phospholipids are dispersed in an aqueous phase, hydration of the polar head groups of the lipid results in a heterogeneous mixture of structures, generally referred to as vesicles, most of which contain multiple lipid bilayers forming concentric spherical shells. These were the liposomes first described by Bangham[1] and are now referred to as multilamellar vesicles (MLVs). Sonication of these lipid dispersions results in size reduction of these liposomes to vesicles containing only a single bilayer with diameters ranging from 25-50 nm. These structures are referred to as small unilamellar vesicles (SUVs). Since MLVs and SUVs have certain limitations as model membrane systems and as drug delivery systems, a number of laboratories have developed single bilayer liposomes which exhibit a size range of 100-500 nm in diameter. These vesicles are referred to as large unilamellar vesicles (LUVs). The nomenclature describing liposomes can be confusing since liposomes have been classified as a function of the number of bilayers (e.g., MLV, SUV), or as a function of the method of preparation (e.g., REV, FPV, EIV) or as a function of size (e.g., LUV, SUV). The following are examples of frequently used nomenclatures:

TABLE 1
Examples of Nomenclature Used to Describe Liposomes.

TYPE OF VESICLE	TERM USED	APPROX. SIZE (μm)
Small, Sonicated Unilamellar	SUV	0.025-0.05
Large, Vortexed Multilamellar	MLV	0.05-10
Large Unilamellar	LUV	0.1
Reverse Phase Evaporation	REV	0.5
French Press	FPV	0.05
Ether Injection	EIV	0.02

MATERIALS USED IN LIPOSOME PREPARATION

The lipids most commonly used to prepare liposomes are shown in Fig. 2.

Phospholipids: Glycerol containing phospholipids are by far the most commonly used component of liposome formulations and represent more than 50% of the weight of lipid present in biological membranes. The general chemical structure of these types of lipids is exemplified by phosphatidic acid. The "backbone" of the molecule resides in the glycerol moiety. At position number 3 of the glycerol molecule the hydroxyl is esterified to phosphoric acid (hence the name glycerolphospholipids). The hydroxyls at positions 1 and 2 are usually esterified with long chain fatty acids giving rise to the lipidic nature of these molecules. One of the remaining oxygens of phosphoric acids may be further esterified to a wide range of organic alcohols including glycerol, choline, ethanolamine, serine and inositol. The phosphate moiety of phosphatidic acid carries a double negative charge only at high pH. The pK values for the two oxygens are 3 and about 7. At physiologically relevant pH values this molecule presents more than one net negative charge, but not quite 2. The most abundant glycerol phosphatides in plants and animals are phosphatidylcholine (PC), also called lecithin, and phosphatidylethanolamine (PE), sometimes referred to as cephalin. These two phosphatides constitute the major structural component of most biological membranes. In phosphatidylserine (PS),the phosphoric acid moiety of phosphatidic acid (PA) is esterified to the hydroxyl group of the amino acid L-serine, and in phosphatidylinositol (PI) to one of the hydroxyls of the cyclic sugar alcohol inositol. In the case of phosphatidylglycerol (PG), the alcohol that is esterified to the phosphate moiety is glycerol. Table 2 shows the fatty acid composition of two common phosphatidylcholines, one extracted from egg yolk and the other from soy bean oil. Notice the difference in the degree of unsaturation between egg and soy PC. Soy PC contains a greater proportion of unsaturated bonds and is thus more susceptible to peroxidation.

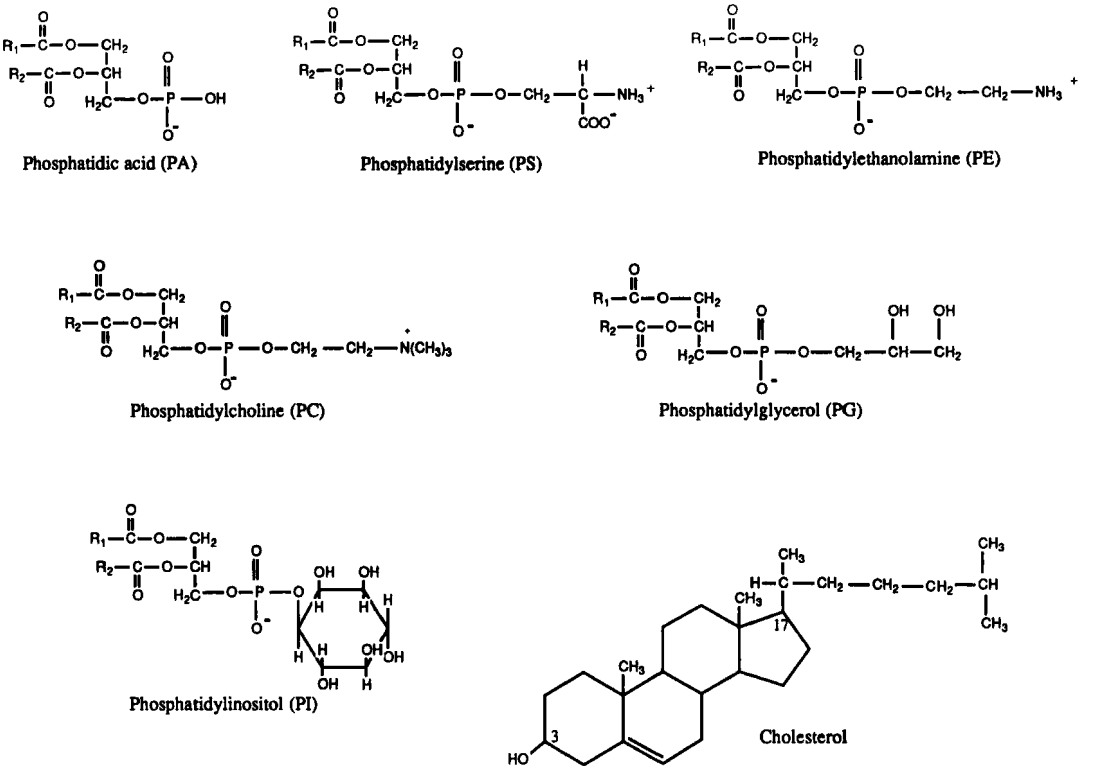

FIGURE 2
Chemical structures of lipids commonly used to prepare liposomes.

TABLE 2
Fatty acid composition of two common phosphatidylcholines, one extracted from egg yolk and the other from soy bean oil.

Fatty Acid Composition		Egg PC	Soy PC
16:0	Palmitic	32	12
16:1	Palmitoleic	1.5	<0.2
18:0	Stearic	16	2.3
18:1	Oleic	26	10
18:2	Linoleic	13	68
18:3	Linolenic	<0.3	5
20:4	Arachidonic	4.8	<0.1
22:6	Dodosapentaenoic	4.0	<0.1

Steroids: The steroid cholesterol and its derivatives are quite often included as components of liposomal membranes. Cholesterol is abundant in animal tissues and is primarily localized in cell membranes. Its inclusion in liposomal membranes has three recognized effects: (i) increasing the fluidity or microviscosity of the bilayer; (ii) reducing the permeability of the membrane to water soluble molecules; and (iii) stabilizing the membrane in the presence of biological fluids such as plasma. This latter effect has proven useful in formulating liposomes for drug delivery applications which use the intravenous route of administration. Liposomes without cholesterol are known to interact rapidly with plasma proteins such as albumin, transferrin and macroglobulins. These proteins tend to extract bulk phospholipids from liposomes thereby depleting the outer monolayer of the vesicles leading to physical instability. Cholesterol appears to substantially reduce this type of interaction. Cholesterol has been called the "mortar" of bilayers because, by virtue of its molecular shape and solubility properties, it fills in empty spaces among the phospholipid molecules, anchoring them more strongly into the structure.

Metabolic Fate of Bilayer Forming Lipids: An attribute of liposomes that translates into unusually high *in vivo* tolerance is the fact that the structural components of the system, phospholipids and cholesterol, are treated no differently than biological membrane lipids. In the body they are broken down by enzyme systems into natural intermediates like glycerol phosphate, fatty acids, ethanolamine, choline and acyl-Co-A and either metabolized further to provide energy, or enter a lipid pools which is drawn upon to build new lipids which replace those that naturally turn over in biological membranes. Phospholipids are hydrolyzed *in vivo* by specific phospholipases (Fig. 3). Phospholipases can be used *in vitro* to modify natural lipids. For example, phospholipase A-2 isolated from snake venom has been used to produce lysophosphatidylcholine from natural phosphatidylcholine. Phospholipase D is being used commercially to produce "semisynthetic" PS, PA and PG from PC. PC dissolved in ether is added and the two phases emulsified. The enzyme catalyzes head group exchange. The rates and yields of the conversion are dependent on the activity of the enzyme and the molar excess of the alcohol to be exchanged for choline.

The liver serves both as the chief source and chief organ for the disposal of cholesterol. A major portion of the cholesterol removed from plasma lipoproteins by the liver is excreted in the bile.

Synthetic Phospholipids: Generally used saturated phospholipids include dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylserine (DPPS), dipalmitoylphosphatidic acid (DPPA) and dipalmitoylphosphatidylglycerol (DPPG). Several unsaturated phospholipids have also been used for preparing liposomes; these include dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG).

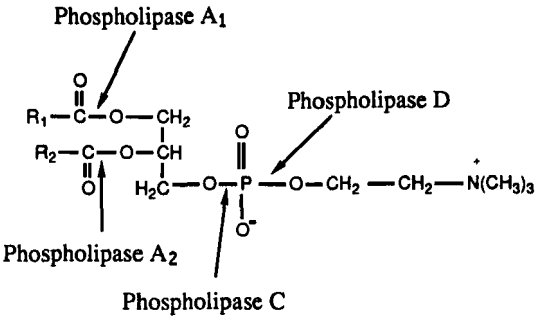

FIGURE 3
Sites of action of phospholipases on phosphatidylcholine.

Other Substances: Diacylglycerol, stearylamine and dicetylphosphate have been incorporated into liposomes so as to impart either a negative or a positive surface charge to these structures. Also, a number of compounds having a single long-chain hydrocarbon and an ionic head group has been synthesized and found to be capable of forming vesicles. These include quaternary ammonium salts and dialkyl phosphates.

Lipid Selection: Many liposome-based pharmaceutical products are entering the clinical trial stage of development; several of these may reach the marketplace in a few years. The lipid component of these products must meet stringent "pharmaceutical" requirements in order to obtain regulatory approval for large-scale human testing and marketing. These include suitable purity, safety and microbial/endotoxin limits, and adequate stability. Currently-available pharmaceutical grade lipid products (such as egg and soy phosphatides) were developed primarily for the parenteral emulsions industry and in general they are not well suited for liposome formulations. Liposomes composed of crude egg yolk phosphatides, for example, are not stable at ambient temperatures for more than a few months. Thus developers of liposome products have been relying upon specialty chemical firms to supply highly purified lipids for their raw material needs. The current cost for these high purity lipids will need to be addressed for large scale production and commercialization of liposome products. Although liposome-based products are within reach, and their market potential is large, successful commercialization depends in part on the willingness of lipid suppliers to differentiate their product lines in response to the needs of the rapidly emerging liposome industry. Since each liposome-based product has its unique stability, safety and purity requirements, it is likely that a range of lipids including natural products, semi-synthetics and synthetics of varying degrees of purity will be needed. The key pharmaceutical and commercial issues that remain to be addressed by both the lipid

suppliers and liposome product developers include the following:

a. Quantities, purities and pharmaceutical attributes of lipids required for liposome products;
b. Detailed specifications for each lipid, including standardized nomenclature and analytical quality control procedures;
c. Introduction of the above into official compendia; and
d. Expansion/centralization of a data base on the stability and safety of key lipids.

WHY LIPOSOMES ARE FORMED

Lipids capable of forming liposomes (or other colloidal structures) exhibits a dual chemical nature. Their head groups are hydrophilic (water loving) and their fatty acyl chains are hydrophobic (water hating). It has been estimated that each zwitterionic head group of phosphatidylcholine has on the order of 15 molecules of water weakly bound to it, which explains its overwhelming preference for the water phase. The hydrocarbon fatty acid chains, on the other hand, vastly prefer each other's company to that of water. This phenomenon can be understood in quantitative terms by considering the critical micelle concentration (c.m.c.) of PC in water. The c.m.c. is defined as the concentration of the lipid in water (usually expressed as moles per liter) above which the lipid forms either micelles or bilayer structures rather than remaining in solution as monomers. The c.m.c. of dipalmitoylphosphatidylcholine has been measured by Smith and Tanford[2] and found to be 4.6×10^{-10} M in water. This value is in agreement with those obtained for similar amphiphiles. Clearly, this is a very small number indicating the overwhelming preference of this molecule for a hydrophobic environment such as that found in the core of a micelle or bilayer.

The large free energy change between a water and a hydrophobic environment (≈15.3 Kcal/mole for dipalmitoylphosphatidylcholine and ≈13.0 Kcal/mole for dimyristoylphosphatidylcholine) explains the overwhelming preference of typical lipids to assemble in bilayer structures excluding water as much as possible from the hydrophobic core in order to achieve the lowest free energy level and hence the highest stability for the aggregate structure. It is also clear from these thermodynamic considerations that bilayer structures do not exist as such in the absence of water because it is water that provides the driving force for lipid molecules to assume a bilayer configuration.

A high degree of surface activity of a given molecule does not guarantee it ability to form bilayer structures in the presence of water. The type of physical structure they attain under a given set of conditions will depend on their interactions with neighboring molecules, their interaction with water and most importantly, whether the surface area of the polar head group, upon hydration, is smaller or larger than the surface area of the hydrophobic group (Fig. 4). For example, phosphatidylcholine, sphingomyelin, phosphatidylserine, phosphatidylinositol and phosphatidylglycerol have a preference for bilayer structures (liposomes). On the other hand, lysophospholipids form micelles

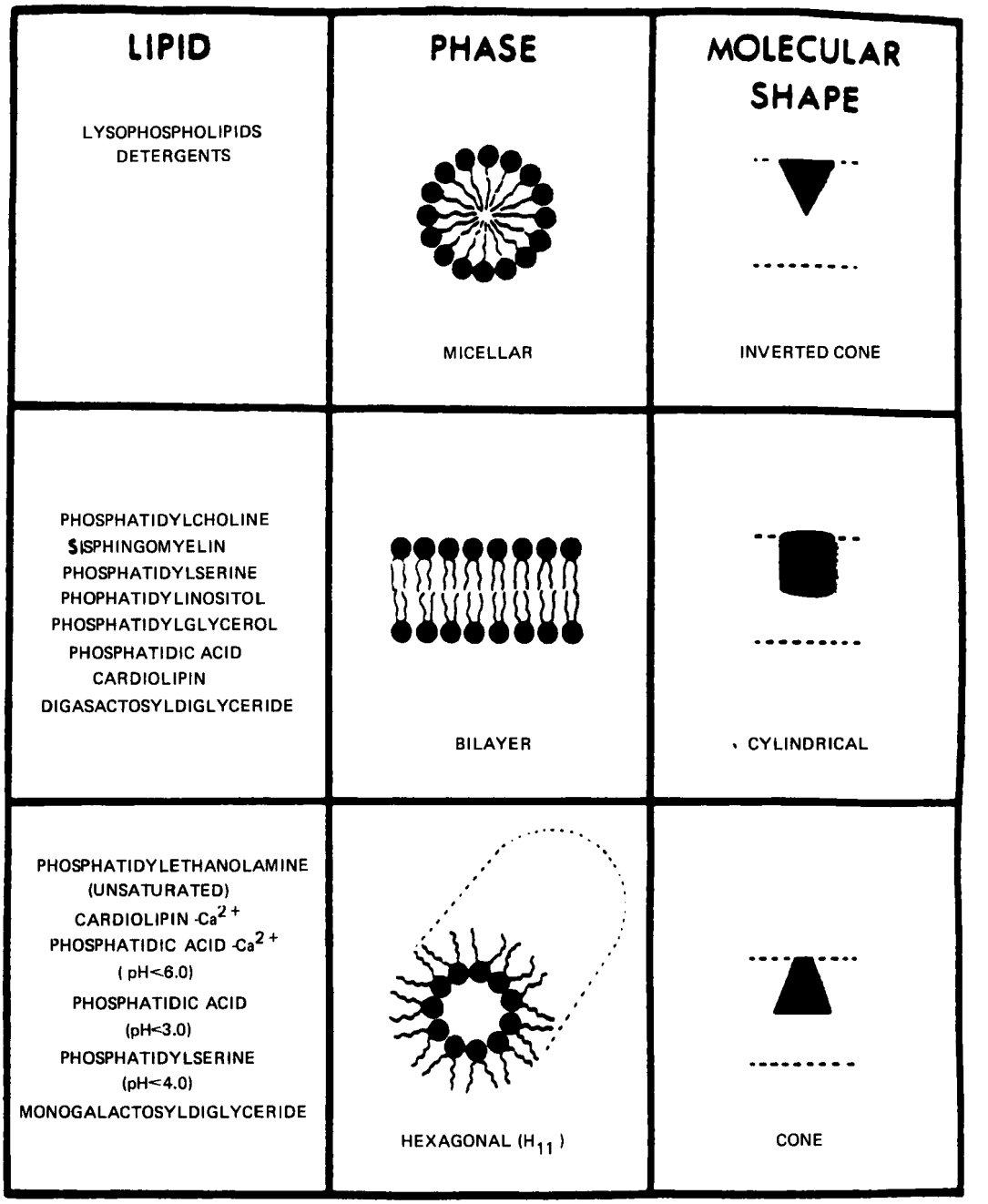

FIGURE 4
Effect of molecular geometry on phase properties of lipids. Source: Cullis, P.R. and Hope, M.J. (1985) "Physical Properties and Functional Roles of Lipids" in *Biochemistry of Lipids and Membranes* , Vance, D.E. and Vance, J.E., eds., Benjamin/Cummings Inc., p. 56.

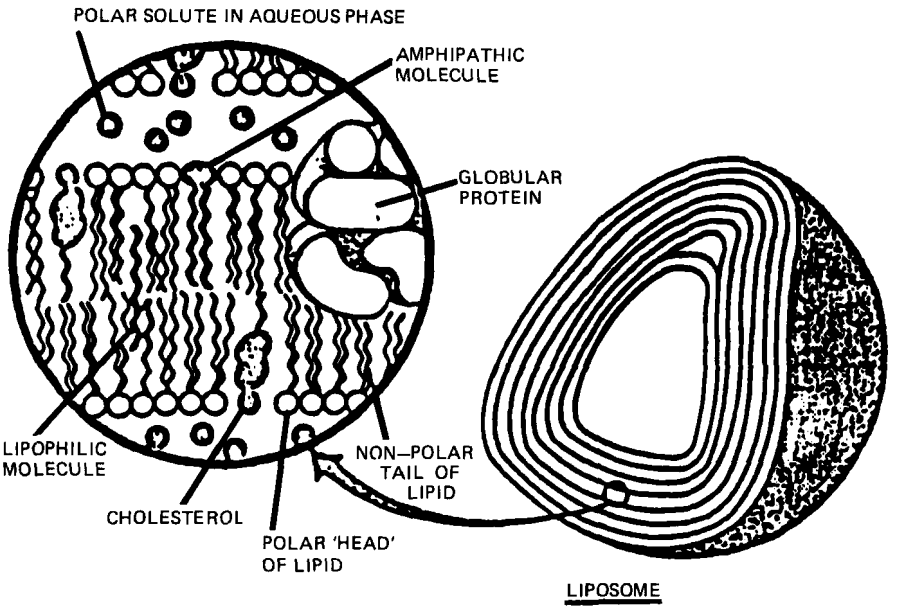

FIGURE 5
Diagrammatic representation the various sites in the liposome available for drug entrapment. Source: Gregoriadis, G. and Allison, A.C. (1980) *Liposomes in Biological Systems,* John Wiley & Sons, Ltd. New York, p. 89.

and phosphatidylethanolamines and negatively charged phospholipids under certain conditions (low pH and in the presence of divalent cations) form hexagonal (H_{II}) structures. It should be pointed out that under proper conditions, relatively large amounts of lipids which normally tend to form hexagonal or micellar structures can be successfully incorporated into liposomes.

CHARACTERIZATION OF LIPOSOMES

Factors Affecting Drug Entrapment: The amount and location of a drug within a liposome is dependent on a number of factors. The location of drug within a liposome is based on the partition coefficient of the drug between aqueous compartments and lipid bilayers, and the maximum amount of drug that can be entrapped within a liposome is dependent on its total solubility in each phase. For example, very little 6-mercaptopurine can be encapsulated in liposomes because this drug has limited solubility in both polar and non-polar solvents. The total amount of liposomal lipid used and the internal volume of the liposome will affect the total amount of non-polar

and polar drug, respectively, that can be loaded into a liposome. Efficient capture will depend on the use of drugs at concentrations which do not exceed the saturation limit of the drug in the aqueous compartment (for polar drugs) or the lipid bilayers (for non-polar drugs). The method of preparation can also affect drug location and overall trapping efficiency. Fig. 5 diagrammatically represents the various sites in the liposome available for drug entrapment.

Incorporation of drugs that have intermediate partition coefficients (significant solubility in both the aqueous phase and the bilayer) may be undesirable. If liposomes are prepared by mixing the drug with the lipids, the drug will eventually partition to an extent depending on the partition coefficient of the drug and the phase volume ratio of water to bilayer. Also, the rate of partitioning will be a function of its diffusivity in each phase. Release rates (a measure of instability) are highest when the drug has an intermediate partition coefficient. Bilayer/aqueous compartment partition coefficients are usually estimated by determining their organic solvent/water (e.g., octanol/water) partition coefficients. They can also be determined precisely by a method described by Bakouche and Gerlier[3] which is based on the physical separation of the aqueous and bilayer phases by ultracentrifugation after mechanical (ultrasonics at low temperatures) disruption of the liposomes followed by analysis of each phase for drug.

Internal Volume and Encapsulation Efficiency: These two parameters are used to describe entrapment of water soluble drugs in the aqueous compartments of liposomes. The internal or trapped or capture volume is expressed as aqueous entrapped volume per unit quantity of lipid (μl/μmol or μl/mg). It is determined by entrapping a water soluble-marker such as 6-carboxyfluorescein, ^{14}C or ^{3}H-glucose or sucrose and then lysing the liposomes by the use of a detergent such as Triton X-100. Determination of the amount of marker that was trapped enables one to back-calculate the volume of entrapped water. The encapsulation efficiency describes the percent of the aqueous phase (and hence the percent of water-soluble drug) that becomes entrapped during liposome preparation. The remaining drug remains outside of the liposome and is therefore "wasted". Encapsulation efficiency is usually expressed as % entrapment/mg lipid.

The internal or trapped volume and encapsulation efficiency greatly depends on liposomal content, lipid concentration, method of preparation and drug used. Some typical values are:

Liposome Type	Internal Volume μl /μmol lipid	Entrapment Efficiency %/mg lipid
SUV	<0.5	<1
MLV	>4	5-15
REV	>10	35-65

Incorporation of charged lipids into bilayers increases the volume of the aqueous compartments by separating adjacent bilayers due to charge repulsion resulting in increases in trapped volume. It should be pointed out that for hydrophobic drugs, entrapment efficiency usually approaches 100% almost irrespective of liposomal type and composition.

Lamellarity: The average number of bilayers present in liposomes can be found by freeze-fracture electron microscopy and ^{31}P-NMR. In the latter technique, the signals are recorded before and after the addition of nonpermeable broadening agent such as Mn^{2+}. Manganese ions interact with the outer leaflet of the outermost bilayer. Thus, a 50% reduction in NMR signal means that the liposome preparation is unilamellar and 25% reduction in the intensity of the original NMR signal means there are 2 bilayers in the liposomes[4].

Size and Size Distribution: The average size and size distribution of liposomes are important parameters with respect to physical properties and biological fate of the liposomes and their entrapped substances. There are a number of methods used to determine this parameter, but the most commonly used methods are:

a. Light Scattering: There are a variety of techniques available to size liposomes based on light scattering. The popularity of this method depends on its ease of operation and the speed by which one can obtain data. The newer instruments are based on dynamic laser light scattering.

If the liposomes to be analyzed were monodisperse, light scattering would be the method of choice; unfortunately, most preparations are heterogeneous, and they require an accurate estimation of their size-frequency distributions. Light scattering methods rely on algorithms to determine particle size distributions and the results obtained can be very misleading. Some complex algorithms have been developed in an attempt to deal with this problem. Furthermore, such methods can not distinguish between a large particle and a flocculated mass of smaller particles. Most importantly, it may is necessary to remove any micron-sized particles that are present in the sample prior to analysis.

The difficulty in interpreting particle size data can be demonstrated by taking a simple example of a dispersion comprised of 97% unilamellar vesicles with a radius of 15 nm and 3% multilamellar vesicles with a radius three times greater (45 nm):

	15 nm Particles	45 nm Particles
Percent Particles	97	3
Percent Surface Area	78	22
Percent Total Volume	54	46
True Statistical Average Radius	15.9 nm	
Instrumental Average Radius	25.3 nm	

Thus, 3% of the particles comprise almost one-half the volume of liposomes. Of course, the same problem of data analysis occurs with other disperse systems such as emulsions and suspensions.

b. Light Microscopy: This method can be used to examine the gross size distribution of large vesicle preparations such as MLVs. The inclusion of a fluorescent probe in the bilayer permits examination of liposomes under a fluorescent microscope and is a very convenient method to obtain an estimate of at least the upper end of the size distribution.

c. Negative Stain Electron Microscopy: This method, utilizing either molybdate or phosphotungstate as a stain is the method of choice for size distribution analysis of any size below 5 μm. It should be used to validate light scattering data that will ultimately be used for quality assurance. For accurate statistical evaluation (±5%), one should count at least 400 particles and not rely on a single specimen for counting.

d. Freeze Fracture Electron Microscopy: This method is especially useful for observing the morphological structure of liposomes. Since the fracture plane passes through vesicles that are randomly positioned in the frozen section, resulting in nonmidplane fractures, the observed profile diameter depends on the distance of the vesicle center from the plane of the fracture. Mathematical methods have been devised to correct for this effect.

For all of the microscopy procedures used, one should always be on the lookout for aggregated particles or flocs.

Application of Double Layer Theory to Liposomes: Once assembled, liposomes behave in much the same way as other charged colloidal particles suspended in water or electrolyte solutions. Under conditions where the charge on each particle is weak, the electrostatic repulsive force among the particles is also weak, increasing the opportunity for close approach. Some neutral particles tend either to flocculate or aggregate and sediment from suspension for this reason. Similarly, two populations of liposomes bearing opposite electric charges will aggregate at a rate that is a function of the electrostatic attractive forces among the particles. Particles bearing net negative charges may be induced to aggregate strongly in the presence of di- or trivalent cations. For example, calcium in the 1-2 mM range will induce liposomes containing more than 50 mole% PS to aggregate. These phenomena have dramatic effects on the physical stability of liposomes and lead to fusion of liposomes with one another resulting in increases in their overall size. Like aggregation, particle size growth, particularly during storage, would be undesirable in most products. Fortunately the tendency of liposomes to aggregate and fuse can be controlled by the inclusion of small amounts of negatively charged lipids such as PS or PG or positively charged amphiphiles such as stearylamine in the formulation. Knowing the number and the sign of charged groups added and the valency and concentration of electrolytes in the

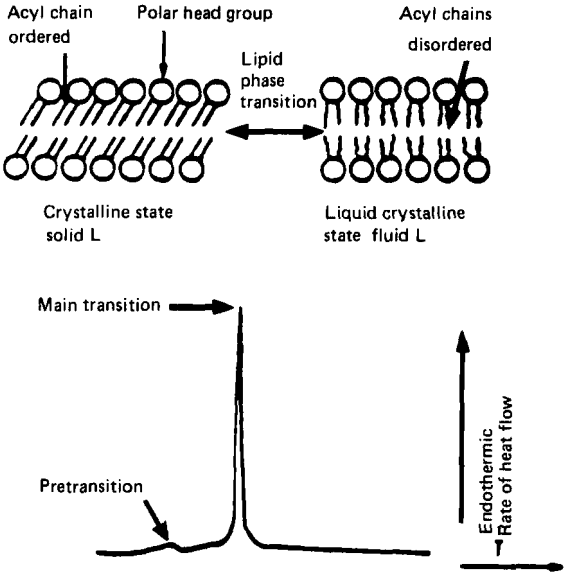

FIGURE 6
Phospholipid gel to liquid crystalline phase transition. Source: Cullis, P. R. and Hope, M. J. (1985) "Physical Properties and Functional Roles of Lipids in Membranes", In *Biochemistry of Lipids and Membranes* Vance, D. E. and Vance, J. E., Ed., The Benjamin/Cummings Publishing Co., California, p. 43.

medium, the magnitude of the electrostatic forces generated by these charged groups can be closely approximated using double layer theory. These results can then be correlated with physical stability of liposomes and used to guide formulation efforts. The amount of charged component and ionic conditions in a particular liposome dosage form can be adjusted to produce a high enough zeta potential to inhibit close approach of the vesicles and prevent aggregation. In practice it is usually necessary to determine empirically the magnitude of the zeta potential required to prevent aggregation in a particular system. However, once this has been done, it is possible to use the zeta potential as a quality control check to insure that each batch of liposomes contains sufficient charged groups to avoid aggregation during storage.

Phase Behavior of Liposomes: An important feature of membrane lipids is the existence of a temperature-dependent reversible phase transition, where the hydrocarbon chains of the phospholipid undergo a transformation from an ordered (gel) state to a more disordered fluid (liquid crystalline) state. These changes have been documented by freeze-fracture electron microscopy but are most easily demonstrated by differential scanning calorimetry (DSC). Fig. 6 illustrates the phase transition region for a typical phospholipid.

TABLE 3
Phase transition temperatures of some synthetic phospholipids used to prepare liposomes.

LIPID	CHARGE	Tm (°C)
Dilauryl Phosphatidylcholine	0	0
Dimyristoyl Phosphatidylcholine	0	23
Dipalmitoyl Phosphatidylcholine	0	41
Dimyristoyl Phosphatidylethanolamine	0	48
Distearoyl Phosphatidylcholine	0	58
Dipalmitoyl Phosphatidylethanolamine	0	60
Dioleoyl Phosphatidylglycerol	-1	-18
Dilauryl Phosphatidylglycerol	-1	4
Dimyristoyl Phosphatidylglycerol	-1	23
Dipalmitoyl Phosphatidylglycerol	-1	41
Distearoyl Phosphatidylglycerol	-1	55

The physical state of the bilayer profoundly affects the permeability, leakage rates and overall stability of the liposomes. The phase transition temperature (T_m) is a function of the phospholipid content of the bilayer.

By proper admixture of bilayer forming materials, one may design liposomes to "melt" at any reasonable temperature. This strategy has been used to deliver methotrexate to solid tumors which are heated to the phase transition temperature of the custom designed liposomal phospholipids. The phase transition temperature can be altered by using phospholipid mixtures or by adding sterols such as cholesterol. The T_m value can give good clues as to liposomal stability and permeability and as to whether a drug is entrapped in the bilayer or the aqueous compartment.

LIPOSOME PREPARATION METHODS

Multilamellar Vesicles (MLV): Multilamellar vesicles are by far the most widely studied type of liposome and, as pointed out by Alec Bangham in 1974, exceptionally simple to make. In general a mixture of lipids is deposited as a thin film on the bottom of a round-bottom flask by rotary evaporation under reduced pressure. MLVs form spontaneously when an excess volume of aqueous buffer is added to the dry lipid. However, in many cases MLVs have not been rigorously characterized with respect to size, polydispersity, number of lamellae, encapsulated volume and stability. Due to their ease of production many investigators have simply made a preparation of MLVs for use in both *in vitro* and *in vivo* experiments without taking the time to fully characterize them. This has led to a great deal of confusion in the interpretation of experimental results because, as will be explained below, minor changes in the method of preparation can lead to major differences in the behavior of liposomes.

Slow vs. Fast Hydration. Thickness of the Lipid Film: The time allowed for hydration and conditions of agitation are critical in determining the amount of the aqueous buffer (or drug solution) entrapped within the internal compartments of the MLV. For example, as pointed out by Szoka and Papahadjopoulos[5], a similar lipid concentration can encapsulate 50% more of the aqueous buffer per mole of lipid when hydrated for 20 hours with gentle shaking, compared to a hydration period of 2 hours with vigorous shaking, despite the fact that the two preparations exhibit a roughly similar particle size distribution. If hydration time is reduced to a few minutes with vortexing, a suspension will exhibit a still lower capture volume and a smaller mean diameter. As pointed out by Bangham[1], the hydration and entrapping process is most efficient when the film of dry lipid is kept thin. This means that different sized round-bottom flasks should be used for different quantities of lipid. Glass beads have been used by some investigators to increase the surface area available for film deposition. Thus the hydration time, method of suspension of the lipids and the thickness of the film can result in markedly different preparations of MLVs, in spite of identical lipid concentrations and compositions, and volume of the suspending aqueous phase.

Effect of Charged Lipids: The presence of negatively charged lipids such PS, PA, PI or PG, or positively charged detergents such as stearylamine will tend to increase the interlamellar distance between successive bilayers in the MLV structure and thus lead to a greater overall entrapped volume. This is particularly true in low ionic strength buffers or non-electrolytes (such as sucrose) since the electrostatic repulsive forces which give rise to the effect are greater under these conditions. Generally about 10-20 mole percent of a charged species is used although it is possible to produce MLVs from a purely charged lipid such as PS. The presence of charged lipids also reduces the likelihood of aggregation following the formation of MLVs.

Hydration in the Presence of Solvent: MLVs with high entrapment of solutes can be produced by hydrating the lipid in the presence of organic solvents. A method introduced by Papahadjopoulos[6] begins with a two-phase system consisting of equal volumes of petroleum ether containing bilayer forming lipids and aqueous phase. The contents of the tube are emulsified by vigorous vortexing and the ether removed by passing a stream of nitrogen gas over the mixture. As the ether is removed in the carrier gas, MLVs form in the aqueous phase. A similar method was reported by Gruner et al.[7] except that diethyl ether was used as the solvent, sonication was used in place of vortexing and the aqueous phase was reduced to a relatively small proportion. Typically the lipids are dissolved in about 5 ml ether and about 0.3 ml of the aqueous phase to be entrapped is added and the two phases are emulsified by sonication while a gentle stream of nitrogen gas is passed over the mixture. The resulting MLV preparation encapsulates up to 40% of the solvent throughout the hydration step, and the concentration of solute molecules is in equilibrium across all the bilayers, a feature that is claimed to translate into greater stability to leakage.

MLVs Formed by Freeze Drying SUV Dispersions: A simple method for preparing MLVs with high entrapment efficiency was developed by Ohsawa et al.[8] and Kirby and Gregoriadis[9]. The aqueous phase containing the molecules to be encapsulated is mixed with a preformed suspension of SUVs and the mixture freeze dried by conventional means. Large MLVs are formed when the dry lipid is rehydrated, usually with a small volume of distilled water. Encapsulation efficiencies up to 40% have been reported for this method.

Small Unilamellar Vesicles (SUV): The classical methods of dispersing phospholipids in water to form optically clear suspensions with a particle weight of about $2x10^6$ daltons involve various mechanical means and began with the sonication method reported in the mid 60's[10] followed by refinements introduced by Hamilton and his colleagues[11] and Barenholz[12] in the mid 70's who employed a high pressure device to produce the same effect in larger volumes. These types of SUV dispersions have been rigorously characterized by Huang[13] and others and shown to consist of rather uniform closed bilayer vesicles of about 25-50 nm diameter. Solvent injection methods have also been devised to produce SUVs. These typically involve the slow injection of a lipid solution in either ethanol or ether into warm water containing a drug or other marker to be entrapped. All of these methods are discussed in greater detail below.

Sonicated SUVs: The preparation of sonicated SUVs has been reviewed in detail by Bangham[14]. Briefly, the usual MLV preparation is subsequently sonicated either with a bath type sonicator or a probe sonicator under an inert atmosphere (usually nitrogen or argon). Although probe sonication leads to more rapid size reduction of the MLVs, degradation of lipids, metal particle shedding from the probe tip and aerosol generation can present problems. Bath type sonicators also have disadvantages (such as the need to pay greater attention to position of the tube and water level in the bath) but temperature can be accurately regulated. Also, the tube containing the specimen is sealed allowing for aseptic operations and little likelihood of personnel exposure to aerosols.

French Pressure Cell: Dispersions of MLVs can be converted to SUVs by passage through a small orifice under high pressure. A French Pressure Cell was used by Hamilton et al.[15] for this purpose. MLV dispersions are placed in the French Press and extruded at about 20,000 psi at 4°C. One pass through the cell produces a heterogeneous population of vesicles ranging from several microns in diameter to SUV size. Multiple extrusions results in a progressive decrease in the mean particle diameter. Following about 4-5 passes, about 95% of the vesicles have converted to SUVs as judged by size exclusion chromatography. The resulting vesicles are somewhat larger than sonicated SUVs ranging in size from 315-500 A. The method is simple, reproducible and nondestructive. However, temperature control is difficult (the pressure cell must be allowed to cool between extrusions or the temperature rise may

damage the lipids) and the working volumes are relatively small (about 50 ml maximum).

Solvent Injection Method

a. Ether Infusion: A method introduced by Deamer and Bangham in 1976[16] provides a means of making SUVs by slowly introducing a solution of lipids dissolved in diethyl ether (or ether/methanol mixtures) into warm water. Typically the lipid mixture is injected into an aqueous solution of the material to be encapsulated (using a syringe-type infusion pump) at 55-65°C or under reduced pressure. Subsequent removal of residual ether under vacuum leads to the formation of single layer vesicles. Depending on the condition used, the diameters of the resulting vesicles ranges from 50-200 nm. The usual lipid concentration is about 2 mg/ml ether and about 2 ml of this solution are infused into 4 ml of the aqueous phase at a rate of 0.2 ml/min. at 50-60° C.

b. Ethanol Injection: An alternative method for producing SUVs that avoids both sonication and exposure to high pressure is the ethanol injection technique described by Batzri and Korn[17]. Lipids dissolved in ethanol are rapidly injected into a vast excess of buffer solution forming SUVs spontaneously. The procedure is simple, rapid and avoids exposure of both lipids and the material to be entrapped to harsh conditions. Unfortunately, the method is restricted to the production of relatively dilute SUV suspensions. The final concentration of ethanol cannot exceed about 10% by volume or the SUVs will not form. Removal of residual ethanol can also present a problem since ethanol forms an azeotrope with water which is difficult to remove under vacuum or by distillation. Various available ultrafiltration apparatus may be used to both concentrate the suspension and remove ethanol, however, these procedures tend to be slow and expensive to scale up. Another limitation of the method is related to the susceptibility of various biologically active macromolecules to inactivation in the presence of even low amounts of ethanol.

Large Unilamellar Vesicles (LUV) and Intermediate Sized Unilamellar Vesicles: Large unilamellar vesicles provide a number of important advantages as compared to MLVs including high encapsulation of water soluble drugs, economy of lipid and reproducible drug release rates. However, LUVs are perhaps the most difficult type of liposome to produce. "Large" in the context of liposomes usually means any structure larger than 100 nm; thus large unilamellar vesicles refers to vesicles bounded by a single bilayer membrane that are above 100 nm in diameter. Some authors have referred to liposomes between the sizes of 50-100 nm as "large" but these would be more appropriately called intermediate sized. Two primary methods are used to produce LUVs, one involving detergent dialysis, the other a sophisticated reverse emulsification technique. Intermediate sized single layered vesicles can be generated from MLV dispersions by sequential extrusion through small pore size polycarbonate membranes under high pressure. A number of other techniques for producing LUVs have been reported including freeze thawing, slow swelling in nonelectrolytes,

dehydration followed by rehydration and dilution or dialysis of lipids in the presence of chaotropic ions. Each of these methods is reviewed below.

LUVs Formed by Detergent Removal: An essentially different approach to produce liposomes is dependent on the removal of detergent molecules from aqueous dispersions of phospholipid/detergent mixed micelles. As the detergent is removed the micelles become progressively richer in phospholipid and finally coalesce to form closed single bilayer vesicles. Three methods of detergent removal appropriate for this purpose have been described in the literature and are treated separately below.

a. Dialysis: Kagawa and Racker[18] were the first to introduce the dialysis method for lipid vesicle preparation. Although these authors were primarily interested in reconstituting biological membranes solubilized with detergents, their method is applicable to the formation of liposomes as well. Detergents commonly used for this purpose exhibit a reasonably high critical micelle concentration (on the order of 10-20 mM) in order to facilitate their removal and include the bile salts sodium cholate and sodium deoxycholate and synthetic detergents such as octylglucoside. The treatment of egg PC with a 2:1 molar ratio of sodium cholate followed by dialysis results in the formation of vesicles in the 100 nm diameter range within a few hours. Another modification of the cholate removal technique is one in which the rate of efflux of the detergent from the mixture is controlled. This procedure described in detail by Milsmann et al.[19] employs a phospholipid:detergent ratio of 0.625 and rapidly removes the detergent in a flow through dialysis cell. The procedure forms a homogeneous population of single layered vesicles with mean diameters of 50-100 nm. A commercial version of the dialysis system is available under the trade name LIPOPREP™.

b. Column Chromatography: The formation of 100 nm single layered phospholipid vesicles during removal of deoxycholate by column chromatography has been reported by Enoch and Strittmatter[20]. The method involves the treatment of phospholipid, in the form of either small sonicated vesicles or a dry lipid film, at a molar ratio of deoxycholate to phospholipid of 1:2. Subsequent removal of the detergent during passage of the dispersion over a Sephadex G-25 column results in the formation of uniform 100 nm vesicles that are readily separated from small sonicated vesicles.

c. Bio-beads™: Another promising method for forming reconstituted membranes reported by Gerritsen et al.[21] may also be applicable to LUV preparation. The system involves the removal of a nonionic detergent, Triton X-100, from detergent/phospholipid mixtures. This method is based on the ability of Bio-beads SM-2 to adsorb Triton X-100 selectively and rapidly. The dried lipid is suspended in 0.5-1.0 % Triton X-100 and washed Bio-beads are added directly to the solution (about 0.3 g wet Bio-beads per ml of dispersion) and rocked for about 2 hours at 4°C.

The beads are removed by filtration. The final particle size is determined by the conditions used including lipid composition, buffer composition, temperature and, most critically, the amount and activity of the beads themselves.

Reverse Phase Evaporation Technique (REV): LUVs can also be prepared by forming a water-in-oil emulsion of phospholipids and buffer in excess organic phase followed by removal of the organic phase under reduced pressure (the so called "Reverse Phase Evaporation or REV method). The two phases are usually emulsified by sonication but other mechanical means have also been used. Removal of the organic solvent under vacuum causes the phospholipid-coated droplets of water to coalesce and eventually form a viscous gel. Removal of the final traces of solvent results in the collapse of the gel into a smooth suspension of LUVs. With some lipid compositions the transition from emulsion to LUV suspension is so rapid that the intermediate gel phase appears not to form. The method which was pioneered by Szoka and Papahadjopoulos in 1978[22] has been used extensively for applications which require high encapsulation of a water soluble drug. Entrapment efficiencies up to 65% can be obtained with this method. The phospholipids are first dissolved in an organic solvent such as diethylether, isopropylether or mixtures of two solvents such as isopropylether and chloroform. The emulsification is most easily accomplished when the density of the organic phase matches that of the buffer (i.e., about 1). For this reason, ether (density of about 0.7) is often mixed with a solvent of higher density such as trichlorotrifluoroethane (density of 1.4) to produce a solvent system with a density close to water. The aqueous phase containing the material to be entrapped is added directly to the phospholipid-solvent mixture. The ratio of aqueous phase to organic phase is usually about 1:3 for ether and 1:6 for isopropylether-chloroform mixtures. Preparations using even greater proportions of organic phase have been reported. The two phases are emulsified by sonication for a few minutes and the organic phase removed slowly under a partial vacuum produced by a water aspirator on a rotary evaporator at 20-30° C. The vacuum is usually maintained at about 500 microns for the first few minutes (using a nitrogen gas bleed to lower the vacuum and a gauge to measure the vacuum) and then raised cautiously to fill the aspirator vacuum to prevent the ether from evaporating too quickly. A typical preparation contains 60 μmol lipid dissolved in 3 ml ether and 1 ml aqueous phase contained in a sealed screw cap tube. The mixture is sonicated in a bath type sonicator for about 5 minutes or until a homogeneous emulsion is formed. For a quick check to determine if emulsification is complete, one can interrupt sonication and allow the tube to stand for about a minute. If a clear layer of ether is observed over the aqueous phase, sonication should be continued for an additional period. Maximal encapsulation (65%) is obtained when the ionic strength of the aqueous phase is low. The method has been used to encapsulate both small and large molecules. Biologically active macromolecules such as RNA and various enzymes have been encapsulated without loss of activity. The principal disadvantage of the method is the exposure of the material to be

encapsulated to organic solvents and mechanical agitation, conditions that lead to the denaturation of some proteins or breakage of DNA stands.

Formation of Intermediate Sized Unilamellar Vesicles by High Pressure Extrusion: As mentioned above, MLV suspensions rich in acidic lipids such as PS or PG tend to have large interbilayer distances and large internal aqueous cores due to electrostatic repulsive forces among the bilayers. Hope, et al.[23] among others have shown that as MLVs are repeatedly extruded through very small pore diameter polycarbonate membranes (0.8-1.0 micron) under high pressure (up to 250 psi) their average diameter becomes progressively smaller reaching a minimum of 60-80 nm after about 5-10 passes. Moreover, as the average size is reduced, the vesicles become more and more single layered. MLVs prepared from pure PG convert to 60-70 nm single layer vesicles following about 10 passes through a 1.0 micron capillary pore membrane. The mechanism at work during such high pressure extrusion appears to be much like peeling an onion. As the MLVs are forced through the small pores, successive layers are "peeled" off until only one remains. For this method to generate truly single layered vesicles, however, the aqueous core of the starting MLV must be greater than about 70 nm in diameter. Although this appears to be the case for vesicles composed predominantly of acidic lipids, neutral vesicles or vesicles with only a few mole percent acidic lipids are not likely to convert to true single lamellar vesicles using this technique because the diameter of the inner most bilayer is probably significantly less than 70 nm. One possible exception to this rule would be neutral lipids hydrated in a nonelectrolyte solution. In this case the small amount of negative charge found as a contaminant of neutral lipids would produce enough charge repulsion to form an inner core greater than 70 nm during hydration.

Miscellaneous Methods

a. Slow Swelling in Nonelectrolyte Solutions: In 1969, Reeves and Dowben[24] reported a method for producing very large (up to several 10's of microns) single layered liposomes by allowing a thinly spread layer of hydrated phospholipids to slowly swell in distilled water or a nonelectrolyte solution. Typically, a mixture of lipids in ether or chloroform is deposited as a thin film on the bottom of a flat-bottomed beaker. The lipid is slowly hydrated by passing nitrogen gas saturated with water vapor over the film for several hours. When the film has completely hydrated it will become opaque in appearance. Following hydration, distilled water or a nonelectrolyte solution (e.g., sucrose) is carefully layered over the film and the beaker is placed in a 37°C water bath for several more hours. During this period very large single walled vesicles are formed by a mechanism which begins with single bilayers swelling and budding from the film, pinching off and eluting into the aqueous medium. The yield of single layered vesicles is good if conditions are right, but the main disadvantage of the technique is its sensitivity to any kind of mechanical agitation during vesicle formation. Also, since a very thin film is required and swelling times are long, this method would be difficult to scale up.

b. Removal of Chaotropic Ions: Oku and MacDonald[25] developed a method of forming giant single lamellar vesicles with diameters in the range of 10-20 microns by removal of sodium trichloroacetate by dialysis or dilution from a solution containing egg phospholipids and molar concentrations of sodium trichloroacetate. The yield of giant vesicles was critically dependent on the starting concentration of the chaotropic ion and temperature. Inclusion of a freeze thaw step reduced the required concentration of trichloroacetate to about 0.1 M. The giant liposomes apparently were formed from concentrations of the ion which induced the transformation of phospholipids from the lamellar phase to the micellar phase. Other chaotropic ions were also shown to be effective, including urea guanidine-HCl.

c. Freeze/Thaw: A method for the reconstitution of membrane proteins based on rapid freezing of sonicated phospholipid mixtures followed by thawing and brief sonication was originally described by Kasahara and Hinkle[26]. In 1981, Pick[27] reported that vesicles formed by this simple procedure exhibited specific trapping volumes of up to 10 µl per µmole lipid with encapsulation efficiencies of 20-30%. Formation of large liposomes by this technique probably results from the fusion of small vesicles during freezing and/or thawing of the suspension of small vesicles. This type of fusion is strongly inhibited by increasing the ionic strength of the medium, e.g., adding sucrose, and by increasing the lipid concentration. For an unexplained reason, pure phosphatidylcholine vesicles do not appear to be good candidates for this type of fusion induced growth, however. Ohu and MacDonald[28] have shown that freeze/thawing of SUVs prepared in high concentrations of alkali metal chlorides also results in the formation of giant single layered liposomes. The method involves the formation of fully hydrated small vesicles in dilute buffer by sonication followed by freeze/thawing in the presence of high concentrations of the electrolyte of interest in order to induce equilibration of the electrolyte across the bilayer membranes of the small vesicles. In the final step of the process the electrolyte concentration is reduced by dialysis against dilute buffer. This results in the influx of water into the small vesicles (driven by the osmotic imbalance) causing them to swell and fuse into giant vesicles. The method is rather involved and not easily scaled up.

d. Dehydration/Rehydration of SUVs: Large unilamellar and oligolamellar vesicles with high entrapment efficiencies have been formed by a clever method reported recently by Shew and Deamer[29]. In this method, sonicated vesicles are mixed in an aqueous solution with the solute desired to be encapsulated and the mixture dried under a stream of nitrogen. As the sample is dehydrated, the small vesicles fuse to form a multilamellar film that effectively sandwiches the solute molecules between successive layers. Upon rehydration, large vesicles are produced which have encapsulated a significant proportion of the solute. The optimal mass ratio of lipid to solute was reported to be approximately 1:2 to 1:3. This method has potential application to large scale production since it depends only on controlled drying and rehydration processes and does not require extensive use of organic solvents, detergents, or dialysis systems.

METHODS FOR CONTROLLING SIZE AND SIZE DISTRIBUTION OF LIPOSOMES

In most studies using liposomes as drug carriers, particle size has not been rigorously controlled. In studies on tissue distribution reported to date, for example, various investigations have used either the initial liposome preparation containing a wide distribution of sizes (ranging from 0.2 to 10's of microns) or sonicated vesicles which, although exhibiting a narrower size distribution, are quite small and thus have a limited capacity to carry drugs. Judging from the few studies utilizing controlled particle size, it is clear that vesicle size can have dramatic effects on the *in vivo* behavior of liposomes. Therefore, before liposome drug carrier systems can be taken seriously for pharmaceutical applications, their size will have to be controlled within reasonable limits. Three possible approaches have been explored for controlling the particle size distribution of liposome preparations: (1) fractionation of the size of interest from a heterogeneous population; (2) homogenization of a polydisperse dispersion to yield a population of smaller vesicles with a narrower size distribution; and (3) extrusion of a heterogeneous preparation through capillary pore membranes of known pore diameter to yield an average size that approximates the pore diameter.

1. Fractionation: Two methods have enjoyed widespread use for fractionating liposomes of the desired size from a heterogeneously sized population: centrifugation and size exclusion chromatography. Both can be used to enrich the product with the desired particle size but are limited in terms of the volumes that can be easily handled.

a. Centrifugation: Liposomes sediment in a centrifugal field at a rate that is dependent on their size and density. Large liposomes composed of neutral lipids such as PC can easily be pelleted at fairly low g forces in a conventional centrifuge. Under proper conditions the smaller liposomes will remain in the supernatant. This method is useful for making gross cuts between small and larger liposomes but not for generating narrow particle size distributions. Also, the volumes that can be handled are limited by the volume capacity of the centrifuge. However, zonal rotors or continuous flow centrifuges may be adaptable to this application. Another disadvantage to centrifugation is that liposomes smaller than about 0.5 micron tend to require high g forces and long spinning times in order to achieve effective separation from particles in the 0.1-0.2 micron range. Also the capacity of the ultracentrifuges normally used for this purpose is limited to a few hundred ml per run.

b. Size Exclusion Chromatography: Column chromatography has been used for many years as an analytical method to assess the particle size of liposomes. Preparative scale chromatography has also been applied to produce liposomes of fairly homogeneous sizes. This method is particularly useful for separating SUVs from larger structures. Typically, a column of Sepharose 4B is equilibrated with a buffer of the same osmolarity as the medium in which the vesicles were prepared and an aliquot of the liposomes is applied to the column. The column is eluted with the same

buffer and fractions are collected. Large liposomes appear in the void volume while SUV size liposomes elute with the included volume. Larger pore size chromatographic media have been used in a similar fashion to fractionate populations of larger particles. In general, however, such chromatographic separations are quite limited in terms of volumes and throughput, must be carried out in batches and result in significant dilution of the product.

2. Homogenization: In those cases where a fairly small particle size is desirable, homogenization has proven to be useful approach. In much the same way as milk is homogenized, the average particle size and polydispersity of vesicle dispersions can be reduced by passage through a high pressure homogenizer. One such devise marketed by the Biological Development Corporation under the trade name Microfluidizer™ has been shown by Mayhew and his colleagues[30] to generate vesicles in the 50-200 nm size range. Such homogenizers are amenable to scale up, and throughput rates are high. As with other high pressure devices, however, heat regulation can sometimes present problems, and the shear forces developed within the reaction chamber can lead to partial degradation of the lipids. Another disadvantage relates to the empirical observation that conditions designed to produce approximately 200 nm particles often results in a bimodal distribution, with the bulk of the vesicles in the desired size range contaminated by a significant proportion of very small vesicles (less than 50 nm)

3. Capillary Pore Membrane Extrusion: A technique that has gained widespread acceptance for the production of liposomes of defined size and narrow size distribution, introduced by Olson et al.[31] in 1979, involves the extrusion of a heterogeneous population of fairly large liposomes through polycarbonate membranes under moderate pressures (100-250 psi). Such membranes have uniform straight-through capillary pores of defined size and polycarbonate does not bind liposomes containing charged species. This simple technique can reduce a heterogeneous population of MLVs or REVs to a more homogeneous suspension of vesicles exhibiting a mean particle size which approaches that of the pores through which they were extruded. MLVs with a mean diameter of 260 nm can be obtained following a single extrusion through 200 nm pore size polycarbonate membranes; 75% of the encapsulated volume resides in vesicles between 170 and 370 nm (as measured by negative stain electron microscopy). Upon additional extrusions through the same pore size membrane the average size is reduced further finally approaching about 190 nm with greater than 85% of the particles in the 170-210 nm range. Compared to SUV preparations this still represents a rather broad distribution of vesicle sizes, but compared to the original MLV population which ranges in size from about 500 nm to several microns, it represents a considerable reduction of both average particle size and polydispersity. In practice it is sometimes preferable to extrude sequentially through membranes of decreasing pore diameter. For example, a concentrated dispersion of MLVs may be difficult to extrude directly through a 200

nm pore size membrane under normal operating pressures (about 90 psi). It is advisable to begin the process by extrusion through a 0.8, 0.6, 0.4 and finally 0.2 micron pore sizes. Alternatively, it is possible to use higher pressures to extrude concentrated dispersions through the smaller pore size membranes directly. A special high pressure filter holder is required, however, since operating pressures may reach 250 psi. One such devise is available commercially under the trade name LUVET™ which can accommodate up to 10 ml and is equipped with a recirculation mechanism which permits multiple extrusion with little difficulty.

STABILITY OF LIPOSOMES

The stability of any pharmaceutical product is usually defined as the capacity of the formulation to remain within defined limits for a predetermined period of time (shelf-life of the product). The first step in designing any type of stability testing program is to specify these limits by establishing parameters defined in terms of chemical stability, physical stability and microbial stability. Next, methods must be established to evaluate each of these parameters. One must treat liposomal drug delivery systems in the same way as the more traditional pharmaceutical dosage forms are treated with respect to the establishment of clearly defined protocols for their characterization, manufacture, stability testing and efficacy. General observations about liposomal stability include:

1. There are very few published reports on long-term stability studies of liposomes.
2. There are no published reports on the establishment of detailed protocols for stability testing.
3. There are no published reports on the establishment of protocols for accelerated stability testing.
4. MLVs and REVs appear to be more stable than SUVs (with respect to leakage on storage).
5. Use of saturated phospholipids and incorporation of cholesterol into the bilayer generally improves stability.
6. Liposomes stored at 4°C, at times, appear to be more stable than liposomes stored at room temperature.

Chemical Stability: Chemically, phospholipids are susceptible to hydrolysis. Additionally, phospholipids containing unsaturated fatty acids can undergo oxidative reactions. Much of the data on liposomes that have appeared in the literature can be considered suspect due to the use of phospholipids containing significant amounts of oxidation and hydrolysis products. These reaction products can cause dramatic changes in the permeability properties of liposomes. Preparative procedures (e.g., sonication) or storage conditions (e.g., exposure to different pH values) can affect the decomposition rate of the liposomal lipids.

a. Lipid Peroxidation: Most of the phospholipid liposomal dispersions used contain unsaturated acyl chains as part of the molecular structure. These chains are vulnerable to oxidative degradation (lipid peroxidation). The oxidation reactions can occur during preparation, storage or actual use. Oxidative deterioration of lipids is a complex process involving free radical generation and results in the formation of cyclic peroxides and hydroperoxides.

Most of the procedures used to measure lipid peroxidation are nonspecific and are either based on the disappearance of unsaturated fatty acids (determined by lipid extraction techniques followed by GLC analysis) or the appearance of conjugated dienes. The latter technique is now widely used since oxidation is accompanied by increased UV absorption in the 230-260 nm range. If unsaturated phospholipids are used to prepare liposomes, and no special precautions are used to minimize oxidation, the reaction will occur readily. Oxidation of the phospholipids may be minimized by a number of ways:

1. Minimum use of unsaturated phospholipids (if appropriate).
2. Use of argon or nitrogen to minimize exposure to O_2.
3. Use of light resistant containers.
4. Removal of heavy metals (EDTA).
5. Use of antioxidants such as α-tocopherol or BHT.

b. Lipid Hydrolysis: The most important degradation product resulting from lecithin hydrolysis is lyso-lecithin (lyso-PC), which results from hydrolysis of the ester bond at the C¤ position of the glycerol moiety. Many workers choose the formation of lyso-PC as a standard measure for the chemical stability of phospholipids since the presence of lyso-PC in lipid bilayers greatly enhances the permeability of liposomes. It is therefore extremely important that the formation of lyso-PC be kept to a minimum during storage. Lyso-PC is usually analyzed by phospholipid extraction followed by separation of PC and lyso-PC by TLC. The spots are then usually scraped and assayed for total phosphorous content.

Although factors such as sonication could affect the degree of lyso-PC formation, probably the single most important method of minimizing this problem is by the proper sourcing of the phospholipids to be used. They should be essentially free of any lyso-PC to start with and, of course, be free of any lipases.

c. Miscellaneous Chemical Stability Concerns: One must not ignore the fact that the other bilayer lipids which may be present can also decompose. For example, cholesterol, in aqueous dispersion, has been shown to oxidize rapidly when unprotected. Finally, the drug itself must be considered. The stability profile of the "free" drug may be quite different from its profile in the encapsulated state. In fact, a number of strategies have been developed which are based on protecting the drug from biological environments by encapsulating them in liposomes. Examples include the protection of insulin from proteolytic enzymes of the gastrointestinal tract and the prolongation of ester hydrolysis of prodrugs (e.g., cortisone hexadecanoate) after intramuscular administration.

STABILITY TESTING (GENERAL CONSIDERATIONS)

Stability testing of liquid disperse systems is one of the most difficult problems faced by formulation chemists. The scientist is often asked to predict the shelf-life of a product or choose between experimental formulations based on estimates of how well they will hold up with time. There are no standardized tests available to determine physical stability, and quite often there is no certainty of what type of stability is being investigated. The first order of priority for solving stability problems of disperse systems is to define clearly the type or types of stability of concern. Categorizing stability as either physical or chemical is not sufficient. The various groups that are concerned with the product (product development, production, analytical, marketing, etc.) must have a clear and precise reference frame of stability.

An understanding of the factors that lead to stability problems can help determine which methods of testing are most likely to yield information applicable to the estimation of the product's shelf-life. Stability tests commonly stress the system to limits beyond those which the product will ever encounter. Typical examples of stress tests include exposure of the product to high temperatures and large gravitational forces. It is important to understand whether these tests are being performed because the product is expected to encounter these conditions or because, even though these conditions will never be approached, the results will help predict shelf-life at more moderate conditions.

High temperature testing (>25°C) is almost universally used for heterogeneous products. Various laboratories store their products at temperatures ranging from 4°C (refrigerator temperature) to 50°C (or perhaps even higher). The temperatures used in heat-cool cycling are also quite varied, often without regard for the nature of the product. What will the increase in temperature likely do to the properties of the systems under study?

For liposomes, higher temperatures may dramatically alter the nature of the interfacial film, especially if the phase transition temperature is reached. If one expects the product to be exposed to a temperature of 45°C for an extended period of time or for short durations, (shipping and warehouse storage), studies at 45-50°C, (long term and heat-cool cycling), are quite justified. A study of a product at these temperatures determines: (1) How is the product holding up at this higher temperature, and; (2) is the damage reversible or irreversible when the product is brought back to room temperature? If temperatures higher than the system will ever encounter are used, even in short-term heat-cool cycling, there is a risk of irreversibly damaging the bilayers so that when it is brought back to room temperature, the membrane can not heal.

If a liposomal dispersion is partially frozen and then thawed, ice crystals nucleate and grow at the expense of water. The liposomes may then be pressed together against the ice crystals under great pressure. If the crystal grows to a size greater than the void spaces, instability is more likely. That is why a slower rate of cooling, resulting in larger ice crystals, produces greater instability. Polymers may retard ice crystal growth.

van Bommel and Crommelin[32] showed that even one freeze-thaw cycle causes almost complete rapid leakage of carboxyfluorescein from liposomes (REVs) prepared from unsaturated phospholipids (even when cholesterol is added). However, liposomes composed of distearoylphosphatidylcholine/dipalmitoylphosphatidylglycerol/cholesterol show slightly better freeze-thaw stability.

Stability testing protocols should be developed for liposomal products on a case-by-case basis. A typical protocol for a product which would be shipped in vehicles not equipped with climate control and stored in warehouses for prolonged periods under similar conditions might include testing under the following conditions:

1. One month at the highest temperature likely to be encountered.
2. One month at lowest temperature likely to be encountered.
3. 12-24 months at room temperature.
4. 12-24 months at various light intensities.
5. Two to three "freeze-thaw" cycles (-20°C to 25°C).
6. Six to eight "heat-cool" cycles (5°C to 45°C, 48 hours at each temperature).
7. 24-48 hours on a reciprocating shaker at 60 cycles/min (estimates transportation conditions).

One should be certain that studies are performed using all types and sizes of containers. Under each of the test conditions, the following data can be collected:

1. Visual and microscopic observations, e.g., flocculation.
2. Particle size profiles.
3. Rheological profiles.
4. Chemical stability.
5. Extent of leakage.

FREEZE DRYING (LYOPHILIZATION) OF LIPOSOMES

Freeze drying involves the removal of water from products in the frozen state at extremely low pressures. The process is generally used to dry products that are thermolabile and would be destroyed by heat-drying.

Lyophilization has great potential as a method to solve long-term stability problems with respect to liposomal stability. Intuitively, one would suspect that liposomes containing drugs entrapped in their bilayers would be better candidates for lyophilization than liposomes containing drugs entrapped in their aqueous compartments since the lyophilization procedure would be expected to cause some bilayer disruption and subsequent leakage.

Various studies have shown that water-soluble markers such as carboxyfluorescein do not survive freeze drying in that even under the best of circumstances (use of saturated lipids and incorporation of cryoprotectants), a significant portion of the marker is lost on reconstitution. On the other hand, liposomes can retain >90% of lipid-soluble

drugs such as doxorubicin on reconstitution. The amount retained depends on the use of cryoprotectants, lipid composition, liposome type and loading dose.

If the leaked out drug is removed and the preparation frozen for a second time, essentially 100% of the drug is recoverable on reconstitution. This indicates that the original loss represents the portion of the drug residing in the aqueous compartment. Thus, when formulating, one must ensure that essentially all the drug is placed in the bilayer or accept a certain percentage of loss to the external medium.

Recently, it was found that trehalose, a carbohydrate commonly found at high concentrations in organisms capable of surviving dehydration, is an excellent cryoprotectant for liposomes. It may work by stabilizing the bilayers, especially at their phase transition temperatures, during both freezing and thawing[33].

Stability of Liposomes in Biological Fluids

The ultimate efficacy of a liposomal dosage form will be judged on the ability of the formulator to reliably control the amount of free drug that reaches the site of action over a given period of time. Generally, the exact "site of action" or receptor site at the molecular level is not known and one relies on attaining reproducible blood levels of the drug. With traditional non-parenteral dosage forms, only the free drug is absorbed, and once the drug is in the blood stream, it has no memory about where it came from. Thus, the only method available to control the pharmacokinetics of a drug is to adjust the amount of drug that enters the blood as a function of time.

Parenteral, especially intravenous, administration of liposomally encapsulated drugs presents the formulator with additional methods to control the pharmacokinetics of the drug. Factors that affect the pharmacokinetics of parenteral liposome administration include:

a. Concentration of free drug in blood;
b. Concentration of liposomes and their entrapped drug in blood;
c. Leakage rate of drug from the liposome in the blood;
d. Disposition of the intact drug-carrying liposomes in the blood.

In order to reliably control the pharmacokinetics of these complex systems, one must be able to separate out the:

a. Stability (leakage rate) of drug from the liposome in the blood.
b. Disposition of the intact drug-carrying liposome in the blood. The pharmacokinetics of intact liposomes is beyond the scope of this review and has been thoroughly reviewed elsewhere[34].

Liposome Stability in Blood and Plasma: The inability of liposomes to retain entrapped substances when incubated with blood or plasma has been known for about a decade. The fact that high molecular weight substances such as inulin and even albumin leak out on incubation with plasma suggests that more than superficial damage is being done to the liposomes even though their gross morphology appears unchanged. The instability of liposomes in plasma appears to be the result of the transfer of bilayer lipids to albumin and high density lipoproteins (HDL). Additionally, some of the protein is transferred from the lipoprotein to the liposome. Both lecithin and cholesterol also exchange with the membranes of red blood cells. Liposomes are most susceptible to HDL attack at their gel to liquid crystalline phase transition temperature. It is therefore worthwhile to determine by DSC whether the formulation has a phase transition temperature close to 37°C.

The susceptibility of liposomal phospholipid to lipoprotein and phospholipase attack is strongly dependent on liposome size and type. Generally MLVs are most stable since only a portion of the phospholipid is exposed to attack and SUVs are the least stable because of the stresses imposed by their curvature. Liposomes prepared with higher chain length phospholipids are most stable both in buffer and in plasma. Incorporation of charged lipid into the bilayer decreases stability in plasma even when cholesterol is included to bring the liposomes to the gel state. Cholesterol and sphingomyelin are generally very effective in reducing the instability of liposomes in contact with plasma. It is believed that the primary reason for this effect is not the increased bilayer tightness produced by cholesterol but the prevention of transfer of phospholipid to the plasma lipoprotein and red blood cell membrane. The following table shows that liposomal stability in plasma increases as the ratio of cholesterol in the liposome increases:

TABLE 4

Release of Solutes From SUVs in the Presence of Plasma.

Liposomes	Sucrose	Inulin	PVP
PC	80.6±10.4	68.9±6.9	26.9±3.7
PC-CH (7:2)	42.2±2.8	31.1±7.2	26.1±3.0
PC-CH (7:7)	4.1±2.1	7.7±0.9	6.6

Source: Kirby, C. and Gregoriadis, G. (1981) Biochem. J., 199, 251-254.

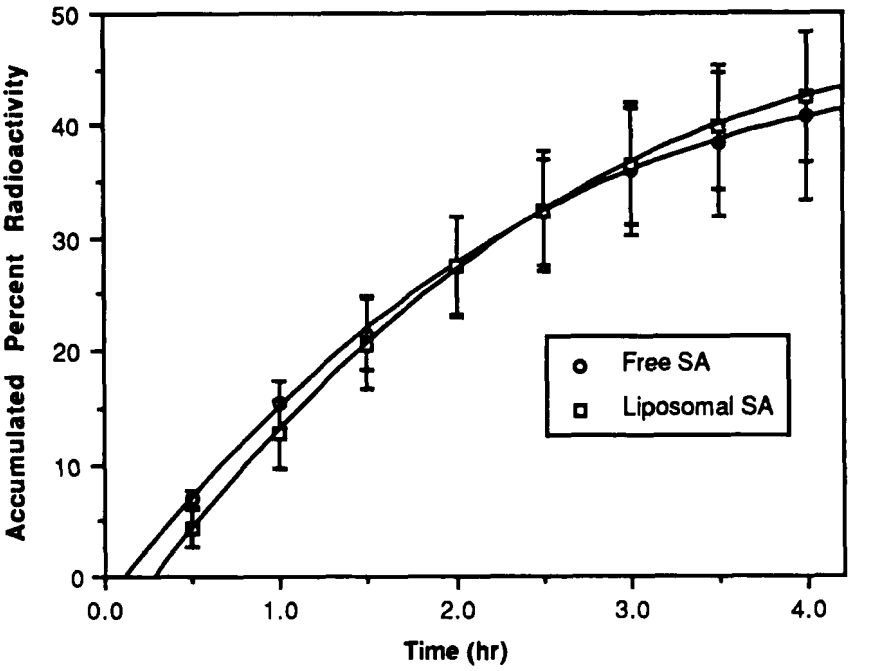

FIGURE 7
Urinary excretion profiles after oral administration of free and liposomally encapsulated (DSPC:CH; 2:1 multilamellar vesicles) salicylic acid to fasted rats. Source: Weiner, N. and Chiang, C.M. (1988) "Gastrointestinal Uptake of Liposomes" in *Liposomes as Drug Carriers,* Gregoriadis, G., Ed., p. 606.

Liposome Stability in the Gastrointestinal Tract: Although about 50 papers have been published on the oral administration of liposomally encapsulated drugs, especially insulin, very little effort has been made to critically assess stability of liposomes in the environment of the gastrointestinal tract. Rowland and Woodley[35] have shown that most of the liposomal formulations that have been used are quite unstable to the g.i. environment (low pH, bile and/or phospholipase). Even distearoylphosphatidylcholine/cholesterol liposomes are very unstable in the gastrointestinal tract and that liposomally encapsulated and free drug give about the same pharmacokinetics when administered by the oral route to rats (Fig 7).

SUMMARY

The therapeutic promise of liposomes as a drug delivery system is fast becoming a reality. One must bear in mind that only in the last five years or so have real advances made in translating progress from university laboratories into pharmaceutically acceptable dosage forms. Pharmaceutical scientists collaborating with process

engineers have been able to produce large volumes of sterile, pyrogen-free liposomes with acceptable shelf-lives. With current emphasis on increasing therapeutic indices of drugs, it appears quite likely that these biocompatible, biodegradable vehicles will receive increased attention from the pharmaceutical industry.

As of this printing, more than 10 companies plan to or have applied to the Food and Drug Administration for approval to test approximately 20 liposomally-entrapped drug entities. These drugs include anticancer and antifungal agents as well as drugs to combat arthritis, glaucoma and dry eye.

Within a short period of time one might expect to see a broad range of liposomal products in various stages of clinical testing. The most promising appear to be liposomal products specifically formulated to facilitate:

a. Site Specific Delivery: Particular emphasis is placed on disease states involving the RES. Examples include antimonial compounds for parasitic disease, immunomodulation using macrophage activating agents and antiviral treatment using ribavirin.

b. Site Avoidance Delivery: The most promising examples are liposomal doxorubicin (reduced cardiotoxicity) and liposomal amphotericin B (reduced nephrotoxicity).

c. Sustained or Controlled Release: Examples include inhalation of bronchodilators, ocular delivery of antibiotics, intramuscular delivery of peptides and topical delivery of a variety of drugs.

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