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LIPOSOME DEPENDENT DELIVERY OF PTERIDINE ANTIFOLATES: A TWO COMPARTMENT GROWTH INHIBITION ASSAY FOR DRUG LEAKAGE AND METABOLISM

by

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Dedicated to

my parents

Chiu Yuk and Lan Fun

my family at home

and

my best friend

Ya-Ling

Their support and encouragement demonstrated in their unconditioned love for me have enabled me to stride forward in times of uncertainty and difficulty. .... I love you all. May God bless you.

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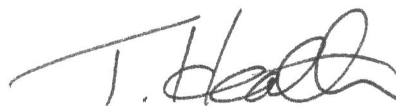
LIPOSOME DEPENDENT DELIVERY OF PTERIDINE ANTIFOLATES: A TWO COMPARTMENT GROWTH INHIBITION ASSAY FOR EVALUATING DRUG LEAKAGE AND METABOLISM

LAWRENCE K. NG

(Under the supervision of Assistant Professor Timothy D. Heath)

A two compartment growth inhibition assay that can provide information about leakage, metabolism and delivery of liposome dependent drugs under cell culture conditions, and at drug concentrations that are relevant to drug delivery has been developed. Two cell lines are grown in separate compartments separated from each other by a 0.1 μm polycarbonate membrane. The membrane allows free drugs to diffuse rapidly from one compartment to another, and does not allow liposomes to diffuse through. Liposomes are added to the first compartment, which contains target cells. The extent of leakage caused by these cells is determined by the growth inhibition of non-target cells in the second compartment. Methotrexate and methotrexate-γ-aspartate leak rapidly and

(67:33) liposomes. In contrast, there is only 42% leakage when the drugs are encapsulated in distearoylphosphatidylglycerol:cholesterol (67:33) liposomes. It is also demonstrated that the target cells (CV1-P) may partially degrade encapsulated methotrexate-γ-aspartate to methotrexate. Therefore, methotrexate-γ-aspartate may be a lysosomally cleaved pro-drug of methotrexate.

APPROVED: 

T. D. Heath

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I. INTRODUCTION

A. Background History of Liposomes

In the early 1960s Bangham and his collaborators at Cambridge, England produced the first liposomes while evaluating the effect of phospholipids on blood clotting. They made the observation that phospholipids dispersed in water formed multilayered vesicles. They showed that each layer was a bimolecular lipid membrane, and that the layers enclosed internal aqueous compartments [1-3]. It rapidly became apparent that these liposomes bore many resemblances to cellular membranes. These new model membranes soon became popular tools for biochemists, and cell physiologists.

Liposomes have proven to be a key tool in the study of the physical characteristics of membrane lipids. Frequently, studies using liposomes composed of simple synthetic lipid mixtures have led to insights concerning the behavior of the more complex lipid arrays of bacterial, fungal, and vertebrate membranes [4-8].

Phospholipid vesicles are also a valuable tool for the study of lipid-protein interaction and, as such, have been applied to problems in the chemistry of biological membranes and of serum lipoproteins. Interesting concepts such as bound lipid around intrinsic membrane proteins, and the existence of specific

lipid requirements for membrane enzymes have derived largely from liposome studies [9,10]. A great deal of effort has also gone into the reconstitution of biological membrane enzyme and transport systems in artificial membranes [9-15]. These reconstituted systems afford an elegant means for understanding the structure and function of membrane enzymes, which often cannot be studied in a meaningful way when they are outside of a membrane environment. Liposomes have also been widely used in the study of the so-called "transport" antibiotics such as valinomycin, gramicidin, and nystatin. These studies have delineated the interesting physical chemistry of the transport antibiotics themselves, and have suggested possible mechanisms for the functioning of natural membrane transport systems [16].

B. Physical Chemistry of Liposomes

Lipids dispersed in water can form a variety of structures. The precise geometry of the structures depends upon the relative concentration of the two principal components (lipid and water), the temperature, the composition of the lipid and the salt concentration of the aqueous phase [17,18]. Over a wide range of such variables, however, the commonest phase structure appears to be that of a layer lattice giving rise to spherulites and myelins, both composite structures consisting of many concentric bimoleclular layers of lipids each separated by an

aqueous compartment. By a variety of techniques phospholipids may be dispersed to form unilamellar liposomes.

The fatty acyl side-chains of the membrane phospholipids progress from a rather closely packed, relatively ordered array (known as the gel state) to a more loosely packed , less ordered state where the side-chains are capable of more rotational motion (known as the fluid state) as the temperature is raised. For membranes composed of homogeneous lipids this order-disorder transition occurs over a rather narrow temperature range and is known as the thermotropic phase transition. The occurrence of the transition can be detected by probes which measure molecular motion or by monitoring the sharp increase in enthalpy of the system by differential scanning calorimetry [7].

The temperature at which a lipid membrane undergoes a phase transition depends on a number of parameters. Short-chain saturated lipids melt at lower temperatures than do long-chain saturated lipids, while unsaturated lipids melt at lower temperature than the equivalent length saturated lipids. For heterogeneous mixed lipids differing in chain length or in degree of unsaturation, the phase transition is broadened. Nonetheless as the temperature rises the membrane lipids change from a more ordered to a less ordered state.

Cholesterol has a very important modulatory effect on the lipid bilayer membrane. If cholesterol is added to a membrane composed of a homogeneous phospholipid, the phase transition gradually diminishes as the mole fraction of

cholesterol increases. Papahadjopoulos and his colleagues have described cholesterol as being a "fluidity buffer," since below the phase transition it tends to make the membrane less ordered while above the transition it tends to make the membrane more ordered, thus surpassing the dramatic shift in order at the phase transition [19]. The basic aspects of membrane phase transitions have been discussed thoroughly elsewhere [20,21].

C. Types of Liposomes and Their Methods of Preparation

At present there are several distinct types of liposome preparations available to the experimeter. The standard preparation of large multilamellar vesicles (MLVs) is prepared by gently dispersing phospholipids in aqueous media [22]. These liposomes are several microns in diameter, are moderately efficient at trapping solutes, and are relatively stable. Sonication of MLV produces small unilamellar vesicles (SUVs) which range in size from 200 to 500 Å [23]. However, this method of preparation is not the method of choice for encapsulating macromolecules such as DNA, because the energy generated during the sonication process may cause sufficient damage to the macromolecules. SUVs are also too small to capture many macromolecules [24]. SUVs can also be prepared by rapid injection of alcoholic lipid solutions into the aqueous phase [25].

Recently, several new approaches for increasing the trapping efficiency of liposomes have evolved. Large unilamellar vesicles (LUVs) have been prepared by ether evaporation. These liposomes are 3-4 times more efficient at solute entrapment than are MLVs [26,27]. LUVs can also be formed from water-in-oil emulsions of phospholipids and buffer in an excess organic phase, followed by removal of the organic phase under reduced pressure [28]. Vesicles formed by this technique, which are referred to as reverse-phase evaporation vesicles (REV), have a high aqueous space-to-lipid ratio and encapsulate a high percentage of the initial aqueous phase. However, the organic solvents that can be used for REV preparation are quite restricted. Ethers [28] and fluorocarbons [29] are the only ones that are used. This technique is applicable to a large number of phospholipids and solvents, either alone or in mixtures. The principle disadvantage of this method is the exposure of the material to be encapsulated to organic solvents and to short periods of sonication --- conditions that may possibly result in denaturation of sensitive proteins or breakage of DNA. The size distributions obtained for most phospholipid mixture are quite uniform, although preparations which contain cholesterol have a heterogeneous size distribution. Size can be reduced and rendered more uniform by extrusion through polycarbonate membrane [30].

D. Liposome-cell Interactions In Vitro: Their Implications In Drug Delivery

Liposomes can interact with cells via four major mechanisms: adsorption to the cell surface, endocytosis by the cell, fusion with the cell plasma membrane, and lipid exchange between liposomal and cellular, or subcellular membranes. The mechanisms are by no means mutually exclusive. A number of experimental criteria have been proposed by Pagano and Weinstein [31] to distinguish between these mechanisms.

1. Adsorption

Adsorption may be defined as the stable association of intact liposomes with the cell surface, either nonspecifically through elecrostatic/hydrophobic forces, or specifically through interaction with cell-surface components. Pagano and Weinstein [31] have proposed a set of experimental criteria for adsorption. The adsorption described by Pagano implies a stable association without liposome internalization and one that resists repeated washings.

The first observations of such an interaction between liposomes and cells in culture was made by Magee and Miller [32]. Pagano et al. [31] have shown the stable adsorption of neutral SUVs to fibroblasts by scanning electron microscopy (SEM). Similar studies showed adsorption of SUVs to lymphocytes, both by transmission electron microscopy (TEM) and by SEM [33,34]. Using fluorescence self-quenching technique, investigators demonstrated that

adsorbed vesicles remained intact and that the vesicles could not be removed by repeated washings [34,35].

The ability of drug carriers to adsorb to cell surfaces is essential in carrier-mediated drug delivery, since the uptake of the drug-carrier complex will first involve the process of carrier-cell interaction regardless of the mechanisms by which the complex gets incorporated into the cells.

2. Endocytosis

Endocytosis refers to the situation where only a small region of the plasma membrane folds inward, or invaginates, until it has formed a new intracellular membrane-limited vesicle that eventually fuse with lysosomes. There are two types of endocytic processes. Pinocytosis is the nonspecific uptake of small droplets of extracellular fluid by such vesicles. Any material dissolved in the extracellular fluid is internalized in proportion to its concentration in the fluid. In receptor-mediated endocytosis, a specific receptor on the surface of the membrane recognizes an extracellular macromolecule and binds with it. The substance bound with receptor is referred to as the ligand. The region of plasma membrane containing receptor-ligand complex undergoes endocytosis. It is a general process by which cells internalize and degrade many extracellular molecules [36,37]. It is worthwhile to note here that phagocytosis is a fundamentally different process. It is the uptake of large particles such as

bacteria or parts of broken cell, and it occurs in a limited class of cells: those that are phagocytic.

Features that are characteristics of endocytosis are as follows: the uptake should have a marked sensitivity to inhibitors of oxidative phosphorylation and glycolysis, and an increased uptake with increased temperature is also to be expected.

Earler studies on the mechanisms by which liposome contents enter cells implicated liposome-plasma membrane fusion [31,34,38,39,40]. But recent evidence suggests that fusion occurs with low frequency (discussed later). Endocytosis also has been advocated as the dominant mechanism of delivery but has not been regared as a useful route by which liposomes could enter nonphagocytic cells or by which large or labile molecules could gain access to the cytoplasm.

Recently, by encapsulating fluorescent probes and colloidal gold, in large negatively charged PS:Chol liposomes, Straubinger et al. [41] presented evidence that such liposomes are endocytosed by coated pits in a similar manner to that of a number of macromolecules with specific surface-bound receptors, such as low density lipoprotein (LDL), transferrin, α_2-macroglobulin, and others [36,37]. Based on the marked difference between the two aqueous fluorescent probes, carboxyfluorescein and calcein, in membrane permeability at low pH, the results by Straubinger indicated that liposomes encountered an

acidic environment after endocytosis. The fate of liposomes is in general agreement to the fate of other endocytosed vesicles. The observation that liposomes encountered an acidic environment was further supported in the same study that lysosomotropic agents such as chloroquine and NH_4Cl, inhibited the glycerol-dependent enhancement of carboxyfluorescein delivery to the cytoplasm, a process which depends on acidification of the liposome contents. A more recent finding by the same authors provides more evidence that endocytosis represents the most likely mechanism by which liposome contents enter cells [Straubinger in press].

When cells take up liposomes through endocytosis, the cells move the liposomes into lysosomes. Inside the lysosome the liposomal lipid components get degraded and probably migrate outward to become part of the cell's membranes. Other liposomal components that resist lysosomal degradation (such as certain medications) may enter the cytoplasm. Other agents that are suitable for encapsulation in liposomes would include weakly acidic molecules which cells do not export efficiently. The rationale is that weakly acidic molecules when encounter with the acidic environment (lysosome) will render themselves more permeable to the lysosomal membrane. Stability against lysosomal degradation may not be important if pH-dependent efflux is rapid.

3. Fusion

This may be described as the insertion of the lipid bilayer of the liposome into the plasma membrane, with the concomitant release of some, if not all, of the liposomal contents into the cytoplasm. Earlier studies implicated that liposome-plasma membrane fusion was the mechanism by which liposome contents enter cells [31,34,38-40]. However, recent evidence suggests that fusion may or may not occur. Recent studies suggests that liposomes do not fuse with the plasma membrane without pertubations such as glycerol treatment [42], or the inclusion of viral proteins thought to promote membrane fusion [43-45]. By encapsulating poliovirus RNA and SV40 DNA in LUVs composed of phosphatidylserine, workers had demonstrated through infectivity assays that these macromolecules were transferred from vesicles to the cells [46-48]. It could be argued that if the liposomes had entered the cells by endocytosis, lysosomes would have digested them and the macromolecules would have been destroyed. Therefore one may suggest that fusion was responsible for the uptake of liposome-encapsulated materials. However, one does not know whether the delivery was due to a low frequency transfer of macromolecules to the cytoplasm during one or more of the steps involved in liposome endocytosis. While high concentrations of glycerol are known to enhance membrane fusion [49], the observation that the infectivity of naked SV40 DNA was also increased to a similar degree by the glycerol treatments suggests that a mechanism

involving a stimulation of endocytosis and/or increase in membrane permeability (either plasma or lysosomal) are also highly possible [47]. Results obtained with metabolic inhibitors are also consistent with the interpretation that the enhancement of liposome delivery in glycerol-treated cells occurs via an energy-dependent endocytotic pathway [48]. Furthermore, because fusion is at best a low frequency event, it cannot be used for the delivery of drug molecules to cells [50].

4. Lipid Exchange

This may be defined as the transfer of liposomal lipids to cellular, or subcellular, membranes, or vice-versa, without any association of the liposome contents. For lipid exchange to take place the liposomal lipid must have a particular chemistry in relation to the target cell. Once a liposomal lipid joins the cell membrane it can either remain in the membrane for a long time or be redistributed to a variety of intracellular membranes. Reviews on lipid exchange have been described elsewhere [31,51].

E. Lipid Vesicles as Carriers for Introducing Biological Active Materials into Cells

The versatility of the methodology for producing lipid vesicles and the ways

with which liposomes are interacting with cells have made them excellent candidate as carrier vesicles. The ability to produce vesicle of widely differing size and surface properties enables a wide range of materials to be incorporated into vesicles, either within the vesicle membrane or encapsulated within the internal aqueous space(s) of the vesicle. In the past several years there have been many reports in the literature on the use of phospholipid vesicles as carriers for introducing biologically active substances into cells in vitro and in vivo. Recent reviews in this area of research include those by Pagano [31] and Poste [39].

Unfortunately, two major problems were not solved, and they persist today. First, liposomes injected i. v. are for the most part unable to leave the general circulation and hence are unlikely to reach most cell types. Unencapsulated, or free, drugs typically diffuse through capillary walls into tissues, but liposomes are too large to pass through the walls of capillaries in most organs. Secondly, the majority of the liposomes injected i. v. are retained by cells that make up the reticuloendothelial system (RES). This system consists of macrophages and other highly phagocytic cells that are distributed throughout the lymph nodes, liver, spleen, bone marrow and lungs and that circulate in the blood and the nymph. Such cells take up liposomes from the circulation. The rate of clearance of a given dose of i. v. injected liposomes from the circulation is known to be influenced by several factors. These include vesicle surface charge and size.

Generally, uncharged (or positively charged) and small vesicles are cleared less repaidly than negatively charged or larger vesicles, respectively [40,52]. For small unilamellar neutral liposomes, the solute retention by liposomes and their half-life in the circulation can be controlled by the appropriate manipulation of liposomal membrane fluidty and composition [53-55]. Uptake by reticuloendothelial cells and organs probably prevents liposomes from circulating long enough to reach many targeted cells and tissues efficiently. However, the localization of liposomes injected i.v. in cells of the RES offers a potentially powerful method for targeting therapeutic agents to these cells, known as passive targeting since it simply exploits the natural fate of liposomes in being taken up by mononuclear phagocytes [56].

Even though the anatomy of the circulation seems likely to frustrate targeting of liposomes to cells outside the vasculature in most tissues and organs, significant opportunities may nonetheless exist for targeting liposomes to cell types within the vasculature. These opportunities fall into three different groups: (1) passive targeting to the RES (which we have just described), (2) active targeting to specific subsets of circulating blood cells, and (3) active targeting to vascular endothelial cells [56]. Remarkable progress has been made over the last few years using monoclonal antibodies and other immunologic probes to classify the major classes of circulating blood cells into subsets bearing defined differential antigens [57,58]. Monoclonal antibodies

currently represent the major class of ligand available for ligand-directed targeting of liposomes and other drug carriers [32,66]

F. Site-Specific Drug Delivery via Injection of Liposomes into Anatomically Isolated Site

Many of the problems associated with systemic adminstration of liposomes are not encountered if liposomes are applied topically to the skin or the eye, used as douche in the female genital tract, or when injected into sites that are anatomically isolated as joints, the urinary bladder, and the cerebral ventricles. In each of these situations liposomes are introduced into the vicinity of the desired target cell(s). Although liposome uptake by phagocytic tissue histiocytes will persumably occur in tissue locations where these cells are present, the mononulcear phagocyte system would not be expected to dominate liposome disposition to the extent observed when liposomes are injected i.v. Review on this area has been described elsewhere [56].

G. The Liposome drug-carrier concept: Its Development and Future

The goal in many of the initial studies on liposomes as drug carriers has been to demonstrate that there is interaction of lipid vesicles with cells.

Questions that are fundamental to the development of methods whereby vesicles and their contents could be directed to specific regions of the cell also have been studied extensively. To understand the factors which may influence the behavior and fate of liposomes in vivo, experiments are usually conducted in a well defined cell-cultured environment. In most cases the convenience of working with well-characterized cells in culture enables liposomes to be added directly to the desired target cells, so that factors affecting drug delivery can be isolated and studied individually.

The ability of liposomal agents, transported by carrier into cell's interior, to act on relevant targets was demonstrated in cell culture [67]. Since this discovery, more and more information related with using liposomes as drug carriers obtained in vitro has been revealed.

Drug retention by liposomes in the biological milieu needs to be maximized if successful application of liposomes as drug carriers [40,68] is desired. Work on the in vivo fate of a number of liposome-entrapped drugs [40,69] indicates that, after intravenous injection, there is an almost immediate release of drug in the blood circulation. The rate at which this occurs is much higher than that expected from solute diffusion through intact bilayers [70,71] and it has been attributed, at least in part, to the loss of the structural integrity of liposomes as a result of phospholipid removal by high density lipoproteins [72-74]. Using in vitro technique, several researchers have demonstrated that in the presence of whole

blood, plasma or serum, stability of small MLVs or small unilamellar liposomes can be controlled by adjusting their cholesterol content. Cholesterol stabilises liposomes in the presence of serum by reducing the loss of their phospholipid component to high density lipoproteins [70,71,75].

Liposomes are able to carry relatively large amounts of encapsulated materials such as drugs in their aqueous spaces, and they have been used in some areas of chemotherapy and cellular biology. Liposomes have been used as passive or slow release capsules [76], or in other models, specific ligands have been attached to liposome surfaces with the aim of promoting drug effects on given cell population [32,59-66]. Since large unilamellar liposomes have a greater encapsulating efficiency than small liposomes, the former type has been used preferentially by some groups. In addition, certain macromolecules which cannot be encapsulated in small liposomes, such as DNA, are easily encapsulated in large liposomes [47]. With smaller molecules such as drugs, large and small liposomes can be employed. Given the higher encapsulation efficiency of large liposomes, one may wonder whether they are also more efficient for specific delivery of their contents to target cells in vitro. Recently, Machy and Leserman have demonstrated a rather unexpected result. They demonstrated that small liposomes are superior for in vitro delivery of antifolate, methotrexate, than large liposomes,presumably because cells are better able to ingest small liposomes by endocytosis [77]. However, other researchers have

observed the opposite phenomenon. When 5-fluoroorotate was encapsulated in liposomes, sonicated liposomes of the same composition as larger liposomes were 10-fold less effective than larger liposomes for drug delivery [78].

In most cases, in order for drug to exert its therapeutic effect, the drug must penetrate the plasma membrane by diffusion through the lipid bilayer, or carrier-mediated uptake, and enter the interior of the target cell; drugs and hormones that act by interacting with cell-surface receptors are an exception to this rule. Many potentially worthwhile drugs are excluded from use because they fail to penetrate membranes. The use of a liposomal carrier offers a way of circumventing membrane barriers and of promoting the entry of certain types of drugs into cells. Recent evidence suggests that liposome contents enter into cells via endocytosis. Early exploitation of these effects concerns the antitumor drug cytosine arabinoside triphosphate (ara CTP); this substance is an inhibitor of DNA polymerase and is the active principal eventually produced by the adminstration of the prodrug cytosine arabinoside (ara C). Tumor cell populations often acquire resistance to ara C by reducing their level of deoxycytidine kinase, the first enzyme in the pathway which activates the drug [79]. Mayhew et al. [80] have shown that they could circumvent the cellular permeability barrier to ara CTP by giving the drug in liposomal form. This approach may also circumvent the problem of drug resistance due to reduced deoxycytidine kinase levels.

Papahadjoupolos et al [81] used liposomes to overcome the permeability barrier of actinomycin-D-resistant tumor cells. The liposomal actinomycin D inhibited RNA synthesis in the drug-resistant cells at concentrations many times lower than did free actinomycin D. This is a rather important observation since many types of tumors become refractory to drugs bcause of the evolution of reduced levels of drug permeation [82].

One should keep in mind that putting a drug into a liposome is not a guarantee of enhanced drug uptake, either in vivo or in vitro. Recently, Heath et al. has introduced a new concept of describing the above phenomena. Drugs that can enter cells rapidly as free compounds will do so after leakage from liposomes, and may be defined as liposome-independent agents. Drugs that cannot pass readily through the plasma membrane will enter cells through uptake of the liposomes, and have been defined as liposome-dependent agents [83]. The in vivo potency of drugs may be improved by either delivery mechanism. Liposome-independent drugs may exhibit enhanced effects through controlled release [84,85], or through increased localisation in target organs [86,87]. Liposome-dependent drugs are potentially capable of highly specific effects, especially if used with ligand-directed liposomes. The concept of liposome-dependent drug is of considerable importance in the field of drug delivery. While there may be drugs which are inherently impermeable to cell plasma membranes, there are circumstances that we want the drugs to be

impermeable to the cell plasma membranes. This is especially true in the field of targeted drug delivery using liposomes as drug carriers. We mentioned earlier that most liposomes leak in the presence of serum, making the drug impermeable to cell plasma membranes will cut down the untoward effects on non-target cells due to leaked compounds. The identification of liposome-dependent drugs is possible for the pteridine antifolates, because detailed information is available about their transport and dihydrofolate reductase-inhibitory properties. Methotrexate-γ-aspartate (see Figure 1) is an effective dihydrofolate reductase inhibitor [88] that enters cells 100-times less effectively, and is 160 times less toxic than the free drug. The discovery of the liposome-dependency of methotrexate-γ-aspartate shows the potency of selecting compounds by evaluating their transport properties [89]. In addition, the identification of liposome-dependent drugs also opens up avenue in search for drugs that requires lysosomal action for their full effects to be seen.

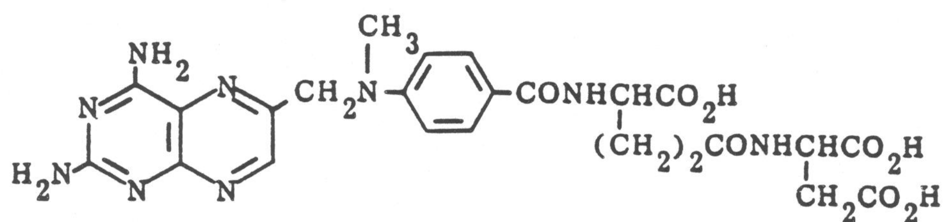


Figure 1: The Structure of Methotrexate-γ-aspartate

II. STATEMENT OF THE PROBLEM

The interest in liposomes as carriers of macromolecules is based on their potential to enclose and protect diverse materials of biological interest and to deliver them, functionally intact and in significant quantities, to the interior of many cell types. Without doubt the mechanism by which drug enters cells is one of the most important considerations for optimizing liposome-mediated drug delivery [90]. Drugs that can enter cells rapidly as free compounds will do so after leakage from liposomes, and are defined as liposome-independent agents [89]. Alternatively, drugs that cannot pass readily through the plasma membrane will enter cells through uptake of the liposomes, and have been defined as liposome-dependent drugs [83]. The potency of a liposome-dependent drug is increased by encapsulation in negatively charged liposomes [89] or neutral antibody-directed liposomes [61-63].

The potency of an encapsulated liposome-dependent drug depends on the uptake of intact liposomes by endocytosis [41]. Consequently, the stability of the liposome is critical to efficient drug delivery. Liposomes are known to lose their contents through adsorption to the cell membrane [91], and through serum induced leakage [92,93]. However, a survey of the literature reveals that there has not been a systematic evaluation of liposome leakage under the conditions

that closely parallel those prevailing at the IC_{50} of encapsulated drug. This stems from the fact that in some cases the drug concentration at the IC_{50} is as low as 0.005 µM [94], and leakage studies with drugs are currently not feasible at such low concentrations (nanomolar) if conventional analytical techniques are followed. Leakage measurement at low drug concentrations is important because one can study the leakage behavior of liposome suspensions in conditions that are most relevant to drug delivery. As a result, untowards effects on non-target cells due to leaked compounds can be reduced further by employing less concentration of liposome-dependent drugs if liposomes can be made more stable to cell- and serum-induced leakage.

The present study was designed to obtain information, under cell culture conditions, about this low concentrations leakage, and about the effects of encapsulated drug on target and non-target cells.

III. EXPERIMENTAL METHODS

A. Lipid and Other Materials

Phosphatidylglycerol and distearoylphosphatidylglycerol were obtained from Avanti Polar Lipids (Brimingham, Al), and were stored as a chloroform solution at -20°C in glass ampoules under argon. Cholesterol was obtained from Sigma Chemical Co. (St Louis, Mo), and was purified by four recrystallizations from methanol. The product was stored in the same way as phospholipids [89]. 6.5 mm Transwell chambers with 0.1 μm pore size polycarbonate membranes (not treated for cell culture) were a custom purchase from Costar (Cambridge, MA). Methotrexate was purchased from Sigma. Methotrexate-γ-aspartate was synthesised and provided by J. R. Piper, Southern Research Institute, Birmingham, AL [88]. Sterile solutions of free or encapsulated drug were stored at 4 °C, and used within one month of preparation. Carboxyfluorescein was obtained from Eastman Chemical Co. (Rochester, NY) and purified with Sephadex LH-20 [95].

B. Experimental Procedures

1. Preparation of Phospholipid Vesicles

Reverse phase evaporation (REV) method of Szoka and Papahadjopoulos [28] was used to prepare both non-loaded and loaded liposomes. 10.0 µmol phospholipids and 5.0 µmol cholesterol, dissolved in chloroform, were transferred to a 13 mm x 100 mm glass tube with a Teflon-lined screw cap. The sample tube was inserted into a conventional evaporation tube and the solvent was evaporated at a specific temperature, which depends on the kinds of phospholipid used (see below), in a rotary evaporator (Rotavapor, Buchi, Switzerland). A few milliliters of H_2O was added to the evaporation tube to ensure thermal contact between the sample tube and the water bath. 1.0 ml of hydrated ether (isopropyl ether for saturated phospholipids and diethyl ether for unsaturated phospholipids) was added to dissolve the lipid film. 0.4 ml of aqueous buffer containing the material to be encapsulated was later added to the tube, resulting in the formation of a two-phase system. The mixture was then sonicated briefly in a bath-type sonicator (G112SP1G, Lab. Supplies Co., Hickville, N.Y.) until an emulsion was formed. The tube containing the emulsion was transferred immediately to the evaporation tube. A vacuum of 300-400 mmHg was established, and the bulk of the ether was removed slowly by rotary

evaporation. During evaporation, a viscous gel-like phase formed, so the sample tube was removed frequently and the gel disrupted by brief vortexing. After the majority of the gel phase had collapsed to form a slightly turbid (liposome) suspension, residual ether was removed by increasing the vacuum to 700-750 mmHg with care to minimize evaporation of the aqueous sample. Liposomes were separated from unencapsulated drug and residual ether by gel chromatograhy with a 1 x 15 cm Sephadex G-50 (Pharmacia, Piscataway, N.J.) column, and 50 mM morpholinoethanesulfonic acid (MES) / 50mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) / NaCl (pH 7.2), 290 mmol/kg buffer as eluating buffer. In our studies, all liposomes were prepared from phospholipid:cholesterol (67:33), and will subsequently be referred to by phospholipid alone.

Methotrexate and methotrexate-γ-aspartate solutions prepared at 10 mM drug in 50 mM MES / 50 mM HEPES / NaCl (pH 7.2), 290 mmol/kg buffer were used to prepare loaded liposomes. Loaded phosphatidylglycerol (unsaturated phospholipid) liposomes were prepared at 37 ^{o}C, while loaded distearoylphosphatidylglycerol (saturated phospholipid) liposomes were prepared at 54 ^{o}C. Since loaded liposomes would later be used for growth inhibition assay, their preparations were carried under sterile conditions. Drug concentration in both types of liposomes was determined from the absorbance at 370 nm after solubilization of a sample of the vesicles in

chloroform:methanol:buffer (1:3:1) assuming a molar extinction coefficient of 7943 at 370 nm [96]. The phospholipid content of the vesicles was measured by phosphorus determination [97].

A solution containing 50 mM MES / 50mM HEPES / NaCl (pH 7.2), 290 mmol/kg buffer was used to prepare non-loaded liposomes. Unloaded phosphatidylglycerol liposomes were prepared at 37 ^{o}C, and non-loaded distearoylphosphatidylglycerol liposomes were prepared at 54 ^{o}C. Non-loaded liposomes which were used mainly for validation of the two compartment system were not prepared under sterile conditions. The phospholipid content of the vesicles was measured by phosphorus determination [98].

2. Two Compartment System Validation

a. Carboxyfluorescein Flux Experiment

Four different systems were tested. Namely, 1) Millicell-HA (Millipore, Bedford, MA) with HATF as membrane in well, 2) a chamber of similar design to the Millicell-HA, but with dialysis tubing as membrane in well, 3) a chamber of similar design to Millicell-HA, but with 0.1 μm pore size polycarbonate membrane in well, and 4) Custom purchased Costar Transwell with 0.1 μm pore size polycarbonate membrane in well. Purified carboxyfluorescein was dissolved in 50 mM HEPES to a final concentration of 1 mM at pH 7.2. The

tonicity of this solution was adjusted to 290 mmol/kg with NaCl. To validate the first three systems, 0.6 ml of HEPES / NaCl (pH 7.2), 290 mmol/kg buffer was first added to three individual wells of a 24-well plate (Costar). Three chambers were placed in the wells , and 0.4 ml of 1.0 mM carboxyfluorescein solution was added to each of the three chambers. The plate was then incubated at 37°C on an American Rotator V shaker (American Scientific Products, McGaw Park, IL) at a rate of 120 rpm. At separate time points, 10 μl of samples were taken from the chambers and the wells. These samples were diluted to 1.0 ml with HEPES / NaCl (pH 7.2), 290 mmol/kg buffer. The amount of carboxyfluorescein that diffused across the polycarbonate membrane of the chambers was determined by the absorbance at 493 nm using a molar extinction coefficient of 70,000. To validate the fourth system, 0.7 ml of HEPES / NaCl (pH 7.2), 290 mmol/kg buffer was first added to three individual wells of a 24-well plate. Three chambers were suspended from the top of the wells, and 0.15 ml of 1.0 mM carboxyfluorescein solution was added to each of the three chambers. The flux of CF across the membrane was followed as described above. We also modified this experiment by adding 0.7 ml of 1 mM carboxyfluorescein in the well and 0.15 ml of HEPES / NaCl (pH 7.2), 290 mmol/kg buffer in the Transwell chamber, and followed their course of diffusion with respect to time.

b. Phospholipid Flux Experiment

The above four systems were tested. Non-loaded liposomes were added to nine individual chambers in the wells of a 24-well plate which contained 0.6 ml of HEPES / NaCl (pH 7.2), 290 mmol/kg buffer for the first three systems and 0.7 ml of the same buffer for the fourth system. 1.0 μmole / 0.4 ml of nonloaded liposomes were used for the first three systems, and 1.0 μmole / 0.15 ml were used for the fourth system. The plate was then incubated at 37°C with shaking at 120 rpm. At 1, 2 and 3 days, the contents of three chambers and wells were assayed for lipids by phosphorus determination [97]. The percent of liposomes retained in the chamber was calculated using the following equation:

$$\%\ \text{Retention} = \frac{\text{Lipid content of chamber}}{\text{Lipid content of chamber} + \text{lipid content of well}} \times 100$$

3. Cell Culture and Growth Inhibition Assay

a. One Compartment Growth Inhibition Assay

CV1-P (adherent cell), an African green monkey kidney cell line [99], was obtained from P. Berg, Stanford University, Palo Alto. EL4 (suspension cell), a T-lymphoma was obtained from W. Ershler, University of Wisconsin, Madison, who originally purchased this cell line from American Type Culture Collection.

Cells were grown in Dulbecco's modified Eagle's minimal essential medium (pyruvate, 3 g/l glucose), with 100 units/ml penicillin and streptomycin, and 10% fetal calf serum (K-C Biologicals).

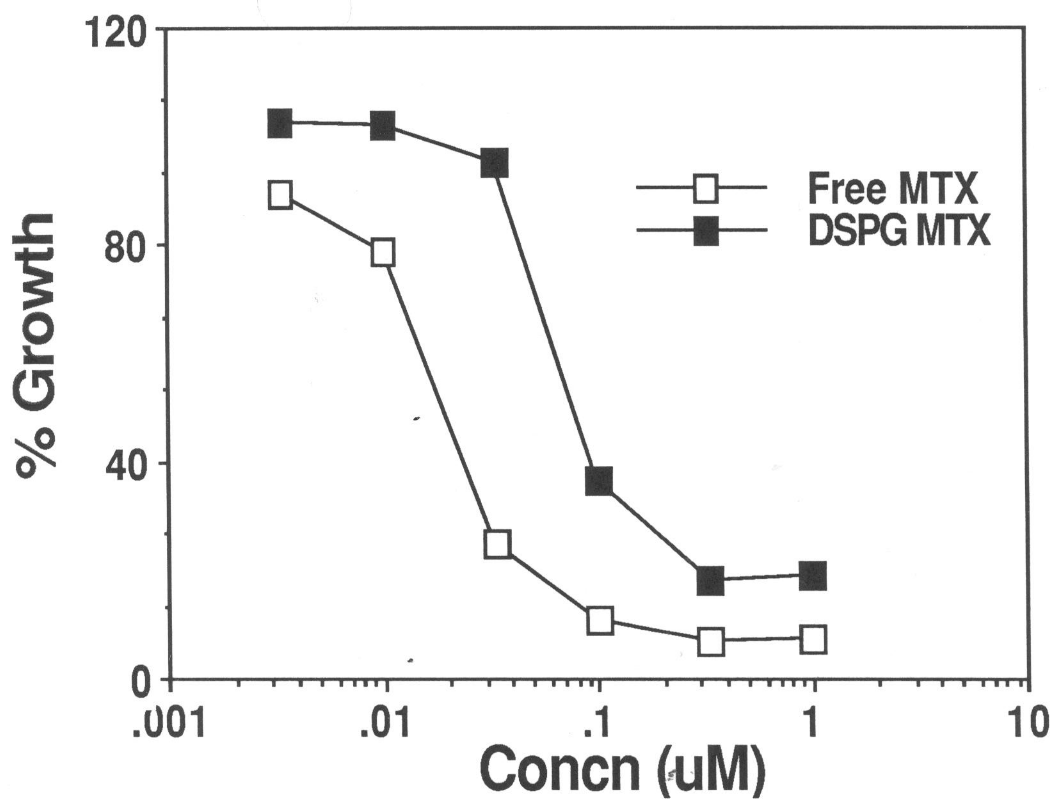
One compartment growth inhibition studies were carried out as previously described [89]. Briefly, cells were suspended for EL4 cells for growth inhibition at 3 x 10^4 cells per well in 24-well plates (Costar). Tripicate wells were treated with drug immediately after EL4 cells were added to the wells. Control well were treated with buffer alone. Three wells were counted at the time of treatment to give the original cell concentration. The cells were allowed to grow for 72 hours before counting with a Coulter counter, model ZM. EL4 cells were resupended directly in medium, diluted 1/50 in isotonic counting fluid (Diadnostic Technology, Inc., Hayward, Calf.) and counted. Percent growth was determined according to the equation:

$$\%\ \text{growth} = \frac{[\text{sample count} - \text{original count}]}{[\text{control count} - \text{original count}]} \times 100$$

The mean percent growth was plotted against the $\log_{10}$ of the drug concentration for the cell line (Figure 2). The concentration of drug required to produce 50% inhibition of growth (IC_{50}), was determined from the plots.

Figure 2

Growth inhibition by free and distearoylphosphatidylglycerol:cholesterol (67:33) encapsulated methotrexate on EL4 cells in a single compartment system. Vesicles were made using REV method. EL4 cells were treated at the concentrations shown.



b. Two Compartment Growth Inhibition Assay

i. In the Presence of CV1-P cells

Two compartment growth inhibition studies with the presence of CV1-P cells were carried out in the following way. 0.7 ml of CV1-P cells at a concentration of 2 $\times 10^4$ per ml were first added to each well of a 24-well plate. The cells were allowed to adhere to the surface of the plate for 6 hours under normal growth conditions. A chamber was then suspended from the top of each well. To each chamber, 0.15 ml of EL4 cells at a concentration of 3.3 $\times 10^4$ per ml was added. Triplicate wells were treated with drug as soon as EL4 cells were added to the chambers. Control wells were treated with buffer alone. Three wells and three chambers were counted at the time of treatment to give the original cell concentration. The cells were allowed to grow for 72 hr at 37°C with shaking, and were then counted with a Coulter Counter, model ZM. The whole content of each chamber was transferred to a counting vial containing 9.8 ml diluent, and was counted to determine EL4 cells. CV1-P cells were freed of medium, and resuspended by treatment with 1 ml of 0.1% trypsin in phosphate buffered saline,1 mM EDTA solution at 37°C for 20 min. The cell suspension was diluted 1 : 50 with isotonic counting fluid and counted. The mean percent growth was plotted against the $\log_{10}$ of the drug concentration for each cell line. The concentration of drug required to produce 50% inhibition of growth (IC_{50}),

was determined from the plots.

ii. In the Absence of CV1-P cells

Two compartment growth inhibition studies with the absence of CV1-P cells were carried out in the following way. 0.7 ml of plain growth medium were first added to each well of a 24-well plate. A chamber was then suspended from the top of each well. To each chamber, 0.15 ml of EL4 cells at a concentration of 3.3 $x10^4$ per ml was added. Triplicate wells were treated with drug as soon as EL4 cells were added to the chambers. Control wells were treated with buffer alone. Three chambers were counted at the time of treatment to give the original cell concentration. The cells were allowed to grow for 72 hr at 37°C with shaking, and were then counted with a Coulter Counter, model ZM. The whole content of each chamber was transferred to a counting vial containing 9.8 ml diluent, and was counted to determine EL4 cells. The mean percent growth was plotted against the $\log_{10}$ of the drug concentration for the cell line. The concentration of drug required to produce 50% inhibition of growth (IC_{50}), was determined from the plots.

iii. Growth Inhibitory Effects of Non-loaded Liposomes

Two compartment growth inhibition studies of the effects of non-loaded liposomes on cell growth were carried out in the following way. 0.7 ml of CV1-P

cells at a concentration of 2 x10^4 per ml were first added to each well of a 24-well plate. The cells were allowed to adhere to the surface of the plate for 6 hours under normal growth conditions. A chamber was then suspended from the top of each well. To each chamber, 0.15 ml of EL4 cells at a concentration of 3.3 x10^4 per ml was added. Triplicate wells were treated with non-loaded liposomes, at the lipid concentrations used when cells were treated with encapsulated methotrexate and methotrexate-γ-aspartate, as soon as EL4 cells were added to the chambers. Control wells were treated with buffer alone. Three wells and three chambers were counted at the time of treatment to give the original cell concentration. The cells were allowed to grow for 72 hr at 37°C with shaking, and were then counted with a Coulter Counter, model ZM. The whole content of each chamber was transferred to a counting vial containing 9.8 ml diluent, and was counted to determine EL4 cells. CV1-P cells were freed of medium, and resuspended by treatment with 1 ml of 0.1% trypsin in phosphate buffered saline,1 mM EDTA solution at 37°C for 20 min. The cell suspension was diluted 1 : 50 with isotonic counting fluid and counted. The mean percent growth was plotted against the $\log_{10}$ of the drug concentration for each cell line. The concentration of non-loaded liposomes required to produce 50% inhibition of growth (IC_{50}), was determined from the plots.

IV. RESULTS

A. Validation of the Two Compartment System

The objective of this research is to obtain information of low concentration liposomal leakage. If liposomes or free drugs are added to one compartment, which contains a cell population, then the extent of leakage can be determined by the growth inhibition of a separate cell population in the second compartment. The following equation can be used to evaluate the extent of leakage:

$$\text{Apparent \% Leakage} = \frac{IC_{50} \text{ of free drug}}{IC_{50} \text{ of encapsulated drug}} \times 100$$

This is called 'apparent leakage', because, in practice, a slower and more extensive loss of liposome contents would give similar results in this system. In other words, when this equation is used to evaluate leakage, one should assume that liposomes leak their contents shortly after addition to the target cell population. Beside this important assumption, this two compartment system for growth inhibition assay must fulfill a number of criteria in order to be successful in evaluating liposome leakage. The two compartments (denoted as chamber and well), which are separated from each other by a semi-permeable

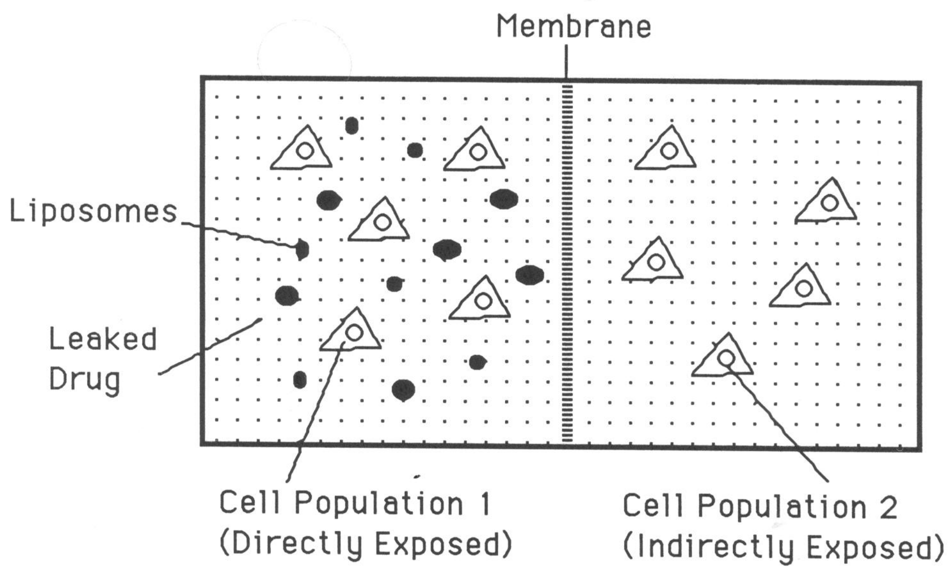
membrane, must only allow free drugs to pass through while retaining the liposomes in the compartment where they are originally placed (Figure 3). In addition, free drug (added or leaked) must reach equilibrium rapidly across the membrane, and the system must be easily used in a standard cell culture environment.

A search for this system began with the commercial available Millicell-HA (Millipore, Bedford, MA) culture plate insert (Figure 4). Figure 5 shows the results of a carboxyfluorescein flux study, in which Millicell-HA with HATF as membrane was used in a two compartment system. When 0.4 ml of 1 mM carboxyfluorescein solution was added to the chambers, it took the carboxyfluorescein approximately 5 hours to reach about the same concentration in the two compartments. However, almost 30% of liposomes leaked through the membrane in 2 days of incubation (Table 1). We then attempted to make our own chamber based on the format of the Millicell-HATF in order to cut down this leakage. A membrane was attached to one end of plastic tubing cut into sections of comparable length to the Millicell-HA, and plastic feet were added as spacers.

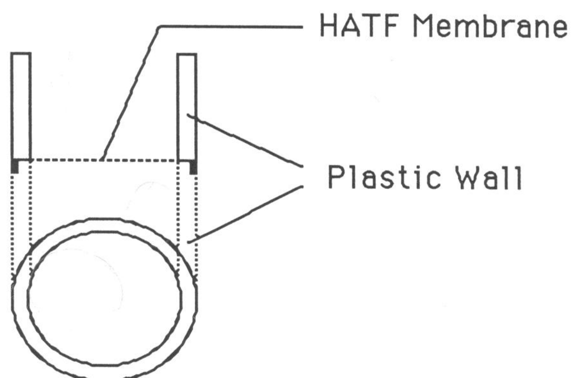
Figure 6 shows the results of a carboxyfluorescein flux study, in which Millicell-HA type chambers with dialysis tubing as membrane were used in a two compartment system. Although it was shown in the phospholipid flux study that almost none of the liposomes (2 %) leaked through the membrane in 3

Figure 3

Idealised two compartment system for growth inhibition assay. Two cell lines are grown in separate compartments separated from each other by a membrane. The membrane must only allow free drugs to pass through while retaining the liposomes in the compartment where they are originally placed.


Membrane
Liposomes
Leaked
Drug
Cell Population 1
(Directly Exposed)
Cell Population 2
(Indirectly Exposed)

A - Millipore Millicell Chamber



B - Millicelll in Well

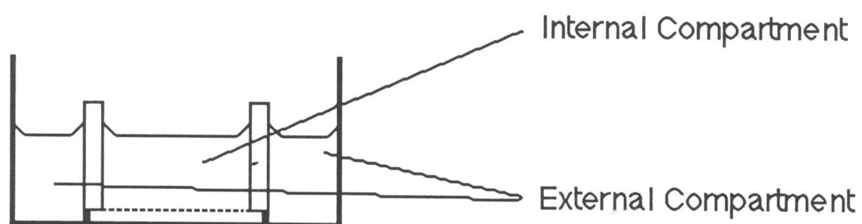


Figure 4. Millipore Millicell chamber, and Millicell in well.

Figure 5

Carboxyfluorescein absorbance vs. time for a two compartment system. 0.4 ml of 1 mM carboxyfluorescein was added to Millicell HATF chambers. The amount of carboxyfluorescein that diffused across the membrane of the chambers into the wells that contained 0.6 ml HEPES buffer was determined by the absorbance at 493 nm using a molar extinction coefficient of 70,000.

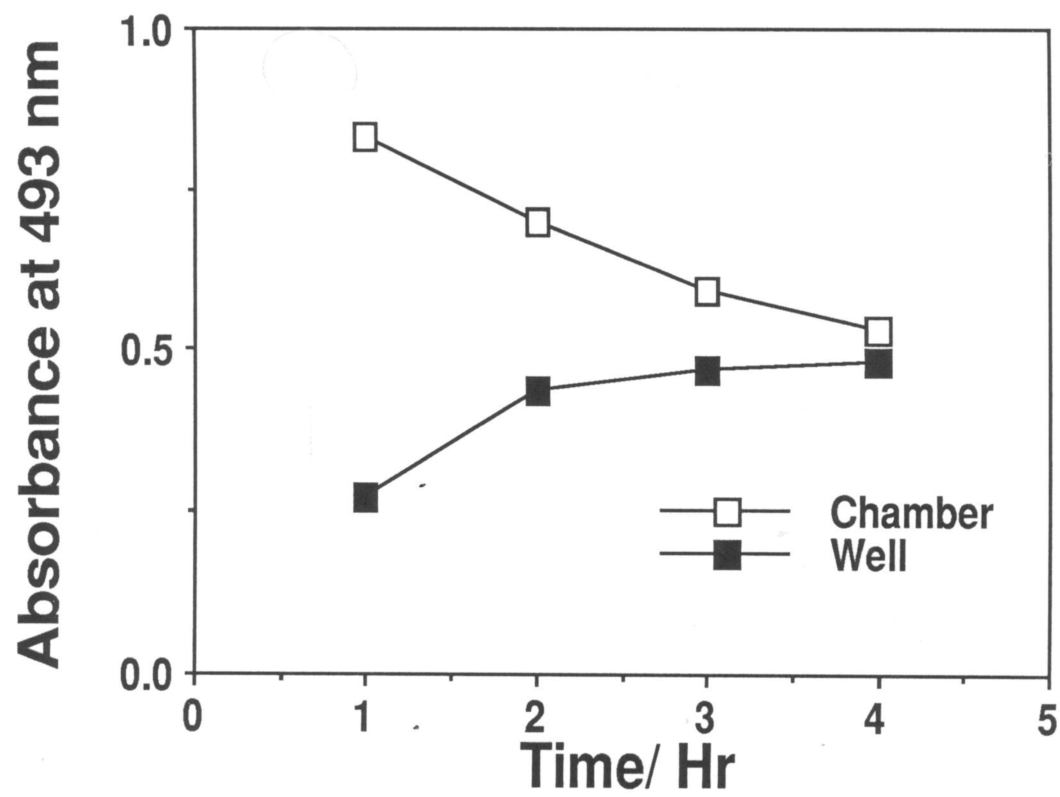

1.0
0.5
0.0
Absorbance at 493 nm
0
1
2
3
4
5
Time/ Hr
Chamber
Well

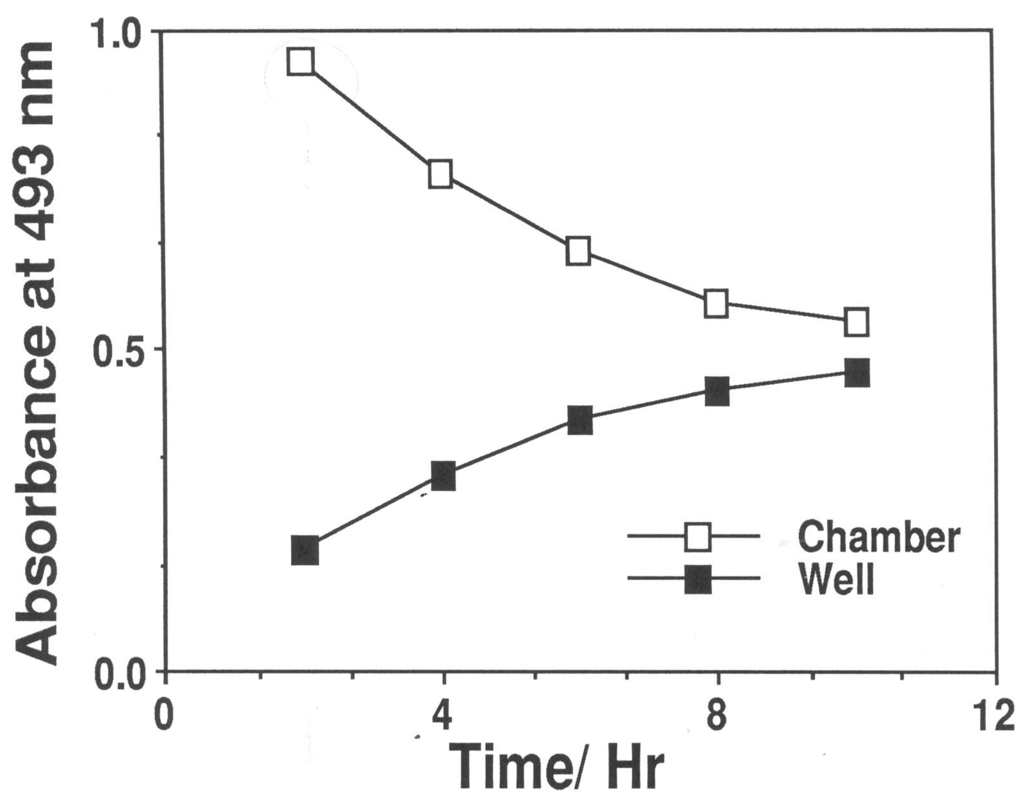
Table 1: THE RETENTION OF LIPOSOMES IN CHAMBER VS. TIME

Time (Hr)	% Phospholipid retained in chamber		
	Membrane type		
	HATF	Dialysis tubing	Polycarbonate (0.1 μm pore size)
48	70	98	97.6

Three types of chambers were compared: (1) Millcell-HA with HATF membrane; (2) a chamber of the form of Millcell-HA, but with dialysis tubing as membrane; and (3) a chamber of the form of Millcell-HA, but with 0.1 μm pore size polycarbonate membrane. Nonloaded phosphatidylglycerol:cholesterol 67:33 liposomes were prepared by reverse-phase evaporation. Liposomes at a concentration of 1.0 μmole phospholipid/0.15 ml were added to the chambers placed in the wells, which contained 0.7 ml of HEPES buffer. The two compartment system was incubated at 37° C with shaking. The percent of liposomes retained in the chamber by the membrane with respect to time was determined by phosphorus analysis.

Figure 6

Carboxyfluorescein absorbance vs. time for a two compartment system. 0.4 ml of 1 mM carboxyfluorescein was added to Millicell-HA type with dialysis tubing as membranes. The amount of carboxyfluorescein that diffused across the membrane of the chambers into the wells that contained 0.6 ml HEPES buffer was determined by the absorbance at 493 nm using a molar extinction coefficient of 70,000.


1.0
0.5
0.0
Absorbance at 493 nm
0
4
8
12
Time/ Hr
Chamber
Well

days of incubation (Table 1), carboxyfluorescein still not reached the same concentration in the well and the chamber after 10 hours.

Figure 7 shows the results of a carboxyfluorescein flux study, in which Millicell-HA type chambers with Nuclepore (Berkeley, CA) 0.1 μm pore size polycarbonate membranes were used in a two compartment study. There was rapid carboxyfluorescein equilibrium between the two compartments, and only 2.4% of liposomes leaked through the membrane in 3 days of incubation (Table 1). Based upon these findings, we used these chambers in a growth inhibition study. However, these chambers were unsuitable, because damage was done to the adherent cells by the chamber's feet which scraped the cells from the bottom of the wells as the plate was agitated. Subsequently, the Costar Transwell became available on the market. Damage to the adherent cells, owing to abrasion between the chamber's feet and the well, is completely eliminated with the Transwell chamber because the Transwell chamber is suspended from the top of the well (see figure 8). The Costar Transwell is a plastic chamber that may be placed in the wells of a 24 well plate. The lower surface is a polycarbonate membrane that can allow the passage of molecules, while retaining particles such as liposomes to an extent that depends on the pore size of the polycarbonate membrane and the liposome size.

Figure 9 & 10 show the results of a carboxyfluorescein flux study, in which 0.1 μm pore size Transwells were used in a two compartment system. When

Figure 7

Carboxyfluorescein absorbance vs. time for a two compartment system. 0.4 ml of 1 mM carboxyfluorescein was added to Millicell-HA type chambers with Nuclepore 0.1 μm pore size polycarbonate membrane as membranes. The amount of carboxyfluorescein that diffused across the membrane of the chambers into the wells that contained 0.6 ml HEPES buffer was determined by the absorbance at 493 nm using a molar extinction coefficient of 70,000.

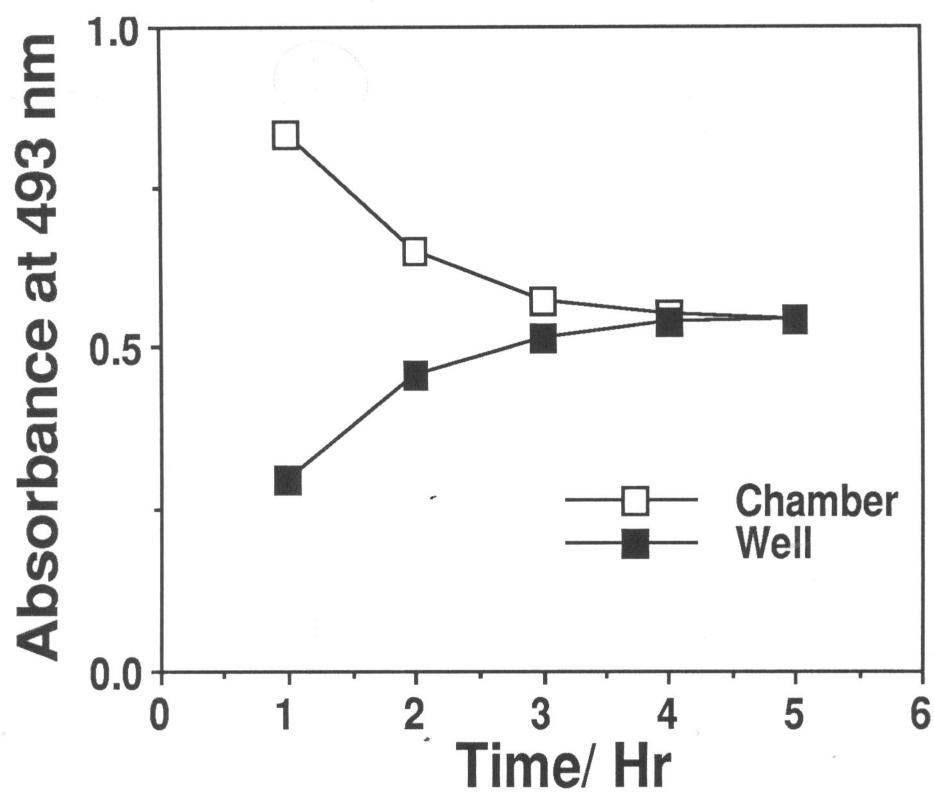
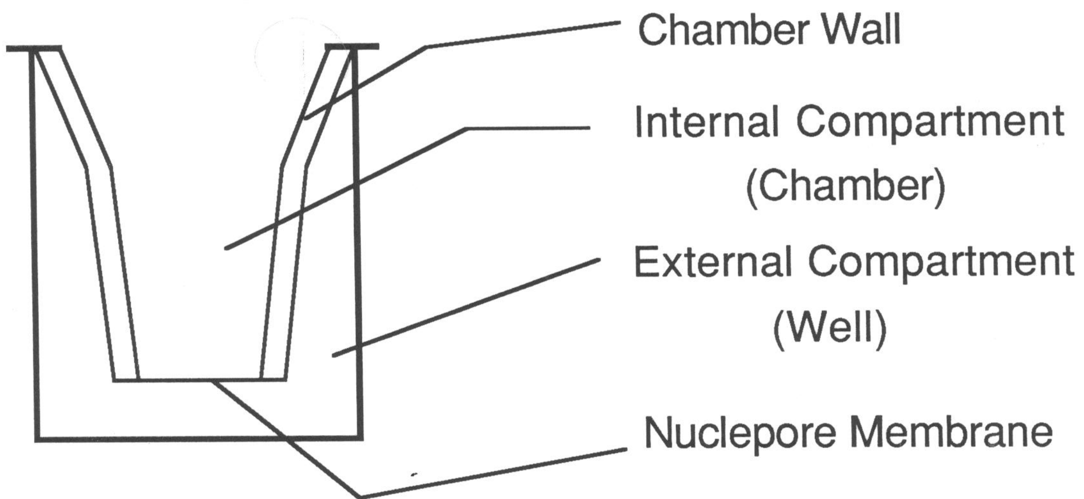

Absorbance at 493 nm
1.0
0.5
0.0
0
1
2
3
4
5
6
Time/ Hr
Chamber
Well

Figure 8

The two compartment growth inhibition assay. The two compartment system is formed by suspending the Costar Transwell in the well of a Costar 24 well plate. The chamber and well of the two compartment system are separated from each other by a 0.1 μm pore size polycarbonate membrane, which allows free drug to diffuse rapidly from one compartment to the other, and does not allow liposomes to diffuse through. CV1-P cells are grown on the bottom surface of the well, and EL4 cells are grown in suspension in the Transwell chamber.


Chamber Wall
Internal Compartment
(Chamber)
External Compartment
(Well)
Nuclepore Membrane

0.15 ml of 1 mM carboxyfluorescein solution was added to the chambers, it took the carboxyfluorescein approximately 4.5 hours to reach the same concentration in the two compartments (figure 9). Similar results were obtained when 0.7 ml of 0.1 mM carboxyfluorescein was added to the wells (figure 10).

Table 2 shows the results of a phospholipid flux experiment in which 0.1 μm pore size Transwells were used in a two compartment system. Using either phosphatidylglycerol or distearoylphosphatidylglycerol empty liposomes prepared by the REV method, we found that less than 5% of the phospholipid diffused into the well in 72 hours for both liposome preparations. This amount of phosphate is neglible, and close to the limits of the assay used. Therefore, we can conclude that a negligible amount of liposomal lipid diffused into the wells in 3 days. In conclusion, decision was made to use custom purchased Costar Transwell as chamber in our two compartment growth inhibition assay.

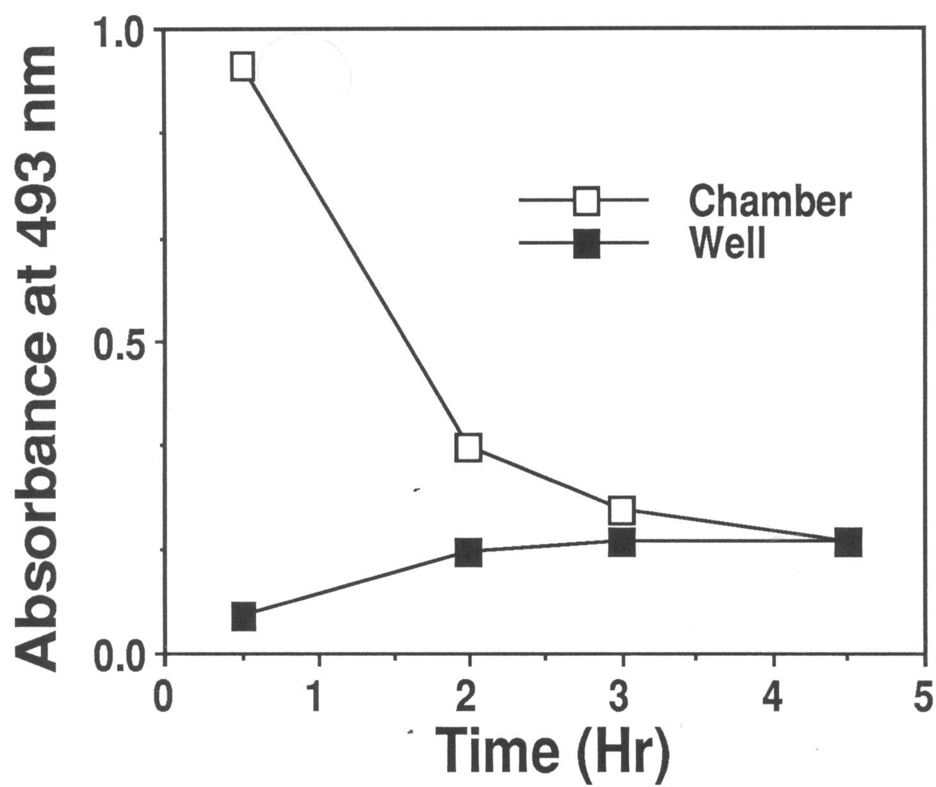
B. Growth Inhibition Assay

1. Free and phosphatidylglycerol encapsulated methotrexate and methotrexate-γ-aspartate

Table 3 shows the results of one and two compartment studies using free and phosphatidylglycerol encapsulated methotrexate. Free methotrexate is a more potent inhibitor of EL4 growth than it is an inhibitor of CV1-P growth.

Figure 9

Carboxyfluorescein absorbance vs. time for a two compartment system. 0.15 ml of 1 mM carboxyfluorescein was added to the Costar Transwell chambers. The amount of carboxyfluorescein that diffused across the membrane of the chambers into the wells that contained 0.7 ml HEPES buffer was determined by the absorbance at 493 nm using a molar extinction coefficient of 70,000.



1.0
0.5
0.0
Absorbance at 493 nm
0
1
2
3
4
5
Time (Hr)
Chamber
Well

Figure 10

Carboxyfluorescein absorbance vs. time for a two compartment system. 0.70 ml of 1 mM carboxyfluorescein was added to the wells. The amount of carboxyfluorescein that diffused across the membrane from the wells into the chambers that contained 0.15 ml HEPES buffer was determined by the absorbance at 493 nm using a molar extinction coefficient of 70,000.

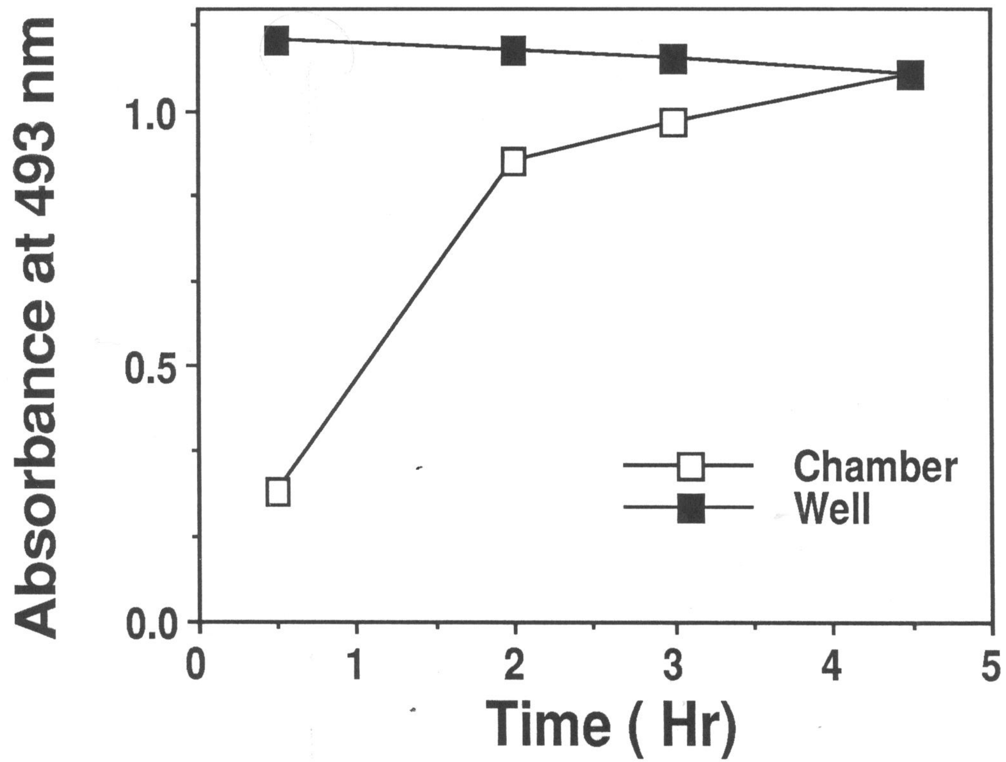

Absorbance at 493 nm
1.0
0.5
0.0
0
1
2
3
4
5
Time (Hr)
Chamber
Well

Table 2: THE RETENTION OF LIPOSOMES IN 0.1 μm TRANSWELL CHAMBERS

Time/Hr	% Phospholipid retained in chamber	
	Phosphatidylglycerol	Distearoylphosphatidylglycerol
24	95.9	100
48	94.4	96.9
72	94.6	94.8

Nonloaded phosphatidylglycerol:cholesterol 67:33 and distearoylphosphatidylglycerol:cholesterol 67:33 liposomes were prepared by reverse-phase evaporation. Liposomes at a concentration of 1.0 μmole phospholipid/0.15 ml were added to the chambers suspended from the in wells, which contained 0.7 ml of HEPES buffer. The two compartment system was incubated at 37° C with shaking. The percent of liposomes retained in the chamber by the membrane with respect to time was determined by phosphorus analysis.

Table 3: GROWTH INHIBITORY POTENCY OF FREE AND PHOSPHATIDYLGLYCEROL ENCAPSULATED METHOTREXATE

Cell type	IC_{50} (μM)							
	One compartment system				Two compartment system			
	free drug	n[a]	encaps	n	free drug	n	encaps	n
CV1-P	-		-		0.060±0.02	(5)	0.013±0.004	(2)
EL4	0.019±0.00	(3)	0.026±0.004	(2)	0.011±0.0007	(5)	0.011±0.00	(2)

Methotrexate was encapsulated in phosphatidylglycerol:cholesterol (67:33) liposomes prepared by reverse-phase evaporation. The final drug:lipid ratio was 122 mmol/mol. Free or encapsulated drug was added to the wells, and cells were allowed to grow for 3 days before counting. [a] n represents the number of determinations. The value given is the mean of all determinations ± the standard deviation.

Moreover, methotrexate is a more potent inhibitor of EL4 growth in a two compartment system than it is in a one compartment system. The reasons for this are unclear, but this observation suggests that the value obtained for the free drug in a two compartment system will be the most appropriate for comparison with values obtained for encapsulated drug. Encapsulated methotrexate is 4.6 times more potent than free methotrexate for growth inhibition of CV1-P cells. Encapsulated methotrexate has an IC_{50} identical to that of free methotrexate for EL4 cells. This is consistent with the hypothesis that the contents of the liposomes undergo virtually complete leakage within a short time of addition to the well.

Table 4 shows the results of one and two compartment studies using free and phosphatidylglycerol encapsulated methotrexate-γ-aspartate. The potency of free methotrexate-γ-aspartate is significantly lower on EL4 cells than it is on CV1- P cells. Moreover, free methotrexate-γ-aspartate is more potent for EL4 cells in the two compartment system than it is in the one compartment system. These observations are very similar to comparable observations with methotrexate. Again, the reasons are unclear, but it is obviously necessary to use values obtained in the two-compartment system for comparison with the encapsulated drug. Encapsulated methotrexate-γ-aspartate is 109 times more potent than free methotrexate-γ-aspartate for growth inhibition of CV1-P cells.

Table 4: GROWTH INHIBITORY POTENCY OF FREE AND PHOSPHATIDYLGLYCEROL ENCAPSULATED METHOTREXATE-γ-ASPARTATE

Cell type	IC_{50} (μM)							
	One compartment system				Two compartment system			
	free drug	n[a]	encaps	n	free drug	n	encaps	n
CV1-P	-		-		1.41±0.58	(9)	0.013±0.007	(7)
EL4	0.45±0.25	(5)	0.28±0.0	(2)	0.33±0.08	(5)	0.28±0.02	(3)

Methotrexate-γ-aspartate was encapsulated in phosphatidylglycerol:cholesterol (67:33) liposomes prepared by reverse-phase evaporation. The final drug:lipid ratio was 125 mmol/mol. Free or encapsulated drug was added to the wells, and cells were allowed to grow for 3 days before counting. [a] n represents the number of determinations. The value given is the mean of all determinations ± the standard deviation.

This is consistent with all previous observations that have demonstrated this drug to be liposome dependent [89,94]. Encapsulated methotrexate-γ-aspartate is slightly more potent than free methotrexate-γ-aspartate for growth inhibition of EL4 cells, although statistical analysis demonstrates that these values are not significantly different. The similarity of these values suggests that a rapid and extensive loss of liposome contents occurs shortly after addition to the medium.

2. Free and Distearoylphosphatidylglycerol encapsulated methotrexate and methotrexate-γ–aspartate

Table 5 shows the results of one and two compartment studies using free and distearoylphosphatidylglycerol encapsulated methotrexate. Encapsulated methotrexate is 3 times more potent than free methotrexate for growth inhibition of CV1-P cells. However, encapsulated methotrexate is about 2.4 times less potent than free methotrexate for growth inhibition of EL4 cells. This value is consistent with the hypothesis that approximately 42% of the liposome contents leak shortly after addition to the medium. This is in contrast to the apparently complete leakage that occurred when phosphatidylglycerol was used to encapsulate the drug.

Table 6 shows the results of one and two compartment studies using free and distearoylphosphatidylglycerol encapsulated methotrexate-γ-aspartate.

Table 5: GROWTH INHIBITORY POTENCY OF FREE AND DISTEAROYLPHOSPHATIDYLGLYCEROL ENCAPSULATED METHOTREXATE

Cell type	IC_{50} (μM)							
	One compartment system				Two compartment system			
	free drug	n[a]	encaps	n	free drug	n	encaps	n
CV1-P	-		-		0.060±0.02	(5)	0.020±0.009	(3)
EL4	0.019±0.00	(3)	0.026±0.004	(2)	0.011±0.0007	(5)	0.026±0.006	(5)

Methotrexate was encapsulated in distearoylphosphatidylglycerol:cholesterol (67:33) liposomes prepared by reverse-phase evaporation. The final drug:lipid ratio was 30 mmol/mol. Free or encapsulated drug was added to the wells, and cells were allowed to grow for 3 days before counting. [a] n represents the number of determinations. The value given is the mean of all determinations ± the standard deviation.

Table 6: GROWTH INHIBITORY POTENCY OF FREE AND DISTEAROYLPHOSPHATIDYLGLYCEROL ENCAPSULATED METHOTREXATE-γ-ASPARTATE

Cell type	IC_{50} (μM)							
	One compartment system				Two compartment system			
	free drug	n[a]	encaps	n	free drug	n	encaps	n
CV1-P	-		-		1.41±0.59	(9)	0.015±0.008	(3)
EL4	0.45±0.25	(5)	0.28±0.0	(2)	0.33±0.08	(5)	0.28±0.06	(3)

Methotrexate-γ-aspartate was encapsulated in distearoylphosphatidylglycerol:cholesterol (67:33) liposomes prepared by reverse-phase evaporation. The final drug:lipid ratio was 56 mmol/mol . Free or encapsulated drug was added to the wells, and cells were allowed to grow for 3 days before counting. [a] n represents the number of determinations. The value given is the mean of all determinations ± the standard deviation.

Encapsulated methotrexate-γ-aspartate is 94 times more potent than free methotrexate-γ-aspartate for growth inhibition of CV1-P cells. This is consistent with all previous observations that have demonstrated this drug to be liposome dependent [89,94]. Distearoylphosphatidylglycerol encapsulated methotrexate-γ-aspartate is slightly more potent than free methotrexate-γ-aspartate for growth inhibition of EL4 cells. Statistical analysis demonstrates that the growth inhibitory potency of distearoylphosphatidylglycerol encapsulated methotrexate-γ-aspartate and free methotrexate-γ-aspartate are not significantly different. The similarity of these values suggests either a rapid and extensive loss of liposome contents shortly after addition to the medium, or a small extent of conversion of the drug to a more potent form, presumably methotrexate.

3. Serum Induction versus Cellular Induction: Two Compartment System in the Absence of CV1-P cells

Table 7 shows the results of two compartment studies in the absence of CV1-P cells using free and phosphatidylglycerol encapsulated methotrexate and methotrexate-γ-aspartate. Both free methotrexate and methotrexate-γ-aspartate

Table 7: THE EFFECT OF CV1-P ABSENCE ON THE GROWTH INHIBITORY POTENCY OF FREE AND PHOSPHATIDYLGLYCEROL ENCAPSULATED METHOTREXATE AND METHOTREXATE-γ-ASPARTATE USING A TWO COMPARTMENT SYSTEM

Cell type	IC_{50} (μM)							
	Methotrexate				Methotrexate-γ-aspartate			
	free drug	n[a]	encaps	n	free drug	n	encaps	n
EL4	0.0111±0.006	(5)	0.0117±0.005	(4)	0.32±0.12	(5)	0.33±0.14	(4)

The drugs were encapsulated in phosphatidylglycerol:cholesterol (67:33) liposomes prepared by reverse-phase evaporation. The final drug:lipid ratio was 122 mmol/mol for methotrexate and 125 mmole/mole for methotrexate-γ-aspartate. Free or encapsulated drug was added to the wells, which contained no CV1-P cells. EL4 cells in the chambers were allowed to grow for 3 days before counting. [a] n represents the number of determinations. The value given is the mean of all determinations ± the standard deviation.

are slightly more potent than encapsulated drugs for growth inhibition of EL4 cells. The similarity of these values demonstrates a rapid and extensive loss of liposome contents (95% and 97% leakage) shortly after addition to the medium. This implies that the serum and growth medium may be responsible for most of the leakage seen in our growth inhibition studies.

Table 8 shows the results of two compartment studies in the absence of CV1-P cells using free and distearoylphosphatidylglycerol encapsulated methotrexate and methotrexate-γ-aspartate. Both free methotrexate and methotrexate-γ-aspartate are more potent than encapsulated drugs for growth inhibition of EL4 cells, suggesting that most of the contents are still retained in the liposomes. If the IC_{50} of free drug is compared with that of encapsulated drug for both methotrexate and methotrexate-γ-aspartate, we can infer that leakage is 35% for both methotrexate and methotrexate-γ-aspartate. When we compare this apparent leakage value for methotrexate with the apparent leakage value obtained for methotrexate in the presence of CV1-P cells, we see a 7% difference between them, suggesting that serum in the growth medium may be responsible for 35% of the leakage, while CV1-P cells cause the residual 7% leakage. However, statistical analysis demonstrates that the difference caused by the presence of CV1-P cells is not significant. Similar comparison of the leakage value for methotrexate-γ-aspartate with the leakage

Table 8: THE EFFECT OF CV1-P ABSENCE ON THE GROWTH INHIBITORY POTENCY OF FREE AND DISTEAROYLPHOSPHATIDYLGLYCEROL ENCAPSULATED METHOTREXATE AND METHOTREXATE-γ-ASPARTATE USING A TWO COMPARTMENT SYSTEM

Cell type	IC_{50} (μM)							
	Methotrexate				Methotrexate-γ-aspartate			
	free drug	n[a]	encaps	n	free drug	n	encaps	n
EL4	0.0110±0.006	(5)	0.031±0.007	(3)	0.32±0.12	(5)	0.91±0.13	(4)

The drugs were encapsulated in distearoylphosphatidylglycerol:cholesterol (67:33) liposomes prepared by reverse-phase evaporation. The final drug:lipid ratio was 30 mmol/mol for methotrexate and 56 mmole/mole for methotrexate-γ-aspartate. Free or encapsulated drug was added to the wells, which contained no CV1-P cells. EL4 cells in the chambers were allowed to grow for 3 days before counting. [a] n represents the number of determinations. The value given is the mean of all determinations ± the standard deviation.

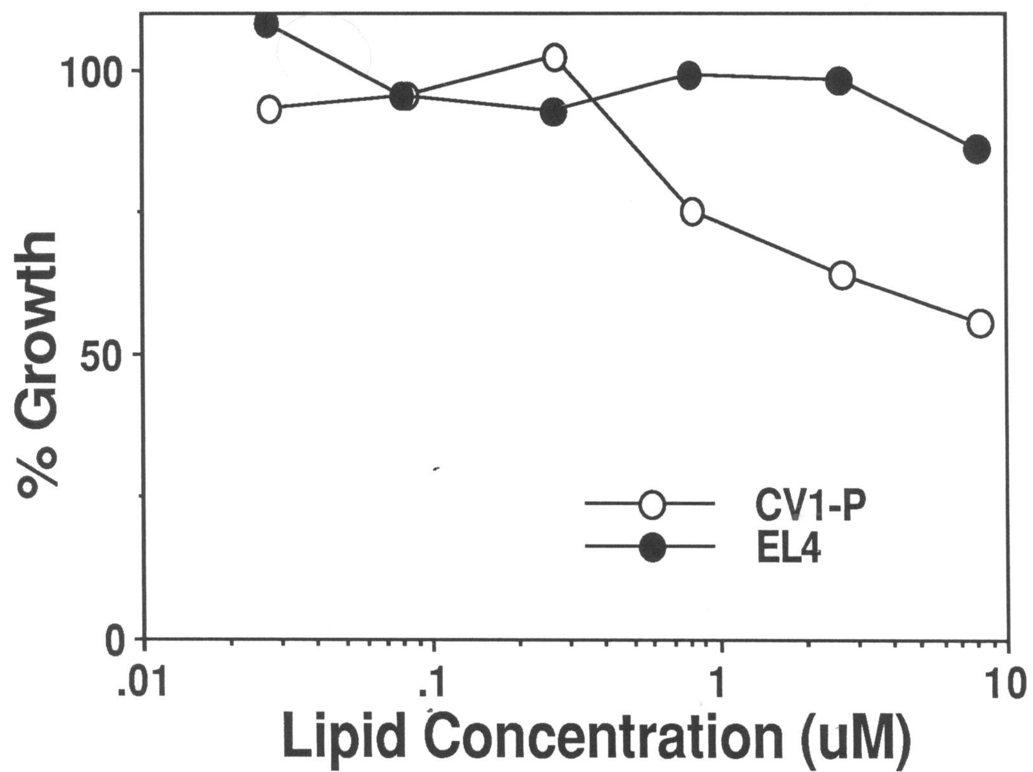
value of methotrexate-γ-aspartate obtained in the presence of CV1-P cells reveals that the leakage value for this drug is apparently increased by 83% to 118% in the presence of CV1-P cells. Statistical analysis demonstrates that this apparent increase in leakage induced by the presence of CV1-P cells is significant ($p < 0.05$). Since methotrexate-γ-aspartate should leak no more than methotrexate, it is more reasonable to suggest that methotrexate-γ-aspartate is partially metabolized to a more potent form, presumably methotrexate.

4. Growth inhibitory effects of non-loaded liposomes

It has been demonstrated that empty liposomes may inhibit the growth of some cell lines [89]. In order to exclude the possibility that lipid metabolites produced by CV1-P cells may inhibit EL4 cell growth in a two compartment system, non-loaded liposomes, at the lipid concentrations used when cells were treated with encapsulated methotrexate and methotrexate-γ-aspartate, were added to the wells where CV1-P cells were located. The growth inhibition of both CV1-P and EL4 cells was then followed. Figure 11 shows that there is no inhibition on the growth of EL4 cells caused by the addition of empty phosphatidylglycerol liposomes to the wells. However, at the highest phosphatidylglycerol lipid concentrations, there was inhibition on CV1-P cell

Figure 11

The effect of nonloaded phosphatidylglycerol liposomes on cell growth. Nonloaded phosphatidylglycerol liposomes, at the lipid concentrations when cells were treated with encapsulated methotrexate and methotrexate-g-aspartate, were added to the wells where CV1-P cells were located. EL4 cells in the chambers and CV1-P cells in the wells were allowed to grow for 3 days before counting.

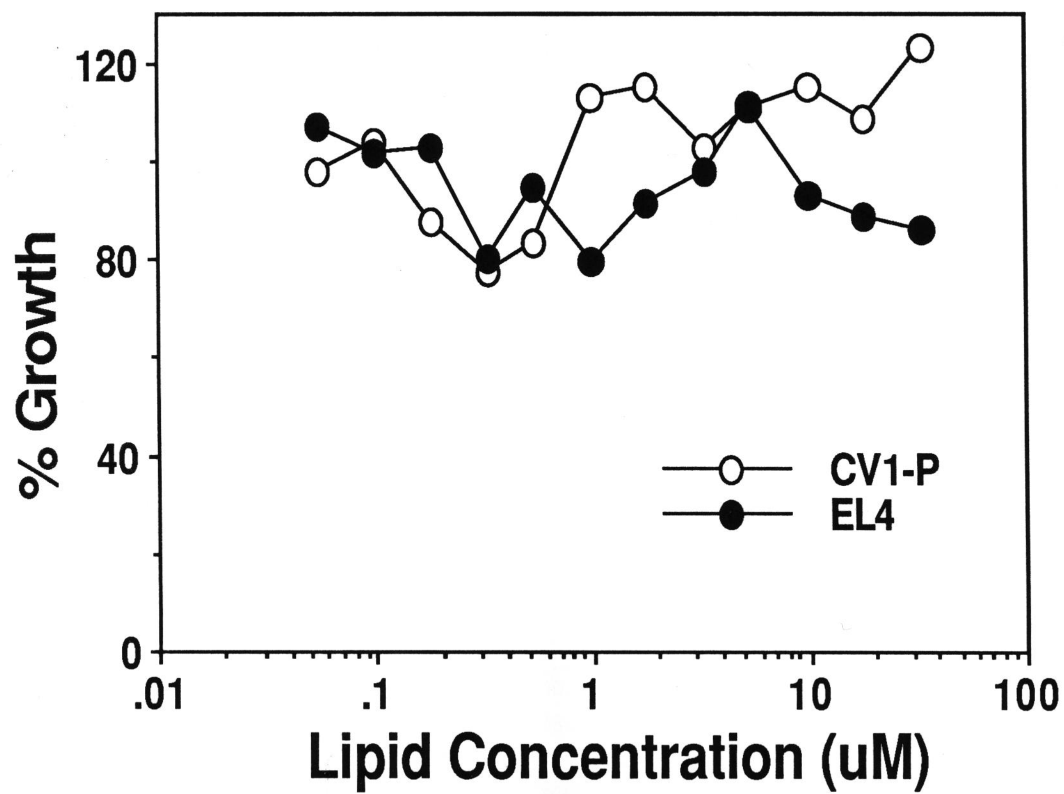

% Growth
100
50
0
.01
.1
1
10
Lipid Concentration (uM)
CV1-P
EL4

growth. Previously we have demonstrated that phosphatidylglycerol liposomes were quite growth inhibitory for CV1-P cells [89]. Despite these effects of phosphatidylglycerol on CV1-P cells , the effects of encapsulated methotrexate and methotrexate-γ-aspartate occur at much lower lipid concentrations. Therefore, we can say that the effects of the encapsulated drugs is entirely caused by the drug.

Figure 12 shows the effects of non-loaded distearoylphosphatidyglycerol liposomes on cell growth. There is no effect of this lipid on either cell line with the range of lipid concentrations used in drug delivery studies.

Figure 12

The effect of nonloaded distearoylphosphatidylglycerol liposomes on cell growth. Nonloaded distearoylphosphatidylglycerol liposomes, at the lipid concentrations when cells were treated with encapsulated methotrexate and methotrexate-g-aspartate, were added to the wells where CV1-P cells were located. EL4 cells in the chambers and CV1-P cells in the wells were allowed to grow for 3 days before counting.



% Growth
120
80
40
0
.01
.1
1
10
100
Lipid Concentration (uM)
CV1-P
EL4

V. DISCUSSION

A simple method to study the leakage, metabolism and delivery of liposome dependent drugs under cell culture conditions, and at drug concentrations that are relevant to drug delivery has been described. Methotrexate and methotrexate-γ-aspartate encapsulated in phosphatidylglycerol liposomes appear to leak rapidly and almost completely. In contrast, these two drugs encapsulated in distearoylphosphatidylglycerol liposomes leak far less extensively. This observation is consistent with the known effect of phospholipid phase transition temperature on the stability of liposomes in the presence of serum [54].

Some of the data obtained in this research suggest that the target cells (CV1-P) may partially degrade encapsulated methotrexate-γ-aspartate to methotrexate. The strongest evidence for this phenomenon was obtained with distearoylphosphatidylglycerol liposomes. When distearoylphosphatidylglycerol liposomes were used, the IC_{50} on EL4 cells for encapsulated methotrexate and for encapsulated methotrexate-γ-aspartate in the absence of CV1-P cells suggest that approximately 35% leakage occurs from those liposomes. Encapsulated methotrexate-γ-aspartate in the presence of CV1-P cells gives an

IC_{50} on EL4 cells that is slightly lower than that of free methotrexate-γ-aspartate. Although this value is not significantly different from that of free methotrexate-γ-aspartate, it is significantly lower than the two compartment IC_{50} of encapsulated methotrexate-γ-aspartate on EL4 cells in the absence of CV1-P cells. Moreover, there is no evidence from the companion studies with distearoylphosphatidylglycerol encapsulated methotrexate to suggest that CV1-P cells processing can increase leakage from 35% to 100%. Consequently, it is concluded that distearoylphosphatidylglycerol encapsulated methotrexate-γ-aspartate is partly metabolised and released into the medium as methotrexate.

A simple way of comparing IC_{50} values in order to estimate leakage has been described above. If methotrexate-γ-aspartate is assumed to be converted in the CV1-P cells to methotrexate, that it is released into the medium by the CV1-P cells, that the conversion is rapid, and that sufficient methotrexate is produced to be the sole cause of EL4 growth inhibition, then the following equation can be used to estimate the extent of metabolism:-

$$\text{Apparent \% Metabolism} = \frac{IC_{50} \text{ of free Methotrexate}}{IC_{50} \text{ of encapsulated Methotrexate-}\gamma\text{-aspartate}} \times 100$$

Using this equation, it was calculated that approximately 4% of the encapsulated methotrexate-γ-aspartate is metabolised by the CV1-P cells to methotrexate, and released into the medium.

A number of pieces of evidence support the possibility that methotrexate-γ-aspartate is metabolised to methotrexate. Previously, it has been demonstrated that liposome-mediated drug delivery involves lysosomal processing of the liposomes and their contents [89,94]. Therefore, the drug is likely to be exposed to the lysosomal degradative enzymes. It has been found that hydrolysis of pteroylpolyglutamates is catalyzed by enzymes that possess peptidase like activity and are referred to γ-glutamyl hydrolases [100]. The majority of the activity of this enzyme is found associated with the lysosomal fraction after differential centrifugation of cell homogenates [101-105]. The hydrolase is nonspecific with regard to the pteridine or pteridine like moiety [106-110], although linkage through the γ- rather than α-carboxyl of of glutamate is essential [109,111-113]. Baugh et al [113] have demonstrated that this enzyme can remove any amino acid from the γ-carboxyl of pteridines, because non-glutamate C-terminal amino acids can all serve as substrates. Consequently, one can say that this enzyme is capable of converting methotrexate-γ-aspartate to methotrexate.

In spite of the extensive leakage of phosphatidylglycerol encapsulated

methotrexate-γ-aspartate, and its partial conversion to methotrexate, by far the greatest effects are seen on the target CV1-P cells. On this cell line, phosphatidylglycerol encapsulated methotrexate-γ-aspartate is 109 times more potent than free methotrexate-γ-aspartate. However, on the EL4 cell line, the encapsulated drug is only 1.2 times more potent than the free drug. It seems remarkable that the potency of the encapsulated drug should be so much increased despite the loss of all but a small fraction, perhaps only 1-2%, of the encapsulated contents. In previous studies, in which CV1-P cells were exposed to encapsulated methotrexate-γ-aspartate for 1-72 hours [94], Heath et al. established that the IC_{50} of encapsulated drug is quite dependent on exposure length. This implies that the small amount of drug retained by the liposomes is largely retained for the entire 72 hours of the growth inhibition assays implying that leakage in the culture medium is both rapid and transient.

Distearoylphosphatidylglycerol liposomes leak far less than phosphatidylglycerol liposomes. If one compare them in terms of how much drug they retain, distearoylphosphatidylglycerol retains about 58% of encapsulated contents, and phosphatidylglycerol no more than 1-2%. If drug delivery efficiency were directly related to retention, one might expect drug in distearoylphosphatidylglycerol liposomes to be 29-58 times more potent than drug in phosphatidylglycerol liposomes. For delivery of fluoroorotic acid [114],

this is indeed what has been observed, suggesting that liposome stability can affect the efficiency with which fluoroorotic acid is delivered. However, the potency of methotrexate-γ-aspartate is quite comparable when encapsulated in liposomes of either composition. The reasons for this are unclear, but it seems possible that liposomes resistant to serum induced leakage may also be resistant to lysosomal degradation. Consequently, they may release their contents more slowly. How this may affect drug potency, and why it affects one drug and not another will require further investigation.

Thus far, the effects of CV1-P cells metabolizing methotrexate-γ-aspartate on non-target cell populations have been discussed. It is also appropriate to consider how the metabolism of methotrexate-γ-aspartate may be involved in its delivery to CV1-P cells themselves. Previously, Heath et al. [63] speculated on the mechanism, by which methotrexate-γ-aspartate is transferred from the lysosomal compartment to the cytoplasm of the cell. One possibilty suggested was that the methotrexate-γ-aspartate might be degraded to methotrexate, and transported across the lysosomal membrane by the folate transport system. The present observations seem to suggest that metabolism does occur, confirm that this mechanism may be important. There is a second reason why conversion of methotrexate-γ-aspartate may be important for full expression of its potency. The retention of antifolates in cells is known to occur through their

polyglutamylation in the cytoplasm [115,116]. Methotrexate-γ-aspartate cannot be polyglutamylated [117,118], and its retention and potency may depend on it first being converted to the methotrexate. However, knowledge on what fraction of the lysosomally processed methotrexate-γ-aspartate is converted to methotrexate is not attainable at this phase of the research. However, it currently appears possible that methotrexate-γ-aspartate may be a lysosomally cleaved pro-drug of methotrexate.

VI. CONCLUSION

A simple two compartment system that can be used for growth inhibition assay has been developed and vaildated. This two compartment system has been proven useful in obtaining information about drug leakage, metabolism, and delivery under cell culture conditions, and at drug concentrations that are relevant to drug delivery. There is close to 100 % leakage when methotrexate is encapsulated in phosphatidylglycerol:cholesterol (67:33) liposomes, but only 42 % when encapsulated in distearoylphosphatidylglycerol:cholesterol (67:33) liposomes, and serum is found to be responsible for causing most of the leakage seen in both liposomes. Also demonstrated in this study is the metabolism of methotrexate-γ-aspartate by CV1-P cells. It is the hope in future work to study these parameters in more detail, and to examine other liposome-dependent drugs.

VII. REFERENCES

1 Bangham, A. D. and Horne, R. W. (1964) J. Mol. Biol. 8, 660.

2 Bangham, A. D., Standish, M. M. and Watkins, J. C. (1965) J. Mol. Biol. 13, 238.

3 Bangham, A. D. (1978) Ann. N. Y. Acad. Sci. 308, 2-7.

4 Melhorn, R. and Keith, A. D. (1972) in Membrane Molecular Biology (Fox, C. E. and Keith, A.D., eds.) Sinauer Association.

5 Smith, I. C. P., Tullock, A. P., Stockton, G. W., Schrier, S., Joyce A., Butler, K., Boulanger, Y., Blackwell, B. and Bennett, L. G. (1978) Ann. N. Y. Acad. Sci. 308, 8-28.

6 Radda, G. K. and Vanderkooi, J. (1972) Biochim. Biophys. Acta 55, 224-230.

7 Ladbrooke, B. D., and Chapman, D. (1969) Chem. Phys. Lipid 3, 304-357.

8 Cronan, J. E. and Gelman, E. P. (1975) Bact. Rev. 34, 232-256.

9 Jost, P., Griffith, O. H., Capaldi, A. A. and Vanderkooi, G. (1973) Proc. Natl. Acad. Sci. 70, 480-484.

10 Kimelberg, H. K. and Papahadjopoulos, D. (1974) J. Biol. Chem. 249, 1071-1080.

11 Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. and Metcalf, J.

C. (1974) Proc. Natl. Acad. Sci. 71, 622-626.

12 Rogers, M. J. and Stittmater, P. (1973) J. Biol. Chem. 248, 800.

13 Kasahara, M. and Hinckle, P. C. (1976) Proc, Natl. Acad. Sci. 73, 396-400.

14 Rothstein, A., Cabantchik, Z. I., Balshin, M. and Juliano, R. (1975) Biochim. Biophys. Res. Comm. 63, 144-150.

15 Cabantchik, Z. I. et al. (1978) Curr. Topics in Membrane and Transport, Vol. 12.

16 Shamoo, A. E. editor (1975) Carriers and Channels in Biological Systems, N. Y. Acad. Sci.

17 Bangham, A. D. (1963) In Advances in Lipid Research, ed. by R. Paoletti & D. Kritchevsky, vol. 1, New York: Academic Press.

18 Haydon, D. A. & Taylor, J. L. (1963) J. Theoret. Biol. 4, 281.

19 Papahadjopoulos, D., Cowden, M. and Kimelberg, H. K. (1973) Biochim.Biophys. Acta 330, 8-26.

20 Lee, A. G. (1977) Biochim. Biophys. Acta 472, 237-281.

21 Chapman, D. (1975) Quart. Rev. Biophys. 8, 185-235.

22 Bangham, A. D., Hill, M. W. and Miller, N. G. A. (1974) Methods in Membrane Biology, Vol. 1. Plenum Press, New York, p. 1.

23 Mason, J. T. and Huang, C. (1978) Ann. N. Y. Acad. Sci. 308, 29-48.

24 Huang, A. et al. (1979) Biochem. 18, 5610-5614.

25 Batzri, S. and Korn, E. D. (1973) Biochim. Biophys. Acta 298, 1015-1019.

26 Deamer, D. W. (1978) Ann. N. Y. Acad. Sci. 308, 250-257.

27 Schierer, H., Rudolph, S., Furkelstein, M., Coleman, P. and Weissmann, G. (1978) Biochim. Biophys. Acta 542, 137-153.

28 Szoka, F. C. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194-4198.

29 Hunt, C. A., Burnette, R., MacGregor, R. D., Strubbe, A. E., Lau, D. T., Taylor, N. and Kawada, H. (1985) Science 230, 1165-1168.

30 Olson, F., Hunt, C. A., Szoka, F. C., Vail, W. J. and Papahadjopoulos, D. (1979) Biochimica Biophysica Acta 557, 9-23.

31 Pagano, R. E. and Weinstein, J. N. (1978) Ann. Rev. Biophys. Bioeng. 7, 435-468.

32 Magee, W. E. and Miller, O. V. (1972) Nature 235, 339-341.

33 Huang, L. and Ritchie, A. K. (1978) Ann. N. Y. Acad. Sci. 308, 439.

34 Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R., and Hagens, W. A. (1977) Science 195, 489-491.

35 Blumenthal, R., Weinstein, J. N., Sharrow, S. D. and Henkart, P. (1977) Proc. Natl. Acad. Sci. 74, 4975-4984.

36 Goldstein, J., Anderson, R. G. W. and Brown, S. (1979) Nature 279, 679-685.

37 Pastan, I. H. and Willingham, M. C. (1981) Science 214, 504.

38 Posta, G. and Papahadjopoulos, D. (1978) The influence of vesicle membrane properties on the interaction of lipid vesicles with cultured cells. In Liposomes and Their Uses in Biology and Medicine, Papahadjopoulos, D., ed. (New York: New York Academy of Sciences), pp. 164-184.

39 Poste, G. (1980) in Liposomes in Biological Systems (Gregoriadis, G. and Allison, A., eds), pp. 101-151.

40 Kimelberg, H. K. and Mayhew, E. (1978) Crit. Rev. Toxicol. 6, 25-79.

41 Straubinger, R. M., Hong, K., Friend, D. S. and Papahadjopoulos, D. (1983) Cell 32, 1069-1079.

42 Szoka, F., Magnusson, K-E., Wojcieszyn, J., Hou, Y., Derzko, Z. and Jacobson, K. (1981) Proc. Nat. Acad. Sci. 78, 1685-1689.

43 Uchida, T., Kim, J., Yamaizumi, M., Miyake, Y. and Okada, Y. (1979) J. Cell Biol. 80, 10-20.

44 Huang, R. T. C., Wahn, K., Klenk, H. D. and Rott, R. (1980) Virology 104, 294-302.

45 Vainstein, A., Atidia, J. and Loyter, A. (1981) Reconstituted Sendai virus envelopes as a biological carrier for microinjection of proteins and DNA molecules into animal cells. In liposomes, Drugs and Immunocompetent Cell Functions, Nicolau, C and Paraf, A. eds. (New

York: Academic Press), pp. 95-108.

46 Papahadjopoulos, D., Wilson, T. and Taber, R. (1980) In Vitro 16, 49-54.

47 Fraley, R., Subramani, S., Berg, P. and Papahadjopoulos, D. (1980) J. Biol. Chem. 255, 10431-10435.

48 Fraley, R., Straubinger, R., Rule, G., Springer, E. L. and Papahadjopoulos, D. (1981) Biochem 20, 6978-6987.

49 Ahkong, Q. F., Fisher, D., Tampion, W., and Lucy, J. A. (1975) Nature 253, 194-195.

50 Wilson, T., Papahadjopoulos, D. and Taber, R. (1979) Cell 17, 77-84.

51 Pagano, R. E. and Huang, L. (1975) J. Cell. Biol. 67, 49-60.

52 Gregoriadis, G. (1981) Lancet ii, 241-247.

53 Gregoriadis, G., Senior, J. (1980) FEBS Letters 119, 43-46.

54 Senior, J. and Gregoriadis, G. (1982) Life Sci. 30, 2123-2136.

55 Senior, J., Crawley, J. C. W. and Gregoriadis, G. (19885) Biochimica Biophysica Acta 839, 1-8.

56 Poste, G., Kirsh, R. and Koestler, T. (1983) in Liposome Technology, Vol. 3 (Gregoriadis, G., ed.), pp. 1-28.

57 Kennett, R. H., McKearn, T. J., and Bechtol, K. B., Eds., Monoclonal Antibodies, Plenum Press, N. Y. 1980.

58 Greaves, M., Delia, D., Sutherland, R., Rao, J., Verbi, W., Kemshead, J.,

Hariri, G., Glodstein, G, And Kung, P. (1981) Int. J. Immunopharmacol. 3, 283.

59 Gregoriadis, G. and Neerunjun, D. (1975) Res. Comm. Chem. Path. Pharmacol. 10, 351-352.

60 Magee, W. E., Cromen, J. H. and Thor, D. E. (1978) Cancer Res. 38, 1173-1176.

61 Leserman, L. D., Machy, P., and Barbet, J. (1981) Nature 293, 226-228.

62 Huang, A., Kennel, S, and Huang, L. (1983) J. Biol. Chem. 258, 14034-14040.

63 Heath, T. D., Montgomery, J. A., and Piper, Papahadjopoulos, D. (1983) Proc. Natl. Acad. Sci. USA 80, 1377-1381.

64 Bragman, K. S., Heath, T. D., and Papahadjopoulos, D. (1983) Biochim. Biophys. Acat 730, 187-195.

65 Matthay, K. K., Heath, T. D., and Papahadjopoulos, D. (1984) Cancer Res. 44, 1880-1886.

66 Bragman, K. S., Heath, T. D., and Papahadjopoulos, D. (1984) JNCI. 73, 127-131.

67 Gregoriadis, G., and Buckland, R. A. (1973) Nature 244, 170-172.

68 Gregoriadis, G. (1979) in Drug Carriers in Biology and Medicine (Gregoriadis, G. ed) pp. 287-341, Academic Press, London, New York.

69 Gregoriadis, G. (1973) FEBS Lett. 36, 292-296.

70 Gregoriadis, G., and Davis, C. (1979) Biochim. Biophys. Res. Commun. 89, 1287-1293.

71 Kirby, C., Clarke, J. and Gregoriadis, G. (1980) Biochem. J. 186, 591-598.

72 Krupp, L., Chobanian, A. V. and Brecher, P. I. (1976) Biochim. Biophys. Res.Commun. 72, 1251-1258.

73 Scherphof, G., Roerdink, F., Waite, M. and Parks, J. (1978) Biochim. Biophys. Acta 542, 296-307.

74 Chobanian, J. V., Tall, A. R. and Brecher, P. I. (1979) Biochemistry 18, 180-187.

75 Davis, C. and Gregoriadis, G. (1979) Biochem. Soc. Trans. 7, 680-682.

76 Juliano, R. L. and Stamp, D. (1978) Biochem. Pharmacol. 27, 21-27.

77 Machy, P. and Leserman, L. D. (1983) Biochim. Biophys. Acta 730, 313-320.

78 Heath, T. D., Lopez, N., Stern, W. and Papahadjopoulos, D. (1985) FEBS187, 73-75.

79 Kremer, W. B. (1975) Ann. Int. Med. 82, 684-688.

80 Mayhew, E., Papahadjopoulos, D., Rustum, Y. M. and Dave, C. (1978) Ann. N. Y. Acad. Sci. 308, 371-384.

81 Papahadjopoulos, D., Poste, G., Vail, W. J. and Biedler, J. (1976)

Cancer Res. 30, 2988-2994.

82 Goldenberg, C. J. (1975) Cancer Res. 35, 1087-1096.

83 Heath, T. D., Bragman, K. S., Matthay, K. K., Lopez, N. G. and Papahadjopoulos, D. (1985) in Receptor-Mediated Targeting of Drugs (Gregoriadis, G., ed.), NATO ASI Series, Vol. A82, p. 407, Plenum Press, New York.

84 Rustum, Y. M., Dave, C., Mayhew, E. and Papahadjopoulos, D. (1979) Cancer Res. 39, 1390-1396.

85 Mayhew, E. W., Rustum, Y., Szoka, F. C. and Papahadjopoulos, D. (1980) Cancer Treat. Rep. 63, 1923-1928.

86 Mayhew, E, Rustum, Y. and Vail, W. J. (1983) Cancer Drug Delivery 1, 43-58.

87 Gabizon, A., Goren, D., Fuks, Z., Barenholz, Y., Dagan, A. and Meshorer, A. (1983) Cancer Res. 43, 4730-4735.

88 Piper, J. R., Montgomery, J. A., Sirotnak, F. M. and Chello, P. L. (1982) J. Med. Chem. 25, 182-187.

89 Heath, T. D., Lopez, N. G. and Papahadjopoulos, D. (1985) Biochim. Biophys. Acta 820, 74-84.

90 Mayhew, E. and Papahadjopoulos, D. (1983) in Liposomes (Ostro, M., ed.), pp. 289-341, Marcel Dekker, New York.

91 Van Renswoude, J. and Hoekstra, D. (1981) Biochemistry 20,

540-546.

92 Allen, T. M., and Cleland, L. G. (1980) Biochim. Biophys. Acta 597, 418-426.

93 Scherphof, G. L., Damen, J. and Wilschut, J. (1984) in Liposome Technology (Gregoriadis, G., ed.), Vol. 3, p. 205, CRC Press, Boca Raton.

94 Heath, T. D., Lopez, N. G., Piper, J. R., Montgomery, J. A., Stern, W. H. and Papahadjopoulos, D. (1986) Biochim. Biophys. Acta 862, 72-80.

95 Ralston, E., Hjelmland, L. M., Klausner, R. D., Weinstein, J. N. and Blumenthal, R. (1981) Biochim. Biophys. Acta 601, 133-137.

96 Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochim. Physiol. 37, 911-917.

97 Chapman, D. (1969) Introduction to Lipids, McGraw-Hill, London.

98 Bartlett, G. R. (1959) J. Biol. Chem. 234, 466-468.

99 Mertz, J. E. and Berg, P. (1974) Virology 62, 112-124.

100 Bird, O. D., Binkley, S. B., Bloom, E. S., Emmett, A. D. and Pfiffner, J. J. (1945) J. Biol. Chem. 157, 413-414.

101 Silink, M. and Rowe, P. B. (1975) Biochim. Biophys. Acta 381, 28-36.

102 Jagerstad, M. and Olsson, I. (1979) Scand. J. Clin. Lab. Invest. 39, 343-349.

103 Baugh, C. M. and Krumdieck, C. L. (1971) Ann. N. Y. Acad. Sci. 186,

7-28.

104 Shin, Y. S., Chan, C., Vidal, A. J., Brody, T. and Stokstad, E. L. R. (1976) Biochim. Biophys. Acta 444, 794-801.

105 Bernstein, L. H., Gutstein, S., Weiner, S. and Efron, G. (1970) Am. J. Med. 48, 570-579.

106 Silink, M., Reddel, R., Bethel, M. and Rowe, P. B. (1975) J. Biol. Chem. 250, 5982-5994.

107 Rao, K. N. and Noronha, J. M. (1977) Biochim. Biophys. Acta 481, 594-607.

108 Priest, D. G., Veronee, C. D., Mangum, M., Bednarek, J. M. and Doig, M. T. (1982) Mol. Cell. Biochem. 43, 81-87.

109 Kaferstein, H. and Jaenicke, L. (1972) Hoppe-Seyler's Z. Physiol. Chem.353,1153-1158.

110 Nair, M. G. and Baugh, C. M. (1973) Biochemistry 12, 3923-3927.

111 Kazenko, A. and Laskowski, M. (1948) J. Biol. Chem. 173, 217-221.

112 Volcani, B. E. and Margalith, P. (1957) J. Bacteriol. 74, 646-655.

113 Baugh, C. M., Stevens, J. C. and Krumdieck, C. L. (1970) Biochim. Biophys. Acta 212, 116-125.

114 Heath, T. D., Lopez, N. G., Stern, W. H. and Papahadjopoulos, D. (1985) Biochim. Biophys. Acta 187, 73-75.

115 White, J. C. (1981) Cancer Treat. Rep. 65, 3-12.

116 Goldman, I. D. (1981) Cancer Treat. Rep. 65, 13-17.

117 McGuire, J. J. and Bertino, J. R. (1981) Mol. Cell. Biochem. 38, 19-48.

118 McGuire, J. J., Hsieh, P., Coward, J. K. and Bertino, J. R. (1983) in Chemistry and Biology of Pteridines (Blair, J. A., ed.), W. de Gruyter, Berlin, p. 615-619.