# FACTORS INFLUENCING THE UPTAKE AND RELEASE OF DOXORUBICIN

BY LIPOSOMES

BY

ς

LINDA C.L. TAI

B.Sc., University of British Columbia, 1986

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Biochemistry

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 1988

© Linda Tai, 1988

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of BIOLHEMISTRY

The University of British Columbia Vancouver, Canada

Date APRIL 26, 1988

#### ABSTRACT

The use of liposomes exhibiting a transmembrane pH gradient (inside acidic) to accumulate doxorubicin into the interior aqueous compartment has been shown to achieve drug trapping efficiencies in excess of 98% in a manner which is independent of lipid composition. Doxorubicin entrapment appears to be most efficient at 60 C with 100% drug accumulation occurring after 5 minutes. An increase in internal buffering capacity and trap volume of the vesicles significantly enhances doxorubicin Initial drug to lipid ratios as high as 2:1 sequestration. (wt:wt) have been used, although trapping efficiencies fall below 95% at drug to lipid ratios in excess of 1:2 (wt:wt). As vesicle size is decreased the initial drug to lipid ratio must be reduced to 1:10 to maintain high trapping efficiencies. In addition to effecting efficient doxorubicin entrapment, the transmembrane pH gradient also reduces the rate of doxorubicin leakage. For example, in liposomes exhibiting a pH gradient than 2 units, release is less than 5% greater of the encapsulated doxorubicin over 24 hours at 37 C whereas release rates are significantly higher in the absence of a pH gradient. Finally, a procedure for a rapid colorimetric test for determining the amount of unencapsulated doxorubicin is described. The test is based on a spectral shift of doxorubicin 480nm to approximately 600nm upon peak absorption from addition of alkali to the liposomal doxorubicin. The resulting color change of untrapped drug from orange to purple can be quantitated spectrophotometrically or visually.

ii

# TABLE OF CONTENTS

ABSTRACT	ii	
TABLE OF CONTENTS iii		
LIST OF TABLES vi		
LIST OF FIGURES	vii	
ABBREVIATIONS	ix	
ACKNOWLEDGEMENTS	x	
1 INTRODUCTION		
1.1 Model Membrane Systems	1	
1.2 Preparation and Properties of Liposomes	2	
1.3 Rationale for Drug Encapsulation in Liposomes	3	
1.4 Factors Affecting Drug Encapsulation in Liposomes	8	
1.5 Doxorubicin	12	
1.5.1 Structure	12	
1.5.2 Mechanism of Action	12	
1.5.3 Therapeutic Uses and Clinical Toxocity	14	
1.5.4 Distribution and Metabolism	15	
1.6 Rationale for Doxorubicin Encapsulation in Liposomes	16	
2 Uptake of Doxorubicin into Large Unilamellar Vesicles in Response to a pH Gradient		
2.1 Introduction	17	
2.2 Materials and Methods		
2.2.1 Materials	18	
2.2.2 Preparation of large unilamellar vesicles	18	
2.2.3 Generation of the pH gradient	19	

	2.2.4	Doxorubicin uptake in response to pH	
		gradients	20
	2.2.5	Passive entrapment of doxorubicin	21
	2.2.6	Trap volume determinations	21
:	2.3 Resul	lts	
	2.3.1	Temperature dependence of doxorubicin uptake	22
	2.3.2	Effect of cholesterol on doxorubicin uptake .	24
	2.3.3	Effect of lyso PC on doxorubicin uptake	27
	2.3.4	Doxorubicin concentration and vesicle permeability and stability	28
	2.3.5	Effect of buffering capacity on doxorubicin uptake	31
	2.3.6	Effect of varying initial drug to lipid ratios on uptake	5 34
	2.3.7	Vesicle size and drug to lipid ratios on doxorubicin uptake	38
:	2.4 Disc	ussion	43
3 F U	actors I nilamella	nfluencing Release of Doxorubicin from Large ar Vesicles	
:	3.1 Intro	oduction	48
:	3.2 Mate	rials and Methods	49
	3.2.1	Materials	49
	3.2.2 3 3	Methods .2.2.1 Release Assay .2.2.2 Colorimetric Assay	49 50
	3.3 Resu	lts	
	3.3.1	Effect of lipid composition on doxorubicin release	51
	3.3.2	Effect of buffering capacity on doxorubicin release	54

3.3.3 Effect of varying initial drug to lipid ratios on doxorubicin release	57
3.3.4 Colorimetric assay	61
3.4 Discussion	66
4 SUMMARIZING DISCUSSION	68
BIBLIOGRAPHY	72

# LIST OF TABLES

•

1.	Uptake of doxorubicin at various cholesterol concentrations	26
2.	Uptake of doxorubicin in vesicles with varying lyso PC concentrations	26
3.	External doxorubicin concentration and vesicle leakiness	29
4.	Trap volumes of vesicles with internally and externally incubated doxorubicin	29
5.	Size distribution of vesicles extruded through filters	40
6.	Characteristics of liposome-encapsulated doxorubicin preparations	46

# LIST OF FIGURES

1.	Freeze-fracture electron micrograph of multilamellar vesicles	4
2.	Freeze-fracture electron micrograph of small unilamellar vesicles	5
3.	Freeze-fracture electron micrograph of large unilamellar vesicles	6
4.	Structure of doxorubicin	13
5.	Temperature dependence of doxorubicin uptake	23
6.	Structure of cholesterol	25
7.	The relationship between entrapped proton concentration and citrate concentration	32
8.	The influence of trap volume and buffering capacity on doxorubicin uptake	33
9.	The effect of varying initial drug to lipid ratios on the trapping efficiency of doxorubicin	36
10	.Comparison between residual transmembrane pH gradients with varying internal citrate concentrations	37
11	.Effect of drug to lipid ratio on the trapping efficier of doxorubicin in various sized vesicles	ncy 41
12	.The influence of drug to lipid ratios on the maintenar of the pH gradient in smaller vesicle systems	nce 42
13	Proposed mechanism of doxorubicin uptake in LUVETs exhibiting a transmembrane pH gradient	44
14	The influence of cholesterol concentration on doxorubicin release in LUVETs	52
15	.The effect of lyso PC on the release of doxorubicin in LUVETs	53
16	.Release of doxorubicin in vesicles derived from MLVs	55
17	.Release of doxorubicin in vesicles derived from FATMLVs	56

.

18. The effect of varying initial drug to lipid ratios on the retention of doxorubicin in liposomes	58
19.Varying initial drug to lipid ratios on doxorubicin in liposomal systems containing 1M citrate	60
20.The absorbance spectra between 400 and 700nm for doxorubicin solutions at pH 7.5 and 10.5	64
21.Comparison of free/total doxorubicin ratios with the absorbance ratio at 600nm before and after addition of triton X-100 to alkalinized sample	65

# ABBREVIATIONS

CL	Cardiolipin
Chol,C	Cholesterol
DCP	Dicetylphosphate
Dox	Doxorubicin
DPPC	Dipalmitoylphosphatidylcholine
EPC	Egg phosphatidylcholine
HBS	Hepes buffered saline
Hepes	[4-(2-Hydroxyethyl)]-piperazineethansulfonic acid
LUVs	Large unilamellar vesicles
LUVETS	Large unilamellar vesicles by extrusion techniques
MLVs	Multilamellar vesicles
OD	Optical density
PG	Phosphatidylglycerol
∆рН	Transmembrane pH gradient
${}_{\Delta}\Psi$	Transmembrane potential
PL	Phospholipid
SA	Stearylamine
SUVs	Small unilamellar vesicles
TX-100	Triton X-100
VET <sub>#</sub>	Vesicles produced by extrusion of a specific size

#### ACKNOWLEDGEMENTS

As previous acknowledgements from this lab are a hard act to follow, I will try to keep mine as simple as possible. First of all, I must thank Pieter Cullis and Mick Hope for fostering an excellent ( and fun ) scientific working environment. Other individuals who I would also like to thank include : Marcel Bally, Colin Tilcock, Tom Madden, Kim Wong, Larry Reinish, Tom Redelmeier , Helen Loughrey, Richard Harrigan, Simon Eastman, Michel Lafleur, Jeffrey Veiro, Neil Kitson, Archie Chonn and Tanquay (whew!). Special thanks to Lawrence Mayer for Diane having the patience to read this thesis more than once (albeit while drinking scotch !) and introducing me to the pleasures of caring for salt water fish. Individuals aside, I would also like to extend my thanks to Lipex Biomembranes Inc. (biotechnology for the future) for organizing the best ski trips possible, providing me with pocket money and most importantly for inducing my elusive alcohol dehydrogenase. All of you have been instrumental in making this thesis both enjoyable and possible.

х

#### INTRODUCTION

#### 1.1 Model Membrane Systems

A11 biological membranes contain а variety of phospholipids, varying amounts of sterols and a wide array of proteins. The ability of lipids to assume a bilayer structure is dictated by the amphipathic character of membrane lipids. These lipids contain a polar or hydrophilic headgroup region and a nonpolar or hydrophobic region. Much of the research in biological membranes involves the use of model membrane systems which contain naturally occurring or synthetic lipid species of interest. Generation of model membrane systems requires three steps. First there is the isolation or chemical synthesis of a appropriate model system must be given lipid. Second, an constructed containing that lipid and lastly incorporation of a non-lipid component to determine its influence on the lipid or vice versa.

Hydration of a dry phospholipid film results in the formation of structures called liposomes (Bangham et al, 1965). The study of liposomes began with Bangham in the mid-1960's. Bangham's pioneering work has resulted in an extensive use of liposomes as models of biological membranes. Liposomes can carry water soluble agents in their aqueous compartments and lipid soluble agents in their bilayers and can be prepared from a variety of phospholipids thus producing vesicles of differing properties. Major components which are generally used include

egg phosphatidylcholine, cholesterol and charged amphiphiles such as phosphatidic acid (negative charge) or stearylamine (positive charge) to provide particular properties to the whole vesicle as a result of the net charge.

## 1.2 Preparation and Properties of Liposomes

Liposomes are spontaneously formed as multilamellar vesicles (MLVs) upon hydration of a dried phospholipid film. Multilamellar vesicles (fig. 1) are composed of a series of concentric bilayers interrupted by narrow aqueous spaces. MLVs are quite heterogeneous ranging in size from about 80nm to 10u (Kaye, 1981) and being easily prepared are frequently utilized for defining the properties of lipids. Use of MLVs is mostly restricted to physical studies on bilayer organization and the motional properties of individual lipid within a membrane structure (Cullis <u>et al</u>, 1985).

Small unilamellar vesicles (SUVs) (fig. 2) can be produced by sonicating MLVs with either a probe or bath-type sonicator. These SUVs are characterized by diameters less than 50nm and a very high degree of membrane curvature (ie outer to inner monolayer phospholipid ratio is generally greater than 1) resulting in packing restraints. Some sonicated vesicle systems are also metastable and tend to fuse into larger vesicles (Parente and Lentz, 1984). This can restrict the use of SUVs for physical studies on various properties of membrane lipids.

Large unilamellar vesicles (LUVs) of diameters greater than 100nm can be generated by a variety of techniques including reverse phase and solvent evaporation (Szoka et al, 1980). The reverse phase and solvent evaporation techniques, however, are both tedious and time-consuming as organic solvents must be In a recent work carefully removed. (Hope et al, 1985), a procedure was developed for the rapid generation of LUVs (fig. 3) via extrusion of preformed MLVs through polycarbonate filters moderate pressure. under a Advantages of the extrusion procedure includes its rapid preparation time and its use in generation of large unilamellar vesicles from a wide variety of lipid species and mixtures. These LUVETs (large unilamellar vesicles by extrusion technique) can be generated at high lipid concentrations and exhibit relatively hiqh trapping efficiencies.

# 1.3 Rationale for Drug Encapsulation in Liposomes

Initially, the goal of liposome encapsulation involved enzymes for replacement therapy in genetic deficiency diseases. Since then a wide range of compounds have been successfully entrapped in liposomes with the aim of modifying their behavior in the body. These compounds include antibiotics (penicillin) (Gregoriadis, 1973), antimonials used in leishmaniasis (New <u>et</u> <u>al</u>, 1978; Alving, 1982; Alving and Schwartz, 1985) and hormones such as insulin (Patel <u>et al</u>, 1976). These aims were broadened to include a considerable emphasis on anticancer drugs

Fig.1 Freeze-fracture electron micrographs of egg PC at 50 mg/ml concentration. (A) MLVs prepared in 150mM NaCl, 20mM Hepes at pH 7.4. (B) MLVs in the same buffer containing 25% (v/v) glycerol. MLVs were subjected to 5 freeze-thaw cycles. (C) FATMLVs were prepared by subjecting MLVs to 5 freeze-thaw cycles in the absence of glycerol. Bar = 200nm. The micrographs were reproduced with permission from M.J. Hope.



Fig.2 Freeze-fracture electron micrographs of egg PC SUVs at 10mg/ml concentration. SUVs were prepared in 150mM NaCl, 20mM Hepes at pH 7.4 by sonication of the MLV preparation. Bar = 200nm. The micrographs were reproduced with permission from M.J. Hope.



Fig. 3 Freeze-fracture electron micrographs of FATMLVs passed 20 times through filters of various pore sizes. Vesicles were prepared from egg PC at 100mg/ml. The pore sizes of the filters employed were A) 400nm, B) 200nm, C) 100nm, D) 50nm, and E) 30nm. The bar represents 150nm and all panels exhibit the same magnification. Electron micrographs were reproduced with permission from M.J.Hope.



(Weinstein and Leserman, 1984; Weinstein, 1984; Poznansky and Juliano, 1984). The direct administration of these drugs can be hampered by immunological reactions, development of drug resistance and uptake of drug by non-diseased tissues often leading to serious side effects. As liposomes are amphipathic by nature it was believed that both polar, hydrophilic drugs as well as nonpolar, hydrophobic drugs could be entrapped within the aqueous compartment or within the bilayer. For polar drugs the entrapment depends on the solubility of the drug in water and on the volume of water encapsulated per mass of lipid. For lipophilic drugs, maximal incorporation into liposomes simply depends on the amount of lipid and the solubility of the drug in the lipid.

There are many possible reasons for the use of liposomes as vivo. drug carriers in Firstly, they are non-toxic, non-immunogenic and biodegradable. In particular, liposomes often protect the host against liposome contents. This is beneficial if the drug, in the free form, has toxic side effects on non-diseased tissues. A liposome can also be used to concentrate the drug as it can carry hundreds to thousands of drug molecules compared to only a few molecules of drug which can be directly coupled to small molecular carriers such as antibodies or hormones. Liposomes tend to circulate in the bloodstream much longer than most free drugs. This is true of vesicles which especially are negatively charged less than 100nm in diameter (Juliano and Layton, 1980). or

There are numerous problems and limitations of liposomes as drug carriers in vivo. A considerable percentage of injected liposomes are removed from circulation the by reticuloendothelial cells in the liver, spleen and elsewhere. Thus a fair proportion of liposomally encapsulated drug never reaches its intended site. Another problem involves the inability of liposomes to escape the circulatory system because of barriers such as the endothelial cell layer and basement membrane in the capillaries (Weinstein, 1984). Thus liposomes are unlikely to reach cells of solid tumours. Also a variety of other factors such as uniformity of liposome size, sterility and stability must also be taken into account in any liposome preparation which is intended for pharmaceutical application.

## 1.4 Factors Affecting Encapsulation of Drugs in Liposomes

Previous studies have shown that highly polar hydrophilic drugs such cytosine arabinoside or penicillin are as encapsulated at lower efficiency than less polar hydrophobic drugs such as actinomycin D or doxorubicin (Fendler, 1980). This is most likely due to the fact that polar drugs are contained only in the internal aqueous compartment while drugs distributed between the aqueous nonpolar may be compartment and lipid membrane compartment of the liposome. This behaviour can be demonstrated by briefly sonicating liposomes containing entrapped polar or lipophilic drugs. The polar molecules are quickly and completely released as the

liposome membrane was disrupted while the lipophilic drug remains associated with the liposome membrane (Stamp <u>et al</u>, 1979).

Since a large variety of techniques are available for the preparation of liposomes (Hope et al, 1986) the selection of the encapsulation protocol is based on the particular demands arising from the type of drug to be entrapped and the biological effects desired. One of the most popular techniques for drug encapsulation involves passive entrapment which relies on the ability of liposomes to capture a certain aqueous trap volume during vesicle formation. Passive drug entrapment can be applied to the 3 types of liposomes previously described. Sonicated vesicles may be easily prepared and virtually any lipid composition can be employed. However, drawbacks to using this system for drug encapsulation include the low trapping efficiencies from 1-5% (Szoka and Papahadjopoulos, 1978; Mayhew et al, 1984) and that sonication may degrade entrapped molecules such as proteins (Lelkes, 1984). Large unilamellar vesicles have been used because of their larger aqueous trap volume enabling superior drug to lipid ratios and trapping efficiencies to be obtained compared to most SUVs and MLVs. Methods for producing LUVs which have gained widespread popularity for entrapping include solvent evaporization/vaporization drugs in LUVs techniques (Szoka et al, 1978) and the extrusion technique (Hope et al, 1985 ; Mayer et al, 1986). Finally multilamellar vesicles

have also been employed as drug carriers because maximal drug retention can be obtained due to the increased number of lamellae the drug must cross to reach the exterior. However, the major problem typically experienced with MLVs is the low aqueous trapped volumes and trapping efficiencies obtained for traditional MLV dispersions. Techniques including solvent evaporation (Gruner <u>et al</u>, 1985) or freeze-thawing MLVs (Mayer <u>et al</u>, 1985) have resulted in an increase in the aqueous trap volume of multilamellar systems.

Based on trapping efficiency it is possible that passive entrapment may not provide an efficient method for entrapment. Amphiphilic compounds are difficult to encapsulate and retain in liposomes since they can readily permeate through lipid bilayers. Alterations in lipid compositions of liposomes have been used to enhance encapsulation efficiency and decrease release rates by enhancing ionic interactions between drug and charged lipid components.

The use of ion gradients offers a more general means for encapsulation of amphiphilic drugs such as doxorubicin. This process is analagous to the ability of various probes of membrane potentials and transmembrane proton gradients to distribute across lipid bilayers in response to  $\Delta \Psi$  or  $\Delta pH$ . Studies have shown that lipophilic cations such as the drug safranine can be accumulated by LUVs exhibiting a K potential (Bally <u>et al</u>, 1985). In these systems the membrane potentials were established by forming LUVETs in potassium glutamate and subsequently exchanging the untrapped potassium glutamate for

NaCl. The ionophore valinomycin, which is specific for potassium, was used to move K out and generate a membrane potential (interior negative).

Bulk accumulation of antineoplastic agents (Mayer et al, 1985, 1986; Bally et al, 1985) and biogenic amines (Nichols and Deamer, 1976; Bally et al, 1988) inside liposomes can be obtained by incubating the drug in the presence of vesicles exhibiting K or H gradients. Although both types of gradients induce efficient entrapment of lipophilic cations, drug uptake in response to  $\triangle pH$  most likely will be of more practical use. to the fact that  $\Delta \Psi$ -dependent encapsulation is due This requires the use of exogenous ionophores, which are usually toxic, whereas ApH-dependent uptake does not. The use of ion gradients is an extremely useful method for entrapment as the transmembrane ion gradients not only accomplishes efficient drug encapsulation but also decrease the rate of drug efflux from the vesicles.

As previously mentioned, doxorubicin is classified as an amphipathic cation. It is among the most widely used antineoplastic agents which is effective against а broad spectrum of tumors. However, cardiotoxic side effects limit the dose which can be administered. Thus it is thought that liposome encapsulation of doxorubicin may provide significant therapeutic benefit while decreasing the dose-limiting toxic side effects by altering the pharmacokinetics of the drug.

#### 1.5 Doxorubicin

## 1.5.1 Doxorubicin-Structure

Doxorubicin, identified by Arcamone <u>et al</u> in 1969, is one of the most important antitumour agents in clinical use today. It displays activity against a wide range of human malignancies, including a variety of solid tumours. Doxorubicin (fig. 4) consists of a tetracycline ring structure attached to an unusual sugar, daunosamine, by a glycosidic linkage. The sugar has a primary amine which has a pKa~9 and is therefore predominantly protonated at physiological pH's. Cytotoxic agents of the anthracyclic antibiotic class all have quinone and hydroquinone moieties on adjacent rings that permit them to function as electron-accepting and -donating agents

# 1.5.2 Doxorubicin-Mechanism of Action

Studies suggest that doxorubicin binds to nuclear DNA and its inhibition of replication and consequent DNA RNA transcription is considered to be one of the major mechanisms of cytotoxicity (Potmesil et al, 1984). However, the exact nature of this process remains unclear and several explanations of the sequence of events leading to DNA damage have been suggested. The damage caused by doxorubicin may be related to the formation of free radicals. Electrons from NADPH are transferred to the likely due to quinone moiety of doxorubicin, most the interactions between one of the anthracycline rings of



doxorubicin and the flavin component of NADPH:cytochrome P-450 reductase (Bachur et al, 1982). Another explanation suggests that the distortion of the DNA helix caused by drug intercalation may activate endonucleases which results in DNA strand scission (Ross <u>et</u> <u>al</u>, 1979, 1981). Recent studies (Potmesil et al, 1984) have provided evidence that both mechanisms may occur but that their detection depends on the concentration of doxorubicin. At high drug concentrations, greater than the peak plasma level achievable after i.v. bolus, it has been shown that DOX-mediated free radicals cause discernible DNA damage. At lower doxorubicin levels, more relevant to clinical use, another type of interaction between drug and DNA seems to occur which is independent of the free-radical mechanism.

#### 1.5.3 Therapeutic Uses and Clinical Toxicity of Doxorubicin

Doxorubicin has been found effective against acute leukemias and malignant lymphomas. When it is used concurrently with other anticancer drugs (cisplatin, cyclophosphamide), it has been found successful in treating carcinoma of the ovary and non-Hodgkin's lymphoma. It is also one of the most active single agents for treatment of metastatic adenocarcinoma of the breast, carcinoma of the bladder and neuroblastoma (DiMarco, 1975; Calabresi <u>et al</u>, 1985)

However, serious toxic manifestations limit the total dose of doxorubicin which can be administered. Acute toxicity

involving myelosuppression is a major dose-limiting complication with leukopenia reaching a maximum during the second week of recovering by the therapy and fourth week. Stomatitis, gastrointestinal disturbances and alopecia are also common acute side effects but are reversible. Tissue necrosis may develop if the intravenous injection misses the vein. Cardiomyopathy is a unique characteristic of anthracycline antibiotics. Two forms of cardiomyopathies may occur, the first type is an acute form which is characterized by abnormal changes in the ECG. This form is brief and rarely presents a serious problem. However, the second type is a chronic, cumulative dose-related toxicity which often results in congestive heart failure that is unresponsive to digitalis. A maximum total dose of 550 mq/m should not be exceeded as the mortality rate above this dose is in excess of 50% (Minow et al, 1977; Bristow et al, 1978; Myers, 1982; Wiemann and Calabresi, 1985).

## 1.5.4 Distribution and Metabolism of Doxorubicin

Once injected intravenously, doxorubicin is cleared rapidly from the plasma with a triphasic disappearance curve. There is a rapid uptake of doxorubicin by the heart, kidneys, lungs, liver, spleen but the drug does not appear to cross the blood-brain barrier. Doxorubicin is mainly metabolized in the liver and excreted in the bile. A significant portion of doxorubicin is excreted unchanged and the rest appears to be converted into multiple metabolites including adriamycinol (Myers, 1982; Myers <u>et al</u>, 1984; Wiemann and Calabresi, 1985).

## 1.6 Rationale for Doxorubicin Encapsulation in Liposomes

The ultimate goal of a liposomal-encapsulation of doxorubicin is to maintain or improve the antitumour properties of the drug while significantly decreasing the toxic side effects. The predominant toxic effects of concern are the myelosuppression and the chronic cardiotoxicity. As liposomes are taken up mainly by reticuloendothelial cells of the liver and spleen the entrapment of doxorubicin in liposomes may reduce amount which is taken up by the heart and decrease the the In this thesis, a novel "active" trapping cardiotoxicity. procedure for doxorubicin was developed using liposomes with a pre-established transmembrane pH gradient. This system has been characterized by examining the variables which may influence uptake and release of doxorubicin including cholesterol content, internal buffering capacity, temperature and vesicle size. In addition, a technique for rapid assaying of the amount of entrapped doxorubicin will be discussed as it has both research and broader clinical applications. It is also hoped that this active trapping procedure will apply to the entrapment of other lipophilic cationic drugs such as daunomycin and vincristine.

# 2. Uptake of Doxorubicin into Large Unilamellar Vesicles in Response to a pH Gradient

#### 2.1 Introduction

Previous studies have shown that doxorubicin can be accumulated into large unilamellar vesicles made up of egg PC in generated membrane potential created by the response to a K ionophore valinomycin (Mayer <u>et al</u>., 1986). In this study, it has been demonstrated that doxorubicin can be accumulated in LUVs which exhibit a pH gradient in the absence of ionophores. The pH gradient is established by hydrating the vesicles in pH 4.0 buffer and exchanging for an outside buffer of pH 7.5. This encapsulation procedure which relies on the ability of doxorubicin to accumulate into liposomal systems in response to a transmembrane pH gradient (inside acidic) allows doxorubicin to be loaded into preformed liposomes immediately prior to use, thus eliminating stability problems. Trapping efficiencies approaching 100% are readily achieved and drug to lipid ratios 3- to 10-fold higher than obtained for previous formulations are straightforward. Various parameters such as lipid composition, temperature, interior buffering capacity and vesicle size were examined in order to elucidate the mechanism of doxorubicin uptake into vesicles.

#### 2.2 Materials and Methods

#### 2.2.1 Materials

Egg phosphatidylcholine was obtained from Avanti Polar Lipids and was greater than 99% pure. Cholesterol, citric acid, lyso phosphatidylcholine, NaCl, Hepes and TX-100 from Sigma Chemical Company. Doxorubicin was obtained from the Cancer 3 14 Control Agency (Vancouver, B.C.). H-DPPC, C-citrate and 14 C-methylamine were purchased from New England Nuclear.

# 2.2.2 Preparation of large unilamellar vesicles

Vesicles of various sizes were prepared by the extrusion technique according to the protocol of Hope et al (1985). A dry lipid film of egg PC or egg PC:cholesterol was hydrated in citrate buffer and vortexed to produce multilamellar vesicles between 25 and 200 umole phospholipid/ml. The resulting dispersion was then frozen in liquid nitrogen and thawed in a 40 C water bath. A total of five freeze-thaw cycles was usually emploved. These frozen and thawed MLVs or FATMLVs were then transferred into a device (produced by Sciema Technical Services sold through Lipex Biomembranes, Inc.) which allowed the and extrusion of the FATMLVs through standard 25mm polycarbonate with pore sizes ranging from 0.2 to 0.015u (Nucleopore Corp., Pleasanton, CA). Unless otherwise stated, two stacked filters were employed and extrusion was repeated 10 times.

The size distributions of the extruded liposomal systems were determined by quasi-elastic light (QEL) scattering utilising a Nicomp Model 200 Laser Particle Sizer with a 5mW Helium-Neon Laser at an exciting wavelength of 632.8 nm. QEL scattering employs digital autocorrelation to analyze the fluctuations in scattered light intensity generated by the diffusion of vesicles in solution. The measured diffusion coefficient is used to obtain the average hydrodynamic radius and hence the mean diameter of the vesicles.

2.2.3 Generation of the pH gradient

The pH gradient (inside acidic) was generated by forming the LUVs in citrate buffers pH 4.0 ranging in concentration from 10mM to 1M and subsequently exchanging the untrapped buffer for 150mM NaCl, 20mM Hepes pH 7.5 buffer (Hepes buffered saline, HBS) employing Sephadex G-50 5ml columns equilibrated in HBS. A faster alternate method which involved diluting the pH 4.0 vesicles in saline (2X) and titrating the exterior pH to 7.5 using NaOH was also employed . The pH gradient of these systems 14 was determined utilising C-methylamine as the pH probe. One C-methylamine was added to vesicles exhibiting pH uCi/ml of gradients and allowed to equilibrate for 15 minutes. Aliquots were passed down Sephadex G-50 columns to remove untrapped 14 C-methylamine. Lipid phosphorous and C-methylamine content liposomes were assayed before and after gel filtration . of

Lipid phosphorous was quantitated using the method prescribed by 14 Fiske and Subbarow (1975). C-methylamine was quantitated using a United Technologies Packard Tri-Carb 2000 Series Liquid Scintillation Analyzer. The pH gradient was determined by calculation of interior and exterior proton concentration according to the following equation: pH gradient =  $\log_{10}[H^+]_i$ ------[H]<sub>o</sub>

2.2.4 Doxorubicin uptake in response to pH gradients

Doxorubicin in either solution or powder form was added to dispersions of defined concentrations in the presence or LUV absence of a pH gradient. The mixture was then heated at 60 C 10 minutes with intermittent vortexing to ensure all for powdered doxorubicin was in solution. After 10 minutes, the non-sequestered drug was removed by loading aliquots of the solution onto a Sephadex G-50 column packed in 1ml disposable syringes (Pick, 1981). Lipid and drug were then assayed. Lipid concentrations were determined by liquid scintillation counting to quantitate H-DPPC (0.05uCi/ umole lipid) or by phosphorous assays (Fiske and Subbarow, 1925). Doxorubicin was quantitated following the mixing of an aliquot of the column eluant with 1% triton X-100 to disrupt the vesicles and release the trapped drug. The absorbance of this solution is monitored employing a Shimadzu UV-160 spectrophotometer and doxorubicin uptake is expressed in nmoles doxorubicin/umole phospholipid or total lipid.

2.2.5 Passive entrapment of doxorubicin

Doxorubicin was trapped passively by preparing vesicles in 150mM NaCl, 20mM Hepes buffer containing the drug. The non-sequestered drug was removed by passing aliquots of the solution over 1ml Sephadex G-50 columns. Lipid and drug concentrations were then assayed according to the protocol in section 2.2.4.

2.2.6 Trap volume determinations

Trap volumes were determined by preparing multilamellar vesicles in the presence of 1 uCi/ml of C-citrate in the citrate buffer pH 4.0 and the LUVETS were made according to the procedure in section 2.2.2. Aliquots were then loaded onto a Sephadex G-50 1ml spin column and vesicles eluted bv centrifugation of this column at 500xg for 3 minutes. Samples obtained before and after the G-50 column were assayed for lipid 14 C-citrate was determined using phosphorous liquid and a scintillation counter. Trapped volumes calculated are expressed as ul of trapped volume per umole of phospholipid or total lipid.

## 2.3 Results

### 2.3.1 Temperature dependence of doxorubicin uptake

described in the protocol of doxorubicin uptake in As section 2.2.4 the sample was heated to 60 C for 10 minutes. This temperature was arrived at after experiments involving uptake at various temperatures showed uptake to be temperature dependent for vesicles composed of egg PC:cholesterol in a 55:45 mole percent ratio. Comparative uptake studies were performed at 21, 37 and 60 C over a time course of 120 minutes to determine the kinetics of doxorubicin uptake. Fig. 5 demonstrates that the rate and efficiency of drug encapsulation is extremely temperature dependent for liposomal systems containing Incubation of doxorubicin in the presence of cholesterol. liposomes exhibiting a transmembrane pH gradient of 3.5 units (inside buffer of 300mM citrate at pH 4.0) at 21 C results in only 50% trapping efficiency. Increasing the temperature of incubation above 37 C yields doxorubicin trapping efficiencies of approximately 100%. This value is achieved within 90 minutes at 37 C and 5 minutes at 60 C.

Fig. 5 Temperature dependence of doxorubicin uptake in Egg PC:cholesterol LUVETs prepared as indicated in section 2.2.2 with 300mM citrate pH 4.0 inside and 150mM NaCl, 20mM Hepes pH 7.5 outside. The temperatures of incubation were ( $\blacksquare$ ) 21 C, ( $\bigcirc$ ) 37 C and ( $\blacktriangle$ ) 60 C.



Time (min)

2.3.2. Effect of cholesterol on doxorubicin uptake

The inclusion of cholesterol (fig. 6) in the bilayer has a very important modulatory effect on the phase transition behaviour of bilayers composed of homogeneous phospholipids (Poznansky and Juliano, 1984). Cholesterol is often used to increase the rigidity of the bilayer and to reduce leakage of entrapped molecules from phosphatidycholine-containing vesicles. Cholesterol has been included in these liposomal systems to improve their applicability as drug delivery vehicles. It has been shown that an incorporation of cholesterol of greater than 30 mole percent into the liposomal bilayer can significantly reduce the tendency of serum components such as lipoproteins to disrupt liposomes (Weinstein, 1984; Finkelstein and Weissmann, Kirby <u>et</u> <u>al</u>, 1980). Studies in which cholesterol 1979; composition in vesicles ranged from 0 to a maximum of 45 mole percent showed that cholesterol did not greatly influence the △pH-dependent uptake of doxorubicin at 60 C. The vesicles were composed of egg PC:cholesterol (100:0 mol%), egg PC:cholesterol (85:15), egg PC:cholesterol (67:33) and egg PC:cholesterol (55:45). At a drug to lipid ratio of 0.3:1 the trapping efficiencies obtained for the different systems were all greater than 90% (Table 1).


Table 1. Uptake of doxorubicin at various cholesterol concentrations. Vesicles were prepared according to the protocol in section 2.2.2. Separation of free from liposomally encapsulated doxorubicin was accomplished using G-50 columns.

% Cholesterol	Before Sep'n*	After Sep'n*	%Trapping eff.
0	336	301	90
15	320	291	91
33	298	272	91
45	289	287	99

\* Before and after separation values are expressed as nmole DOX/umole lipid

Table 2. Effect of lyso PC on uptake of doxorubicin. Vesicles were prepared according to the protocol in section 2.2.2 except that the pH gradient was established by titrating the exterior pH to 7.5 with 1M NaOH and the interior buffer was 150mM citrate at pH 4.0.

% lyso PC	Before Sep'n*	After Sep'n*	%Trapping eff.
0	289	287	99
1	288	281	98
5	222	221	100
_			

\* Before and after separation values are expressed as nmole DOX/umole lipid.

2.3.3 Effect of lyso PC on doxorubicin uptake

The effect of addition of lyso PC into egg PC:cholesterol vesicles was examined to determine if uptake was affected by the presence of small quantities of lyso PC. The structure of lyso PC is identical to that of egg PC except that only one fatty acyl chain is ester-linked to the glycerol backbone instead of two chains for egg PC. It is known that the ester linkage between the fatty acid chains and the glycerol backbone of the phosphatidylcholine is susceptible to acid hydrolysis. Since the interior buffer is citrate at pH 4.0, it was postulated that lyso PC may be produced. This increase in lyso PC may result in increase in vesicle permeability due to the micellar and an solubilizing character of lyso phospholipids (Weiner, 1987). It has been found that the presence of lyso PC at acidic pH markedly increased the permeability of mucosal cells in the distal ileum ( Bolin et al, 1981). Thus experiments were done to determine if lyso PC increased vesicle leakiness such that uptake was decreased due to rapid efflux of doxorubicin after uptake. The systems that were examined included lyso PC:EPC:cholesterol in the following mole percent ratios a) 1:51:48, b) 5:47:48 . It was found that the lipid composition did not influence the ApH-dependent uptake of doxorubicin. Both systems achieved a trapping efficiency of approximately 100% as shown in Table 2.

# 2.3.4 Effect of doxorubicin concentration on vesicle permeability and stability

The actual effect of doxorubicin on liposome permeability not well characterized. Determination of vesicle leakiness is and stability was accomplished using C-inulin as a trap volume and permeability probe. Inulin is a neutral molecule (molecular weight of approx. 5000) which is not normally permeable to membranes. In this experiment the egg PC:cholesterol was hydrated in Hepes buffered saline pH 7.5 containing 1uCi 14 C-inulin/ml of buffer. The vesicle preparation was sized through 0.2um filters and then passed down a 5ml LKB Ultrogel column and trap volume was determined. Liposomes that were passed down the ultrogel columns were then incubated with various concentrations of doxorubicin from 0.1 to 40mM and a saline control at room temperature over a time course of two 14 hours. Table 3 shows that leakage of C-inulin from the vesicles is negligible for samples incubated with up to 40mM suggest that the doxorubicin. These results presence of doxorubicin does significantly not alter the membrane permeability.

The effect of passively entrapped doxorubicin on the membrane permeability and trapped volume of FATMLVs was also studied. The lipid film was hydrated in Hepes buffered saline 14 containing 1uCi C-inulin/ml of buffer and 34mM doxorubicin. Trap volumes were determined by taking aliquots of the solution

External [DOX](mM)	15	Time (min) 45	60	120	
0	5630	5920	6244	6217	
0.1	5810	6195	6287	5677	
1.0	5473	5436	5872	5470	
5.0	5803	5682	6074	5870	
10.0	5654	6138	6157	5474	
20.0	5955	5774	5755	5739	
40.0	6461	5731	6172	6187	

Table 3. Effect of external doxorubicin concentration on vesicle leakiness. Vesicles were prepared according to the protocol in section 2.2 and 2.3.4. Leakage of probe is expressed as DPM  $^{14}$ C-inulin/umole PC.

Table 4. Trap volumes of vesicles with internally and externally incubated doxorubicin. The aqueous trap volume of the vesicles were determined according to the protocol in section 2.2.6.

	Trap volume*		
	Before	After	
Externally added DOX	2.33	2.30	
Passively entrapped DOX (FATMLVs)+	4.84	4.72	

\* Trap volume values are expressed as ul buffer/umole lipid + For the passively entrapped drug FATMLVs were used instead of vesicles extruded through 0.2um filters. after passive entrapment and after dialysis of the vesicles over 14 24 hours to remove untrapped C-inulin. Quantitation of the inulin and phosphorous and trap volume determinations were completed as described in section 2.2.6. Table 4 shows the trap volumes obtained for the passively entrapped doxorubicin and externally added doxorubicin. The passively entrapped doxorubicin does not appear to affect vesicle size or integrity as the trap volumes did not change after dialysis. 2.3.5 Effect of buffering capacity on doxorubicin uptake

In the characterization of  $\triangle pH$ -dependent doxorubicin uptake it was hypothesized that the buffering capacity of the interior buffer plays a large role in uptake. This hypothesis was first tested by determining the buffering capacity of the various concentrations of citrate buffers that were to be used. As by fig. 7 there is a linear relationship between shown increasing citrate concentration and entrapped proton pool. The entrapped proton reserves for 10,50,100,300 and 500mM citrate systems were calculated by titrating each of these buffers with NaOH to pH 7.5. Doxorubicin trapping efficiencies in vesicles containing various concentrations of citrate buffer (10-500mM) were examined to assess the role of the interior proton pool on drug uptake. Quantitation of doxorubicin uptake was accomplished using two vesicle systems. One of which was LUVs derived from MLVs in the presence of increasing concentrations of citric acid as well as from frozen and thawed MLVs (FATMLVs). The FATMLVs have larger aqueous trap volumes (Mayer et al., 1985) and therefore increased amounts of entrapped citric acid. For an initial drug to lipid (wt:wt) of 0.3:1, increasing the citrate concentration from 10 to 100mM produces an increase in the doxorubicin trapping efficiencies for both MLVs and FATMLVs (fig. 8). It was also noted that the trapping efficiency for vesicles produced from MLVs are lower than that observed for liposome systems produced from FATMLVs using citrate

Fig. 7 The relationship between entrapped proton concentration and citrate concentration. Titration of various concentrations of citrate with NaOH were conducted to pH 7.5.



Fig. 8 The influence of trap volume and buffering capacity on doxorubicin uptake. Vesicles were prepared according to section 2.2.2 with the indicated concentrations of citrate buffer. LUVETS of 0.2um diameter were prepared from ( $\blacktriangle$ ) FATMLVs and ( $\blacksquare$ ) MLVs.



concentrations between 10 and 300mM. These results suggest that doxorubicin accumulation in response to a transmembrane pH gradient causes a decrease of the internal H pool and this reduction may result in decreased drug trapping efficiencies if entrapped buffering capacities are not adequate.

2.3.6 Effect of varying initial drug to lipid ratios

At high initial drug to lipid ratios (greater than 0.3:1) doxorubicin trapping efficiencies are observed to decrease. For these high drug to lipid ratios the transmembrane pH gradient may be depleted thus limiting uptake. Studies were undertaken optimize trapping efficiencies and drug:lipid ratios for to EPC:cholesterol (55:45) containing 300mM citrate pH 4.0 as well as to identify conditions which maintain the transmembrane pH gradient subsequent to doxorubicin entrapment. Drug to lipid (wt:wt) were varied from as high as 2:1 down to 1:10 ratios (where it was known that trapping efficiencies of 100% could be readily achieved). It was found that between drug to lipid ratios of 1:10 and 1:3 there was no effect on doxorubicin trapping efficiencies and values of 100% were achieved in this Increasing the initial drug to lipid ratio range (fig. 9). above 1:3 resulted in entrapped doxorubicin:lipid ratios as high as 1:1. However, trapping efficiencies decrease significantly as the initial drug: lipid is increased above 1:2. Measurements C-methylamine as the probe (protocol of pH gradients using

section 2.2.3) were done to determine the effect of high drug to lipid ratios on the trapping efficiencies. The decreased trapping efficiencies observed for the high drug to lipid ratios can be rationalized in fig.10 where the transmembrane pH gradient after encapsulation is monitored as a function of initial drug to lipid ratio. Controls involving vesicles with pH gradients of 3.5 units and no gradients were performed to determine the validity of the method and determine the background binding of the methylamine. It was found that systems exhibiting drug to lipid ratios below 1:3 maintain transmembrane pH gradients in excess of 2.0 units. Such pH gradients would predict [dox] in/[dox] out ratios greater than 100 and thus trapping efficiencies greater than 99% as is observed experimentally. However, doxorubicin entrapment does deplete some of the internal H pool in all systems as the ApH values calculated were always lower than the initially imposed  $\Delta pH$  of 3.5. The effect becomes most pronounced for initial drug to lipid ratios greater than 1:2 where the residual pH gradient falls below 1.5 and corresponding trapping efficiencies also decrease accordingly (fig. 9,10)

In a comparative study the same drug to lipid ratios were examined but instead of 300mM citrate as the internal buffer 1M citrate( pH 4.0) was employed to increase the buffering capacity and perhaps maintain the pH gradient of the higher initial drug to lipid ratios. The increased buffering capacity did have a significant effect on increasing trapping efficiencies as 100%

Fig. 9 The effect of varying initial lipid:drug ratios on trapping efficiency of doxorubicin in vesicles exhibiting a transmembrane pH gradient. Vesicles were prepared according to section 2.2 and incubated with various amounts of doxorubicin. comparison trapping efficiencies between vesicles Α of containing 300mM citrate pH 4.0 (■) and 1M citrate pH 4.0 (▲) at the different drug: lipid ratios was examined.



Fig. 10 Comparison between residual transmembrane pH gradients of vesicles with internal buffers of 300mM citrate pH 4.0 ( $\blacksquare$ ) and 1M citrate pH 4.0 ( $\blacktriangle$ ) at different initial drug:lipid ratios. Determination of pH gradients involved the use of <sup>14</sup>C-methylamine according to the protocol in section 2.2.3.



efficiencies were possible for drug to lipid ratios as high as 1:1 and maintenance of pH gradients also improved for these ratios (fig. 9). In particular, vesicles containing 300mM citrate at drug:lipid 1:1 the trapping efficiency achieved was 86% with no pH gradient remaining while at 1M citrate the trapping efficiency was increased to 97% with a  $\triangle$ pH of 1.11 remaining after entrapment (fig.10).

2.3.7 Effect of vesicle size and drug to lipid ratios on uptake

Previous studies have shown that decreasing vesicle size will increase the longevity of liposomes in circulation (Abra and Hunt, 1981; Senior and Gregoriadis, 1982). In the case of doxorubicin smaller liposomes liposomal may increase the efficacy of the drug compared to free drug which is cleared very rapidly from the plasma. However, smaller liposomes have reduced trap volumes which lowers the internal buffering capacity perhaps limiting drug uptake. The effect of vesicle size and drug to lipid ratio on doxorubicin uptake and maintenance of the pH gradient after uptake were examined. The vesicles were sized according to the protocol in section 2.2.2 and extruded 10 times through a double stack of either 100, 50, or 30 nm filters. The sizes and trap volumes (Mayer et al, 1985) of vesicles generated through these filters are given in Table 5 . An initial drug to lipid ratio of 1:10 for all 3 systems gave 100% trapping efficiencies while maintaining a pH gradient greater than 2.0

units. However as the drug to lipid ratio was increased to 1:5 the systems still achieved high trapping efficiencies but the residual pH gradient dropped dramatically in the  $VET_{50}$  and  $VET_{30}$  systems. At a ratio of 1:3 the trapping efficiencies started to decrease as vesicle size decreased and there was no remaining pH gradient in each system (fig.11,12). These results suggest that the reduced trap volume decreases the internal pool of protons thereby limiting the ability of these systems to maintain the pH gradient after doxorubicin encapsulation.

Table 5. Size distribution of vesicles extruded through the indicated filter sizes. Vesicles were prepared according to the procedure outlined in section 2.2.

Filter size (nm)	QEL size (Gaussian analysis) (nm)
100	93.8 <sup>±</sup> 25.3
50	64.7 <sup>±</sup> 14.4
30	$55.2^{\pm}14.2$

Fig. 11 Effect of lipid:drug ratio on the efficiency of trapping of doxorubicin in various sized vesicles. Vesicles were prepared according to the protocol described in section 2.2 and were extruded through ( $\blacksquare$ ) 100nm filters, ( $\bullet$ ) 50nm filters or ( $\blacktriangle$ ) 30nm filters.



Fig. 12 The influence of lipid to drug ratio on the maintenance of the pH gradient in smaller vesicle systems. Determination of the remaining pH gradient after doxorubicin uptake was calculated according to protocol in section 2.2.3. Vesicles were extruded through ( $\blacksquare$ ) 100nm, ( $\odot$ ) 50nm or ( $\triangle$ ) 30nm filters.



#### 2.4 Discussion

The results presented here provide new information on the mechanism of doxorubicin uptake in liposomes exhibiting a pH gradient (inside acidic). transmembrane The proposed mechanism whereby doxorubicin is accumulated into LUV systems (Fig. 13) involves the penetration of doxorubicin in the neutral form. The unprotonated form of the drug would be expected to be in equilibrium with the protonated form and accumulation of doxorubicin should therefore follow the Henderson-Hasselbach type of transmembrane distribution. Upon reaching the inner aqueous compartment the drug is subsequently reprotonated. With this model of uptake, one would expect uptake to be largely dependent upon buffering capacity of the interior buffer and the trap volume of the vesicles. As reprotonation of the amine functionality would deplete the inner aqueous medium of protons, an interior buffer with a larger buffering capacity and/or a vesicle system with a larger trap volume is expected to accumulate a greater amount of doxorubicin.

This model is supported by the results obtained when varying concentrations of citrate buffer were employed. Increasing the citrate concentration from 10 to 100mM produced increases in doxorubicin trapping efficiencies for vesicles derived from MLVs and FATMLVs. As predicted, the trapping efficiencies for vesicles derived from MLVs were lower than that observed for liposomes produced from FATMLVs at the equivalent

Fig.13 Proposed mechanism of doxorubicin uptake in LUVETs exhibiting a transmembrane pH gradient.





PH 7.5

citrate concentrations between 10 to 300mM. The increased aqueous trapped volumes of the FATMLV-derived vesicles leads to increased amounts of internal citric acid thereby increasing the interior buffering capacity.

The inclusion of cholesterol and lyso PC did not appear to alter the  $\Delta pH$ -dependent uptake of doxorubicin. However, the addition of cholesterol does influence the temperature dependence of uptake. This influence correlates well with the suggested role of cholesterol in controlling membrane fluidity.

involving various initial drug:lipid ratios Studies supports the proposed mechanism of uptake. As the initial concentration of doxorubicin was increased, the pH gradient remaining after drug uptake decreased in a proportional manner. suggests that increasing the initial drug concentration This interior buffering capacity thus increasing the depletes the internal pH and eliminating or decreasing the pH gradient. From these studies the maximum drug to lipid ratio which exhibits properties appropriate for therapeutic use ( >98% trapping efficiency) appears to be approximately 0.3:1. This value is 4.3 to 75 fold higher than drug to lipid ratios obtained for previous liposomal doxorubicin preparations (Table 6). This drug lipid ratio is obtained in conjunction with trapping to efficiency approaching 100% whereas existing entrapment techniques typically yield trapping efficiencies of 50% or less The use of higher drug to lipid ratios is (Table 6). potentially important in two aspects of liposomal drug therapy.

Ref.	Liposom Type	e Size (nm)	Composition	Ratio (m	Drug:lip ol:mol)	oid (wt:wt)	≿Trap eff.
17	SUV	135 <sup>±</sup> 70	PS:PC:C	3:7:10	1:18.6	0.05:1	25
16	MLV MLV MLV MLV SUV SUV SUV SUV SUV	N.D. N.D. N.D. N.D. N.D. N.D. N.D. N.D.	PC:C PC CL:PC:C CL:PC PS:PC:C PC:C CL:C CL:PC:C CL:PC:C PS:PC:C	1:1 1:4:5 1:4 3:7:10 1:1 5:2.5 1:4:2 1:4:5 3:7:10	$1:33 \\ 1:31.2 \\ 1:21.2 \\ 1:14.8 \\ 1:23 \\ 1:14 \\ 1:18 \\ 1:21.2 \\ 1:26.7 \\ 1:44.2$	0.028:1 0.022:1 0.039:1 0.040:1 0.040:1 0.066:1 0.027:1 0.033:1 0.031:1 0.021:1	14 10 62 58 42 15 90 47 45 22
53	SUV SUV SUV	N.D. N.D. N.D.	PC:C PC:C:DCP PC:C:SA	7:2 7:2:1 7:2:1	1:130 1:37 1:225	0.006:1 0.021:1 0.004:1	6.6 25.7 4.0
47	SUV SUV	N.D. N.D.	PC:C:PS PC:C:SA	10:4:1 10:4:3	1:11.6 1:18.4	0.069:1 0.049:1	55 35
22	SUV	90 <sup>±</sup> 20	CL:PC:C:SA	1:5:3.5:2	1:12.4	0.068:1	55
43	LUV	150	PG:DC:C	1:4:5	1:30	0.031:1	50

Table 6 Characteristics of liposome encapsulated doxorubicin preparations.

First, large lipid doses ( in excess of 5g for administration of  $\frac{2}{2}$  a typical 60mg/m doxorubicin dose to a 60kg human) required for many liposomal doxorubicin preparations could be significantly reduced to reasonable levels (approximately 400mg for an equivalent human dose). Second, there are now indications (Mayer <u>et al</u>, 1988) that increased drug to lipid ratios may provide enhanced buffering of toxicity effects.

By decreasing vesicle size it was also shown that uptake at higher drug to lipid ratios was limited by reduced trap volumes and therefore reduced buffering capacities. Thus the maximum drug to lipid ratio appears to be approximately 0.1:1 for systems 100nm or smaller. Although a lower drug: lipid ratio is in smaller systems it is thought that they may be more used smaller liposomes efficacious than the larger systems. As in the circulation longer the liposomally encapsulated remain drug (still in a biologically active form) will give prolonged exposure to tumour cells and perhaps enhanced antitumour activity.

# 3. Factors Influencing Release of Doxorubicin from Large Unilamellar Vesicles

# 3.1 Introduction

The stability of liposomally-encapsulated doxorubicin over an extended period of time is of therapeutic importance in drug delivery. An indication of liposome integrity after encapsulation is the rate of release of the drug from liposomes. The reduced toxicity exhibited by liposomal doxorubicin (Mayer et al, 1988) may be due to decreased toxic levels of the free drug. Release of doxorubicin in the pH-driven encapsulation technique is thought to be dependent on the size of the pH gradient and the form of doxorubicin within the liposome. The retention of the pH gradient should depend on the trap volume of the vesicle, the interior buffering capacity and the amount of drug that is entrapped. The model of uptake in chapter 2 form of doxorubicin permeates the that the neutral proposes liposome and is protonated upon reaching the aqueous interior which has a pH around 4.0. Vesicles with large trap volumes should provide a larger buffering capacity as more citrate can This increased buffering capacity may prove to be be trapped. important in the maintenance of the pH gradient.

There is also some uncertainty as to the form of doxorubicin that is present within the vesicle. Doxorubicin may be predominantly in the protonated form with a small proportion

in the neutral form. Doxorubicin may also be forming a complex with the citrate inside the vesicle. It has been shown that doxorubicin in the presence of citrate at pH 7.0 will form a precipitate (unpublished observations). The purpose of this chapter is to examine the factors which govern doxorubicin release from liposomes prepared according to the protocol described in chapter 2. These factors include the effect of varying lipid composition, internal citrate concentration and initial drug to lipid ratios. In addition, a protocol of a the colorimetric assay for rapid determination of the proportion of free doxorubicin in liposomal preparations will be discussed.

#### 3.2 Materials and Methods

3.2.1 Materials See section 2.2.1

3.2.2 Methods

3.2.2.1 Release Assay

Release of doxorubicin, subsequent to the active trapping procedures described in section 2.2.4, involved dilution of the vesicles remaining after uptake with 150mM NaCl, 20mM Hepes pH 7.5 to concentrations 2-10mM. These samples were then placed in Spectrapor membrane tubing (MW 12000-14000) and dialyzed for 24

hours against 1000 volumes of the Hepes buffered saline at 37 C on a Precision six place Mag-mix. Aliquots of 150ul were removed at the indicated times and passed down a 1ml Sepadex G-50 spin column to remove free doxorubicin and entrapped doxorubicin was determined as described in section 2.2.4.

## 3.2.2.2 Colorimetric Assay

Vesicles were prepared as in section 2.2.2 in 300mM citrate buffers of pH ranging from 4.0 to 7.2. The pH gradient for the active entrapment was achieved by raising the exterior pH to 7.5 with 1M Na<sub>2</sub>CO<sub>2</sub>. Doxorubicin was also passively entrapped by adding doxorubicin to the Hepes buffered saline prior to the lipid hydration step. With the active entrapment procedure the transmembrane pH gradient drives the accumulation of the doxorubicin inside the vesicles such that Dox in/Dox out reflect the [H]  $_{in}/[H^+]_{out}$  (Mayer <u>et al</u>, 1986). The spectrophotometric measurements of the free and liposome encapsulated doxorubicin were made with a Shimadzu UV-160 spectrophotometer. The uptake samples were diluted with HBS pH 7.5 to achieve doxorubicin concentrations between 0.05 to 0.10mM. For each preparation the following sequence of measurements were made. First, the absorbance at 600nm of the diluted sample was established as the "reagent blank". Second, the sample was alkalinized to pH 10.5 (0.02ml/1.0ml sample). Third, with 1.0N NaOH the spectrophotometer was zeroed against a 0.2% triton X-100 solution. Lastly, the absorbance at 600nm of the liposomal

doxorubicin sample to which 0.2% triton X-100 had been added was recorded (0.01ml 20% triton X-100/1.0ml sample). The ratios of free:total doxorubicin were calculated as the absorbance at 600nm upon NaOH addition divided by the absorbance after triton X-100 addition.

#### 3.3 Results

#### 3.3.1 Effect of lipid composition on release of doxorubicin

The in vitro release of doxorubicin in these systems displays a minor dependence on lipid composition. Increasing is the cholesterol content known to decrease membrane permeability of certain entrapped drugs (Ganapathi et al, 1984). Experiments in which cholesterol composition ranged from 0 to 45 mole percent of the total lipid showed similar release patterns (fig. 14). The systems with 0 and 15 mole percent cholesterol both showed a similar rate of release and after 24 hours, both 67% of the doxorubicin. retained With higher amounts of cholesterol, 33 and 45 mole percent, long-term release rates were slower and the vesicles maintained 77% of the doxorubicin after 24 hours. In each case, the release curves seem to follow a biphasic pattern with an initial guick release in the first hours followed by a slower, steady state release. few

The inclusion of lyso PC in small quantities was used to determine the deleterious effects, if any, of the acidic interior buffer. As lyso PC is produced from egg PC by acid

Fig. 14 The influence of cholesterol concentration on doxorubicin release in LUVETS. Vesicles were prepared according to sections 2.2 and 3.2. Vesicles were composed of EPC:chol in the following mole percents : ( $\Box$ ) 100:0, ( $\bullet$ ) 85:15, ( $\blacktriangle$ ) 67:33 and (O) 55:45.



Fig. 15 The effect of lyso PC on the release of doxorubicin in LUVETs exhibiting a transmembrane pH gradient. Vesicles were prepared according to the protocol described in sections 2.2 and 3.2. Lyso PC concentration was present in the following molar percentages: (O) 0%, ( $\blacktriangle$ ) 1% and ( $\blacksquare$ ) 5% lyso PC.



Time (hours)

hydrolysis of a fatty acid side chain, it was thought that the presence of lyso PC might increase the permeability of the vesicles. However, results here suggest that the presence of lyso PC in quantities as high as 5 mole percent induces no increase in rates of release (fig.15). The two systems which were examined had the following lipid compositions of lyso PC:egg PC:cholesterol a) 1:51:48 b) 5:47:48 and c) control (egg PC:chol 52:48).

## 3.3.2 Effect of buffering capacity on release

As shown by the results in the previous chapter the uptake of doxorubicin was profoundly affected by the internal buffering capacity of the vesicles. The comparison of vesicles derived from FATMLVs and MLVs showed that the vesicles derived from FATMLVs had larger trap volumes and thus larger trapping efficiencies. Release experiments were performed on each uptake sample to determine the effect of the various amounts of entrapped citrate buffer. In vesicles which were derived from both MLVs and FATMLVs it was found that increasing entrapped citrate concentration from 10 to 500mM provided greater retention of doxorubicin after 24 hours. The 0.2um diameter vesicles derived from MLVs showed a release rate which was related almost directly to the internal buffering capacity (fig.16).As the citrate concentration is increased from 10-500mM the doxorubicin retained increased from 42-97% after 24 hours.

Fig. 16 Release of doxorubicin from vesicles derived from MLVs. vesicles with various citrate buffers were Multilamellar The sized vesicles were then through 0.2um filters. extruded and 3.2. The interior prepared according to sections 2.2 citrate concentrations included: (O) 10, (△) 50, (□) 100, (◊) 300, (O) 500mM citrate pH 4.0.



Fig. 17 Release of doxorubicin from vesicles derived from FATMLVS. Multilamellar vesicles containing various concentrations of citrate buffer were frozen and thawed prior to sizing of vesicles and then prepared according to sections 2.2 and 3.2. The internal citrate concentrations ranged from (O) 10, ( $\Delta$ ) 50, ( $\Box$ ) 100, ( $\diamond$ ) 300 and (O) 500mm citrate pH 4.0.



The vesicles which were derived from FATMLVs showed a similar release pattern to the vesicles derived from MLVs; however as the trap volume is larger for the FATMLVs the retention was either higher or equal to vesicles derived from MLVs at equivalent citrate concentrations (fig. 17). This trend is readily observed with the higher citrate concentration, 300 and in which virtually no doxorubicin is released. These 500mM, results suggest that doxorubicin retention is dependent upon the In the case of the internal proton pool. lower citrate concentration buffers, the H pool is depleted as doxorubicin accumulation occurs such that retention of doxorubicin is decreased. With citrate at 300 and 500mM, the interior buffering is maintained such that doxorubicin efflux is negligible.

3.3.3 Effect of varying drug:lipid ratios on release

Varying drug:lipid ratios while maintaining the interior buffer concentration constant demonstrated that efficiency of doxorubicin uptake was dependent upon maintenance of the pH gradient. Release experiments were carried out to determine the effect of the transmembrane pH gradient on doxorubicin retention. Using 300mM citrate (pH 4.0) as the internal buffer, drug to lipid ratios from 1:10 to 2:1 were used in the entrapment protocol and residual pH gradients were measured using 14C-methylamine. It was shown that as the initial drug to lipid ratio was increased the residual transmembrane pH

Fig. 18 The effect of varying initial drug to lipid ratios on the retention of doxorubicin. Vesicles were prepared with an internal citrate concentration of 300mM citrate pH 4.0 according to the protocol in sections 2.2 and 3.2. The initial drug to lipid ratios which were used included: (O) 1:10, ( $\Delta$ ) 1:5, ( $\Box$ ) 1:3, ( $\Diamond$ ) 1:2, (O) 1:1 and (x) 2:1.



Time (hre)

gradients decreased. A release study was performed on each drug:lipid ratio and the results showed a similar trend to that obtained in the uptake study. In the low drug to lipid samples 1:10, 1:5, 1:3, 1:2 (wt:wt) where the remaining pH gradient exceeded 1.5 units the release was generally slow with less than 30% of the doxorubicin released after 24 hours in each case (fig.18). However, as the drug to lipid ratio was increased to 1:1 and 2:1 the pH gradient was reduced to zero and doxorubicin release rates were increased significantly such that after 24 the doxorubicin remained in the 30% of hours only 49% and initial drug:lipid of 1:1 and 2:1. vesicles with an respectively. In a separate experiment in which the interior buffer was increased to 1M citrate pH 4.0 the same drug to lipid ratios were examined for their retention of doxorubicin after As expected, the increased buffering capacity entrapment. provided a greater maintenance of the pH gradient in each of the The release experiments conducted also drug:lipid ratios. indicated that the increased buffering capacity allowed greater retention of doxorubicin. Initial drug to lipid ratios ranging 1:10 to 1:2 showed greater than 95% retention after 24 from hours (fig.19) At the high drug to lipid ratios of 1:1 and 2:1 is approximately a 20% increase in retention after 24 there hours over the vesicles containing 300mM citrate (pH 4.0). These suggest that efflux doxorubicin after active results of entrapment is dependent upon the remaining transmembrane pH gradient.

Fig. 19 The effect of varying initial drug to lipid ratios on doxorubicin retention in liposomal systems containing 1M citrate. Vesicles were prepared with an internal citrate concentration of 1M citrate pH 4.0 according to the protocol outlined in sections 2.2 and 3.2. The initial drug to lipid ratios used were: (O) 1:10, ( $\Delta$ ) 1:5, ( $\Box$ ) 1:3, ( $\diamond$ ) 1:2, (O) 1:1, and (x) 2:1.



Time (hrs)

#### 3.3.4 Colorimetric assay

As the ultimate goal of the liposomal doxorubicin formulation is for clinical use, it is imperative that toxic levels of free drug are not present outside of the liposomes. a procedure (section 3.2.2) for determining free and Thus vesicle entrapped doxorubicin was developed which is based on a pH-dependent spectral response. Figure 20 shows the absorbance spectra between 400 and 700nm for doxorubicin at pH 7.5 and Increasing the pH from 7.5 to 10.5 causes a shift in the 10.5. absorbance peak wavelength from 480nm to 550 and 592nm. It is important to note that the relative absorbance above 590nm for doxorubicin at pH 7.5 is negligible when compared to that Because of this, the optical density obtained at pH 10.5. from the liposomes can be readily adjusted to baseline arising values. The pH-dependent spectral response provides the basis for determining the proportion of free and vesicle entrapped doxorubicin in liposomal preparations. At neutral Hα no absorbance is observed at 600nm, subsequent alkalinization of the solution to pH 10.5 induces the spectral shift of free doxorubicin and not vesicle entrapped drug as the lipid bilayer isolates the encapsulated doxorubicin from the alkaline external The resulting OD<sub>600</sub> therefore reflects the amount of media. untrapped doxorubicin in the liposomal preparation. The total concentration of doxorubicin can then be quantitated by monitoring the absorbance at 600nm after the addition of triton
X-100 (compared to a triton X-100 blank solution). The detergent solubilizes the vesicles and thus exposes all the doxorubicin to the alkaline conditions. Using this assay, the proportions of unencapsulated doxorubicin can be determined as the ratio of the absorbance obtained after alkalinization with NaOH divided by that observed in the presence of detergent.

analysis The spectroscopic of liposomal doxorubicin preparations was compared to column chromatography methods which directly measure free and vesicle entrapped drug to correlate absorbance ratio values to actual free dox/total dox ratios over a wide range of trapping efficiencies. Since a transmembrane pH uptake of doxorubicin, EPC:Chol vesicles gradient induces (inside acidic) with pH gradients of varying magnitude were utilized construct liposome systems with trapping to efficiencies ranging from 10 to 99%. Figure 21 demonstrates that ratio at 600nm described here accurately the absorbance represents the ratio of free/total doxorubicin in the vesicle preparations over the full range of trapping efficiencies studied. This assay was also performed on vesicles in which doxorubicin had been passively trapped during vesicle formation ensure that these results were not specific to liposomal to doxorubicin obtained by active entrapment. As shown in fig. 21 the absorbance ratio at 600nm for this sample correlates well with the free/total doxorubicin value obtained by column chromatography.

The absorbance characteristics of the spectral shift also allows the relative amount of free doxorubicin in liposome preparations to be assessed visually. Although such analysis is clearly qualitative, the presence of just 5% free drug can be detected and a dramatic color change is observed for systems exhibiting greater than 15% free drug. Although trapping efficiencies below 50% can be determined spectrophotometrically, little difference in the color change can be discerned by eye and therefore the effective range for visual analysis of doxorubicin trapping efficiencies is 50-95%. Fig. 20 The absorbance spectra between 400 and 700nm for doxorubicin solutions adjusted to pH 7.5 (a) and 10.5 (b).



Fig. 21 Comparison of free/total doxorubicin ratios with the absorbance ratio at 600nm before and after addition of triton X-100 to alkalinized liposomal doxorubicin. Doxorubicin was actively encapsulated ( $\bullet$ ) employing 10mM lipid (EPC:cholesterol, 55:45 mole percent) and 2mM drug while passive entrapment (O) utilized 50mM lipid (EPC) and 2mM drug as described in section 3.2.2.2. Spectroscopic analysis and quantitation of free and total doxorubicin by column chromatography was completed as described in section 2.2.



## 3.4 Discussion

in the previous chapter, doxorubicin can be As shown actively trapped in liposomes exhibiting a transmembrane pH gradient. The factors which govern uptake also play an important role in maintaining doxorubicin within the vesicles. Results have shown that lipid composition plays a minor role in doxorubicin retention. Increasing the cholesterol content of the vesicles slowed the rate of release. These results are consistent with those of Ganapathi et al, 1984 in which anionic liposomes with varying amounts of cholesterol were used to determine the in vitro efflux. The inclusion of lyso PC in small quantities proved to have no effect on doxorubicin release.

Characterization of doxorubicin release from the liposomaldoxorubicin preparation was important for the development of an <u>in vivo</u> system in which retention of doxorubicin is maximal. Factors which influenced efflux rates included the buffering capacity of the vesicles and the initial drug to lipid ratios. The buffering capacity of the vesicles, which can be altered by using different concentrations of citrate or by changing the trap volume, plays a large role in the maintenance of the pH gradient across the vesicles. The rate of doxorubicin leakage was slowed by increasing the trap volume within the vesicles. This was accomplished by freeze-thawing the vesicles prior to extrusion. Increasing the citrate concentration also decreased

the rate of doxorubicin efflux. It is postulated that with low citrate concentrations such as 10mM, the accumulation depletes the internal pool of protons such that the equilibrium between  $DOX-NH_3^+$  and  $DOX-NH_2$  would be shifted towards  $DOX-NH_2$  which is capable of crossing the bilayer.

Varying the initial drug to lipid ratios provided further evidence that depletion of the internal proton pool results in increased release rates. At lower drug to lipid ratios the release is generally slow due to the presence of a substantial residual pH gradient. The high drug to lipid ratios showed much faster release of doxorubicin as the remaining transmembrane pH gradient was negligible.

Lastly, the protocol described for a colorimetric assay simple and rapid method for determining provides a the proportion of free doxorubicin in liposome preparation. Advantages of this procedure are that it does not require the chromatography equipment or sophisticated assay use of be completed within minutes. procedures and can More importantly, as the liposomal doxorubicin can be assessed visually by this procedure without the use of any scientific equipment and samples can be checked prior to in vivo use to determine if there are toxic levels of the free drug present.

## SUMMARIZING DISCUSSION

The studies presented in this thesis illustrate the utility of liposomes as carriers of toxic drugs such as the anticancer agent doxorubicin. There is increasing evidence that liposome encapsulation may provide a significant therapeutic benefit by decreasing dose-limiting toxic side effects while maintaining or, in some cases, increasing its efficacy (Mayer et al, 1988. Bally et al, 1988). Numerous independent studies from several laboratories have demonstrated that entrapment of doxorubicin in liposomes reduces drug accumulation in and subsequent damage to organs such as the heart (Rahman et al, 1980; Gabizon et al, 1982) and kidney (Rahman et al, 1985; van Hoesel et al, 1984) While these studies have established the clinical potential of liposomally encapsulated doxorubicin, a clear indication of the optimal liposome preparation to employ is not so readily The difficulties associated with identifying an available. optimal preparation are implicit in the wide range of vesicle lipid compositions and drug to lipid ratios previously types, employed (Table 6). In particular MLVs, LUVs and SUVs have been utilized with lipid compositions incorporating various amounts positively and negatively charged lipids in addition to of phosphatidylcholine and cholesterol. The variations in lipid composition largely stem from the requirements for trapping doxorubicin, as systems containing only positive or neutral lipids exhibit low trapping efficiencies and drug to lipid

ratios. In liposomes containing negatively charged lipids such as cardiolipin (Rahman <u>et al</u>, 1986) and phosphatidylglycerol, higher drug to lipid ratios are achievable due to the association of the positively charged doxorubicin with the negatively charged lipid (Table 6).

Other protocols, because of interdependence of lipid composition and doxorubicin entrapment precludes a systematic analysis of the characteristics of liposomal doxorubicin which determine <u>in vivo</u> drug toxicity and efficacy. By employing the  $\Delta pH$ -dependent doxorubicin encapsulation with citrate buffer systems, parameters can be independently varied enabling the effects of specific properties of liposomal doxorubicin to be investigated <u>in vivo</u>. Parameters which were examined included the effect of cholesterol and lyso PC concentration on the entrapment of doxorubicin. In both cases the lipid composition did not effect the trapping efficiency of doxorubicin.

The investigations of Chapter 2, which introduced a new encapsulation procedure, relies on the ability of doxorubicin to accumulate into liposomal systems in response to a transmembrane pH gradient (inside acidic). In this preparation, citrate at pH 4.0 provided the internal buffering as opposed to glutamic acid (Mayer et al, 1986) which was used in earlier experiments. The result of this buffer change was an increase in doxorubicin uptake into liposomes. Based on this observation, it was proposed that the internal buffering capacity played a major role in the uptake of doxorubicin into liposomes in response to Thus the main focus of Chapter 2 was in pH gradient. a

determining the factors which influence doxorubicin uptake and the mechanism of uptake in response to the pH gradient.

obtain hiqh The ability to doxorubicin trapping efficiencies appears to be closely related to the capacity of the liposomes to maintain a significant transmembrane pH It was found that depletion of the vesicle internal gradient. buffering capacity caused by either reducing the amount of citrate or dramatically increasing the initial drug to lipid ratio collapses the pH gradient and results in lower trapping efficiencies. As the vesicle size was decreased, a noticeable decrease in trapping efficiencies was found to occur. This was probably due to decreased trap volumes in the smaller vesicle systems and therefore a reduction in the amount of citrate entrapped.

doxorubicin stability of retention The by active appears to be closely related to encapsulation also the maintenance of the pH gradient. The investigations of Chapter 3 determined that reducing the amount of entrapped citrate or dramatically increasing the initial drug to lipid ratio results in a faster leakage of doxorubicin from liposomes. Manipulation of retention is important in drug delivery as release of drug prior to reaching the tumor site would result in increased toxicity due to elevated levels of free drug in the blood. Rapid release of doxorubicin from the liposomes may also lead to decreased efficacy as less drug reaches the tumor.

The colorimetric assay described in Chapter 3 provides a simple method of determining the amount of unentrapped doxorubicin. It was found that alkalinization of a liposomal doxorubicin sample from pH 7.5 to 10.5 shifted the peak absorbance from 500 to 600nm. It is the absorbance at 600nm which determines the amount of free doxorubicin which is present in the preparation. The main advantage of this procedure lies in its qualitative use as the occurrence of as little as 5% free drug can be detected visually and a dramatic colour change can be seen with 15% free drug.

The results of this thesis demonstrates the importance of the vesicle internal buffering capacity in doxorubicin encapsulation and retention. Observations made in this thesis define other of areas research that warrant further Firstly, the exact mechanism for pH gradient investigation. doxorubicin encapsulation dependent needs to be better understood and this requires the determination of the form of doxorubicin which is present in the vesicle. Secondly, while the in vivo toxicity and efficacy of this preparation has been studied (Mayer et al, 1988; Bally et al, 1988) further analysis of the importance of various liposome properties needs to be characterized. Lastly by utilizing liposomes with transmembrane pH gradients it is hoped that other lipophilic cationic drugs which are toxic in the free form can be encapsulated in this manner to reduce toxicity.

## BIBLIOGRAPHY

- 1. Alving, C.R. (1982), Targeting of Drugs, ed. G. Gregoriadis and A. Tromet, 337-353.
- Alving, C.R. and Schwartz, G.M. (1985) G. Gregoriadis (Ed.) Liposome Technology Vol. II, CRC Press, Inc. Boca Raton, Florida, pp 55-68.
- 3. Bally, M.B., Hope, M.J., Van Echteld, C.J.A. and Cullis, P.R. (1985), Biochim. Biophys. Acta, **812**, 66-76.
- 4. Bally, M.B., Mayer, L.D., Madden, T.D., Wong, K.F., Redelmeier, T.E., Loughrey, H.C., Harrigan, P.R., Hope, M.J., and Cullis, P.R. (1988) Chem. Phys Lip. (In press).
- 5. Bally, M.B., Mayer, L.D., Ginsberg,R.S., Masin, D., and Cullis, P.R. (1988) Submitted to Cancer Res.
- 6. Bachur, N.R., Gee, M.V., and Friedman, R.D. (1982) Cancer Res., 42, 1078.
- 7. Bangham, A.D., Standish, M.M., and Watkins, J.D. (1965) J. Mol. Biol., **13**, 238-244.
- 8. Bolin, T., Sjodahl, R., Sundqvist, T., and Tagesson, C. (1981) Scand. J. Gastroent., **116**, 897-901.
- Bristow, M.R., Billingham, M.E., Mason, J.W. and Daniels, J.R. (1978) Cancer Treat. Rep., 62, 873-879.
- 10.Calabresi, P., Schein, P.S. and Rosenberg, S.A. (eds.) (1985) Medical Oncology, Macmillan Publishing Co., New York.
- 11.Cullis, P.R. and Hope, M.J. (1985) Biochemistry of Lipids and Membranes (Vance, D.E., and Vance, J.E. eds.), 25-73.
- 12.DiMarco, A. (1975) Daunomycin and adriamycin. In, Antineoplastic and Immunosuppresive Agents, Pt.ll. Sartorelli and Johns, eds.) Handbuch der Experimentellen Pharmakologie, <u>38</u>, 593-614.
- 13.Fendler, J.H. (1980) Liposomes in Biological Systems, ed G. Gregoriadis and A.C. Allison, 87-100.
- 14.Finkelstein, M.C., and Weissmann, G. (1979) Biochim. Biophys. Acta, **587**, 202-216.

- 15.Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem., 66, 375-400.
- 16.Gabizon, A., Dagan, A., Goren, D., Barenholz, Y. and Fuks, Z. (1982) Cancer Res., 42, 4734-4739.
- 17.Gabizon, A., Goren, D., Fuks, Z., Barenholz, Y., Dagan, A., and Meshoren, A., (1983) Cancer Res., **43**, 4730-4735.
- 18.Ganapathi, R. and Krishan, A. (1984) Biochem. Pharmacol., 33, 698-700.
- 19.Goodman, A.G., Goodman,L.S., Rall,T.W., and Murad,F. (1985) The Pharmacological Basis of Therapeutics (seventh edition), 1283-1285.
- 20.Gregoriadis, G. (1973) FEBS Letters, 36, 292-296.
- 21.Gruner, S.M., Lenk, R.R., Janoff, A.S. and Ostro, M.J. (1985) Biochemistry, **24**, 2933-2842.
- 22.Herman, E.H., Rahman, A., Ferrans, V.J., Vick, J.A. and Schein, P.S. (1983) Cancer Res., 43, 5427-5432.
- 23.Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta., **812**, 55-65.
- 24.Hope,M.J., Bally, M.B., Mayer, L.D., Janoff, A.S., and Cullis, P.R. (1986) Chem. Phys. Lipids, 40, 89-107.
- 25.Juliano,R.L. and Layton,D.(1980) Drug Delivery Systems:Characteristics and Biomedical Applications (ed R.Juliano), 189-236.
- 26.Kaye, S.B. (1981) Cancer Treat. Rev., 8, 27-50.
- 27.Kirby, C., Clarke, J., and Gregoriadis, G. (1980) FEBS Lett., 111, 324-328.
- 28.Lelkes, P.I. (1984) Liposome Technology Vol. 1, CRC Press, Inc., Boca Raton, Florida, 51-65.
- 29.Mayer, L.D., Bally, M.B. and Cullis, P.R. (1985) Biochim. Biophys. Acta, **816**, 294-302.
- 30.Mayer, L.D., Hope, M.J., Cullis, P.R., and Janoff, A.S. (1985) Biochim. Biophys. Acta, **817**, 193-196.
- 31.Mayer, L.D., Bally, M.B., Hope, M.J., and Cullis, P.R. (1985) J. Biol. Chem., **260**, 802-808.

- 32.Mayer, L.D., Bally, M.B., and Cullis, P.R. (1986) Biochim. Biophys. Acta, 857, 123-126.
- 33.Mayer, L.D., Hope, M.J. and Cullis P.R. (1986) Biochim. Biophys. Acta, **858**, 161-168.
- 34.Mayer, L.D., Bally, M.B., Hope, M.J., and Cullis, P.R. (1986) Chem. Phys. Lipids, 40, 333-345.
- 35.Mayer, L.D., Ko, D.S.C., Thomas, N., Masin, D., Bally, M.B., Ginsberg, R.S., and Cullis, P.R. (1988) Submitted to Cancer Res.
- 36.Mayer,L.D., Tai,L.C.L., Bally, M.B., Ginsberg, R.S., and Cullis, P.R. (1988) Submitted to Cancer Res.
- 37.Mayhew, E., Lazo, R. and Vail, W.J. (1984) G.Gregoriadis (Ed.), Liposome Technology, Vol.II, CRC Press, Inc. Boca Raton, Florida, 19-31.
- 38.Minow,R.A., Benjamin,R.S.,Lee,E,T. and Gottlieb, J.A. (1977) Cancer, **39**, 1397-1402.
- 39.Myers, C.E. (1982) Pharmacologic Principles of Cancer Treatment (Chabner, B.A. ed.), 416-434.
- 40.Myers, C.E., Corden, B., and Gianni,L. (1984) The Cancer Pharmacology Annual 2, 66-79.
- 41.New, R.C., Chance, M.L., Thomas, S.C. and Peters, W. (1978) Nature, 272, 55-56.
- 42.Nichols, J.W. and Deamer, D.W. (1976) Biochim. Biophys. Acta, 455, 269-271.
- 43.Olson, F., Mayhew, E., Maslow, D., Rustum, Y., and Szoka, F., (1982) Eur. J. Cancer Clin. Oncol., **18**, 167-176.
- 44.Parente, R.A. and Lentz, B.R. (1984) Biochemistry, 23, 2353-2362.
- 45.Patel, H.M. and Ryman, B.E. (1976) FEBS Letters, 62, 60-63.
- 46.Pick, U. (1980) Arch. Biochem. Biophys., 212, 186-194.
- 47.Poznansky, M.J. and Juliano, R.L. (1984) Pharmacol. Rev., 36, 277-336.
- 48.Rahman, A., Kessler, A., More, N., Sikic, B., Rowden, G., Wooley, B. and Schein, P.S. (1980) Cancer Res., **40**, 1532-1537.
- 49.Rahman, A., White, G., More, N., and Schein, P.S. (1985) Cancer Res., **45**, 796-803.

- 50.Rahman, A., Carmichael, D., Harris, M., Roh, J.K. (1986) Cancer Res., **46**, 2295-2299.
- 51.Ross, W.E., Glaubinger, D., and Kohn, K.W. (1979) Biochim. Biophys. Acta, **562**, 41.
- 52.Ross, W.E. and Bradley, M.O. (1981) Biochim. Biophys. Acta, 654, 129.
- 53.Shinozawa, S., Araki, Y. and Oda, T. (1981) Acta Med. Okayama, **35**, 395-405.
- 54.Stamp, D., and Juliano, R.L. (1979) Can J. Physiol and Pharmacol., **57**, 535-539.
- 55.Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA, **75**, 4194-4198.
- 56.Szoka, F., and Papahadjopoulos, D. (1980) Ann. Rev. Bioeng., 9, 467-508.
- 57.Weiner, A.L. (1987) Liposomes From Biophysics to Therapeutic (Ostro, M. ed.), 339-369.
- 58.Weinstein, J.N. (1984) Cancer Treatment Reports, 68, 127-135.
- 59.Weinstein, J.N. and Leserman, L.D. (1984) Pharmacol. Ther., 24, 207-233.
- 60.Wiemann, M.C. and Calabresi, P., (1985) Medical Oncology (Calabresi, P, Schein, P.S., and Rosenberg, S.A., eds.), 292-362.
- 61.van Hoesel, Q.G.C.M., Steerenber, P.A., Crommelin, D.J.A., van Djik, A., van Oort, W., Klein, S., Douze, J.M.C., de Wildt, D.J. and Hillen, F.C. (1984) Cancer res., **44**, 3698-3705.