A COMPARATIVE STUDY OF NIOSOMES (NON-IONIC SURFACTANT VESICLES) AND LIPOSOMES : THEIR STABILITY IN BIOLOGICAL ENVIRONMENTS.

A THESIS

submitted to

THE UNIVERSITY OF STRATHCLYDE

by

LISBETH R. HUME

in fulfilment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

October 1987.

DEPARTMENT OF PHARMACY

UNIVERSITY OF STRATHCLYDE

204, GEORGE STREET

GLASGOW G1 1XW.

ACKNOWLEDGEMENTS

I would like to thank Dr. Alan J. Baillie, my supervisor and Professor Alexander T. Florence, my head of Department, for their support, encouragement and helpful discussions during the course of this work. I extend my sincere thanks to all my friends and colleagues in the Department of Pharmaceutics.

I am greatly indebted to my wonderful parents, Tom and Jean, whom I love very much. They have continually encouraged me and this thesis is dedicated to them.

Finally, an extra special thanks to Mohan, my dearest friend.

Of all those arts in which the wise excel
Nature's chief masterpiece is writing well.

John Sheffield "Essay on Poetry", 1682.

This thesis is divided into four main areas; Introduction, Experimental, Results and Discussion and Conclusions and Future Work.

The work presented here is part of a major study on novel drug delivery systems undertaken within the Department of Pharmaceutics, University of Strathclyde. This project details the study of a new generation of surfactants in the formation of vesicles suitable for drug delivery. The well-documented liposome system was used as a model throughout this work.

TABLE OF CONTENTS

INTRODUCTION

RESULTS AND DISCUSSION

CONCLUSIONS AND FUTURE WORK

REFERENCES & APPENDICES

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CF	carboxyfluorescein
DCP	dicetyl phosphate
DMPC	dimyristoylphosphatidylcholine
DNA	deoxyribonucleic acid
DPPC	dipalmitoylphosphatidylcholine
DSPC	distearoylphosphatidylcholine
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
EI	ether injection
HS	hand shaken
IgG	immunoglobulin G
LUV	large unilamellar vesicle
m.wt.	molecular weight
MLV	multilamellar vesicle
NaCl	sodium chloride
PAGE	poylacrylamide gel electrophoresis
PC	phosphatidylcholine
PG	phosphatidylglycerol
PS	phosphatidylserine
R*	registered trade mark
RES	reticuloendothelial system
SDS	sodium dodecyl sulphate
SUV	small unilamellar vesicles
T_c	transition temperature

ABSTRACT

Submicron sized vesicles consisting of single and double chain non-ionic surfactant mixtures were prepared by simple dispersion of surfactant dissolved in aqueous medium, or alternatively, injecting the surfactant dissolved in organic solvent into the aqueous phase.

Drug entrapment values were measured by using a fluorescent marker, 5,6-Carboxyfluorescein, and drug release characteristics were evaluated in biological media (serum and plasma) as a function of surfactant composition and in the presence or absence of cholesterol. Surface charge measurements, zeta-potential, as a function of pH, gel electrophoresis and immunoblotting (ELISA) were performed in order to measure the interaction of components of the biological fluid with the prepared vesicles. It was found that all vesicles carried a negative charge and rapidly bound plasma protein, which included albumin and immunoglobulin G, thus affecting the latency of the entrapped marker.

Uptake and degradation of niosomes (non-ionic surfactant vesicles) in a living, unicellular, eukaryotic micro-organism was also investigated. It was found that the rate of release of contents depended on the composition of the vesicles and was a function of enzymatic degradation within these organisms rather than an intracellular pH effect of the digestive organelle.

An identical protocol was carried out with the well- characterised liposome system and their inherent stabilities under a variety of conditions directly compared with niosomes.

SECTION 1

INTRODUCTION

1.1 PRINCIPLES OF DRUG DELIVERY

The past fifty years have witnessed major advances in the control of disease brought about with the use of drugs. These advances are particularly apparent in the treatment of infectious disease by means of vaccines and antibiotics. Some successful anti-cancer drugs are available but the overall failure of cancer chemotherapy has been an important stimulus to drug delivery research.

With accessible targets, it is possible to directly "titrate" the drug to the need of the patient on the basis of biological response. Temporal administration of drug in these situations is straight forward. However, when the target tissue is not accessible, for example, a tumour, drug placement becomes difficult, especially when more than a certain minimum drug concentration has to be maintained for a significant time course. Many factors contribute to the complexity of drug localisation at the target tissue; these include the behaviour of the drug in the body as well as patient compliance.

Controlled Release Systems

Drug administration or delivery must continue at an appropriate rate until the condition is cured or controlled with a minimum of side effects. In some situations this might mean that the drug is delivered more promptly for short periods of time and in other cases it would mean prolongation of drug levels. In the latter category the terms, "sustained release" and "prolonged release" are employed interchangeably; this designates only one aspect of controlled release, namely, to produce adequate levels of drug in the body.

Previous efforts *(Chien,1980)* have focussed on the use of polymers or pumps, to provide "controlled" rather than "sustained" release. Controlled release systems are designed to continuously deliver and maintain the drug concentration at a desired

level in the body. In general, a controlled release system utilises a polymer matrix or pump as a rate controlling device to deliver the drug in a fixed, predetermined pattern for a desired time period. Ideally, the use of this type of drug delivery system should result in a steady drug concentration as a function of time, require fewer and smaller dosages and cause fewer side effects. For the drug to be taken up by the target tissue in the body, several events must occur:

1. The drug must first be released from the carrier,
2. It must then diffuse from the surface of the carrier through the surrounding environment, and eventually,
3. It must access the target.

Drug Carriers

The concept of carrier mediated drug delivery has gained considerable interest in the last decade or so. Directing drugs to specific target organs is an old idea discussed by Paul Ehrlich *(1906)*in the beginning of the century.

To be efficient, the drug-carrier should fulfill several criteria:

1. The carrier should be pharmacologically inactive and the drug must be released in an active form after interaction of the carrier with the target cells.
2. The drug carrier complex must be stable in plasma and extracellular spaces.
3. The carrier should have the ability to take the drug through those anatomical barriers which separate the site of administration from the target; it must also be specifically recognised by receptors or antigens present on the outer membrane of the target cells.
4. The carrier should be non-toxic, non-immunogenic and biodegradable to avoid cellular overload during long term repetitive treatment.
5. Production of the drug loaded carrier in the amounts and conditions required

for clinical use, for example, sterility, apyrogenicity, should be easily achieved and pharmaceutically acceptable in terms of stability and reproducibility.

Several systems for achieving some of these goals have been proposed. Liposomes *(Gregoriadis,1983)*, resealed erythocytes *(Ihler,1983)*, microparticles *(Sjoholm and Edman,1984)*, nanoparticles *(Marty et.al,1978;Oppenheim,1981)* and albumin microspheres *(Tomlinson et.al,1984)* are examples of particulate colloid carriers which are used as targetable drug delivery systems.

Drug Targeting

Drug targeting aims to limit the access of the pharmacological agent to selected cells or tissues. In theory, such a strategy should decrease unwanted side effects by decreasing drug levels in non-target cells and enhance therapeutic activity by increasing the concentration of administered drug within target cells. Targeting can be achieved in two, principally different, ways depending on the characteristics of the carrier in the body, that is, by passive or active targeting *(Poste,1983)*. Passive targeting utilizes the natural homing of the carrier in the body after intravenous (i.v.) administration.

It has been irrevocably shown *(Poznansky and Juliano,1984)* that in fact most carriers, irrespective of their material, nature and composition, will accumulate in the reticuloendothelial system (RES) after i.v. injection. This innate behaviour can be used to drug load the RES. In some tropical parasitic diseases which involve the RES, this phenomenon may be used for real therapeutic benefit *(Alving et.al,1978;Black et.al,1977; New et.al,1978)*. Active targeting implies an attempt to circumvent the RES by some means and using, for example, monoclonal antibodies with specificity for certain cells or receptors, they are intended to deliver the drug to targets in a specific manner. A prerequisite for successful active targeting

is that the targets are in contact with the compartment to which the carrier is administered.

Biological Strategy

Several drug delivery approaches make use of biological entities, such as antibodies, red cells, and liposomes, as drug carriers. The biologically based delivery systems share some of the characteristics of polymeric and prodrug systems and can be used for similar purposes. Implicit in the work on biological carriers is the goal of using highly specific physiological recognition mechanisms as the basis of targeted delivery.

Carriers such as liposomes and red cells can be used to achieve sustained and controlled drug administration. Drug loaded liposomes *(Ostro,1987)* and erythrocytes *(DeLoach,1986)* can be introduced directly into the systemic vasculature or for localised action into the appropriate organ or body cavity. Such carriers can, in addition, perform some tasks which are beyond the capability of the synthetic delivery systems. For example, liposomes, with their membrane mimetic structure, can promote the cellular uptake of drugs that do not readily penetrate cell membranes. This feature may be particularly important in cancer chemotherapy where neoplastic cells often become drug resistant because membrane alterations occur leading to reduced drug permeation.

Biological carriers can be used to deliver active agents which are of macro-molecular dimensions, such as enzymes and nucleic acids; this property may be of value in enzyme replacement therapy of inherited disease and in the genetic manipulations of recombinant DNA technology. These carriers can, in theory, be fitted with highly specific recognition ligands, for example antibodies, so that

they appear prime candidates for the eventual development of "targetable" drug carrier complexes.

Controlled delivery is the desired effect of all drug delivery systems and presently all sustained and prolonged drug delivery systems provide some degree of control, albeit, incomplete. Thus, whereas second generation sustained release products have made significant advances over their first generation counterparts, none of the commercially available systems presently on the market is, in truth, a controlled drug delivery system *(Ihler,1986)*.

1.2. APPLICATIONS OF DRUG DELIVERY TECHNOLOGY

This section deals only with liposome based carrier systems, and concentrates on their potential use as drug delivery vehicles in medicine.

Enzyme therapy

The earliest proposal as carriers of therapeutic agents was made by Gregoriadis and co-workers who studied the liposomal entrapment of amyloglucosidase (E.C.3.2.1.3) from Aspergillus niger *(Gregoriadis et.al,1971)*. They proposed that liposomes might be ideal vehicles for introducing enzymes into deficient cells in genetically inherited metabolic disorders, especially in glycogen storage diseases.

Although promising results were obtained in tissue culture, in experimental animals and in a few preliminary clinical trials, the widespread applicability of liposomes as enzyme carriers is limited. The major reason is the rapid uptake of liposomes by the liver and spleen after intravenous injection. This would tend to eliminate

the use of liposomes in disorders affecting other tissues unless a means can be found to direct them to such tissue. This applies in particular to diseases affecting specialised cells, such as neurological tissues, which are unlikely to take up liposomes. Considerable advances need to be made in liposome technology to introduce the required degree of tissue specificity in vivo. However, drug delivery technology may be potentially applicable to many other types of pharmacologic agent and in different therapeutic situations, such as cancer chemotherapy.

Cancer Chemotherapy

Cancer chemotherapy is severely limited by the intrinsic toxicity of anticancer drugs, hence it is important to control their pharmacokinetic behaviour and tissue distribution. A great deal of research effort has been expended on the study of liposomes, antibodies and other carriers for anticancer drugs. This topic has been well reviewed *(Gregoriadis,1977)*. Many such drugs have been entrapped in liposomes and these include; actinomycin D, vinblastin, daunomycin and cytosine arabinoside *(Juliano and Stamp,1978)*, 5- fluoro-uracil *(Gregoriadis,1974)* and the closely related floxuridine *(Mathias et.al,1977)*, methotrexate *(Kimelberg and Tracy,1978)*, 8-azaguanine and 6-mercaptopurine *(Kano and Fendler,1977)*, bichloroethylnitrosurea *(Mayhew et.al,1976)*, cisdichlorobiscyclopentylamine platinum *(Deliconstantinos et.al,1977)* and doxorubicin, also known as Adriamycin^{R*} *(Olson et.al,1982)*.

Since drug tissue accumulation is a function of the concentration of free drug in plasma, tissue toxicity of anticancer agents including cardiac, nephro- and pulmonary toxicity may be reduced by confining the drug in a carrier. Peak plasma concentrations and tissue accumulation can therefore be avoided and a prolonged therapeutic plasma concentration achieved, due to "slow" drug release.

This concept has been employed successfully when doxorubicin was encapsulated within liposomes.

Doxorubicin is the most widely used agent against various solid tumours and leukaemias *(Blum et.al,1979)*. Despite the potency, its clinical usefulness is hampered by delayed, irreversible cardiotoxicity *(Ugoretz ,1976)* and acute nausea and vomiting. Various researchers *(Forssen and Tokes,1981;Shinozawa et.al,1981;Gabizon et.al,1982&1986;Mayhew et.al,1983;Rahman et.al,1986)* in a number of laboratories have demonstrated in experimental animals that liposomal doxorubicin is as effective as the free drug and is several times less toxic to the heart. More recently investigators have shown that the encapsulated drug also causes significantly fewer side effects in human studies *(Gabizon et.al,1986)*.

These results may be attributed to a modification of organ distribution of drug; accumulation of liposome associated doxorubicin in cardiac tissue is greatly reduced, while drug-containing liposomes accumulate in the liver *(Gabizon et.al,1986;Mayhew et.al,1983;Rahman et.al,1986)*, a phenomenon known as RES loading. This leads to the working hypothesis that liver cells, following sequestration and degradation of liposomes, provide a "depot" for the drug that gives rise to prolonged low plasma concentrations and presumably, to the improved efficacy of doxorubicin against liver metastases (due to high local concentrations).

Antifungal Agents

Systemic fungal infections are frequently seen in patients whose resistance is depressed by disease or medications that supress the immune system. These infections are a common cause of death in victims of acquired immune deficiency syndrome, AIDS, and are common in cancer patients undergoing chemotherapy.

They often resist treatment because use of the antifungal drugs is limited by toxicity.

In studies with mice , liposomal amphotericin B, a powerful antifungal agent, cured systemic fungal infections more effectively than the free drug *(Lopez-Berestein et.al.1983)*. Amphotericin B binds more strongly to ergosterol, the primary fungal sterol, than to cholesterol, it's mammalian cell conterpart *(Medoff et.al.1983;Hamilton-Miller.1973;Chen and Bittman;1977;Readio et.al;1982;Edwards.1980)*. However, the clinically utilised formulation of amphotericin B has a number of serious adverse side effects, most especially severe nephrotoxicity *(Medoff et.al.1983;Pratt.1977)*. The adverse actions of the drug in humans are likely to be due, at least partly, to the ability of amphotericin B to interact with cholesterol and form pores in the cellular membranes of the kidney and cardiovascular system. Amphotericin B binds readily to the cell wall of fungi, is lipid soluble and therefore is incorporated into the membrane of liposomes. It is postulated that the fungi "pulls" the drug out from the liposome membrane, in a process similar to lipid exchange and are destroyed.

It has been shown that incorporation of amphotericin B into certain types of liposomes can markedly reduce the toxicity of the drug without loss of antifungal potency *(Juliano et.al.1985)*. Studies in animals and in patients *(Lopez-Berestein et.al.1983&1985)* support the idea that amphotericin B in multilamellar vesicles composed of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidyl-glycerol (DMPG) remain therapeutically effective and significantly less toxic than the free drug. The reduced toxicity in vivo may be due to various causes including sustained or slow release of the drug, altered tissue kinetics and distribution or effects on the immune system *(Poznansky and Juliano.1984; Juliano.1981)*.

Antimicrobials

The recognition that liposomes accumulate in the RES led to their use in the treatment of certain parasitic diseases where microbes reside within the macrophages of the liver and other tissues of the RES. The access of free drug to such sites is restricted, making infections there difficult to treat by conventional means. Several workers have described the efficiency of liposomal drugs against visceral leishmaniasis in animals (for a review, see *Alving,1986*). Leishmaniasis is a parasitic disease infecting many individuals throughout the world. The parasites invade cells of the liver and spleen and can be lethal if the infection remains untreated. Therapy involves multiple dosing with drugs (intra-muscularly), organic antimonials, which are toxic and in sensitive individuals there is a risk of damage to the heart,liver and kidney. Alving et.al *(1978)* and Black et.al *(1977)* independently found that encapsulating antimonial drugs in liposomes reduced the dosage required to treat leishmaniasis. This therapy takes advantage of the passive targeting of the carrier to the liver and spleen.

Liposomes have also been used in the treatment of other infections that involve macrophages, including brucellosis *(Fountain et.al,1985)*, listeriosis *(Bakker-Woudenberg et.al,1985)* and acute salmonellosis *(Desiderio and Campbell,1983a; and 1983b)*.

Diagnostic Radiology

Because of the preferential delivery of liposomes to the liver and spleen in diagnostic radiology, liposomes containing either radio-opaque water soluble dyes or radio-opaque lipids have been used successfully for causing image enhancement of liver and spleen by computed tomography *(Ryan et.al;1984;Caride et.al,1984; Seltzer et.al,1984)*.

Miscellaneous Uses

Liposomes have extensive use outside the medical field. One novel example is in the maturation of cheeses by proteases contained within liposomes *(Piard et.al. 1986)*.

1.3. LIPOSOMES: PREPARATION, STABILITY, INTERACTIONS WITH BODY FLUIDS AND CELLS

In the early 1960's Bangham and his collaborators at Cambridge made the observation that phospholipids dispersed in water formed multilayered vesicles. Each layer was formed from a bimolecular lipid membrane, and these layers enclosed internal aqueous compartments *(Bangham et.al.1965)*. It rapidly became apparent that these artificial structures, which came to be known as liposomes, closely resembled cellular membranes. These new model membranes soon became popular tools for biochemists, cell physiologists and more recently, for investigators concerned with drug therapy. Their various applications have been extensively investigated *(Gregoriadis,1982;Kaye,1981;Ryman et.al,1980)*. A simplified diagram of a liposome is shown in Figure 1.

Liposomes are of potential interest for the following reasons:

1. They are made of phospholipids which are natural constituents of all cell membranes so that toxicity would not be expected.
2. Entrapped drugs are physically separated from the environment so long as the liposomes remain intact. This can be used to protect drugs from enzymatic degradation. In addition, toxic compounds, eg.doxorubicin, can be carried in the bloodstream thus reducing cardiotoxicity (see page 7).

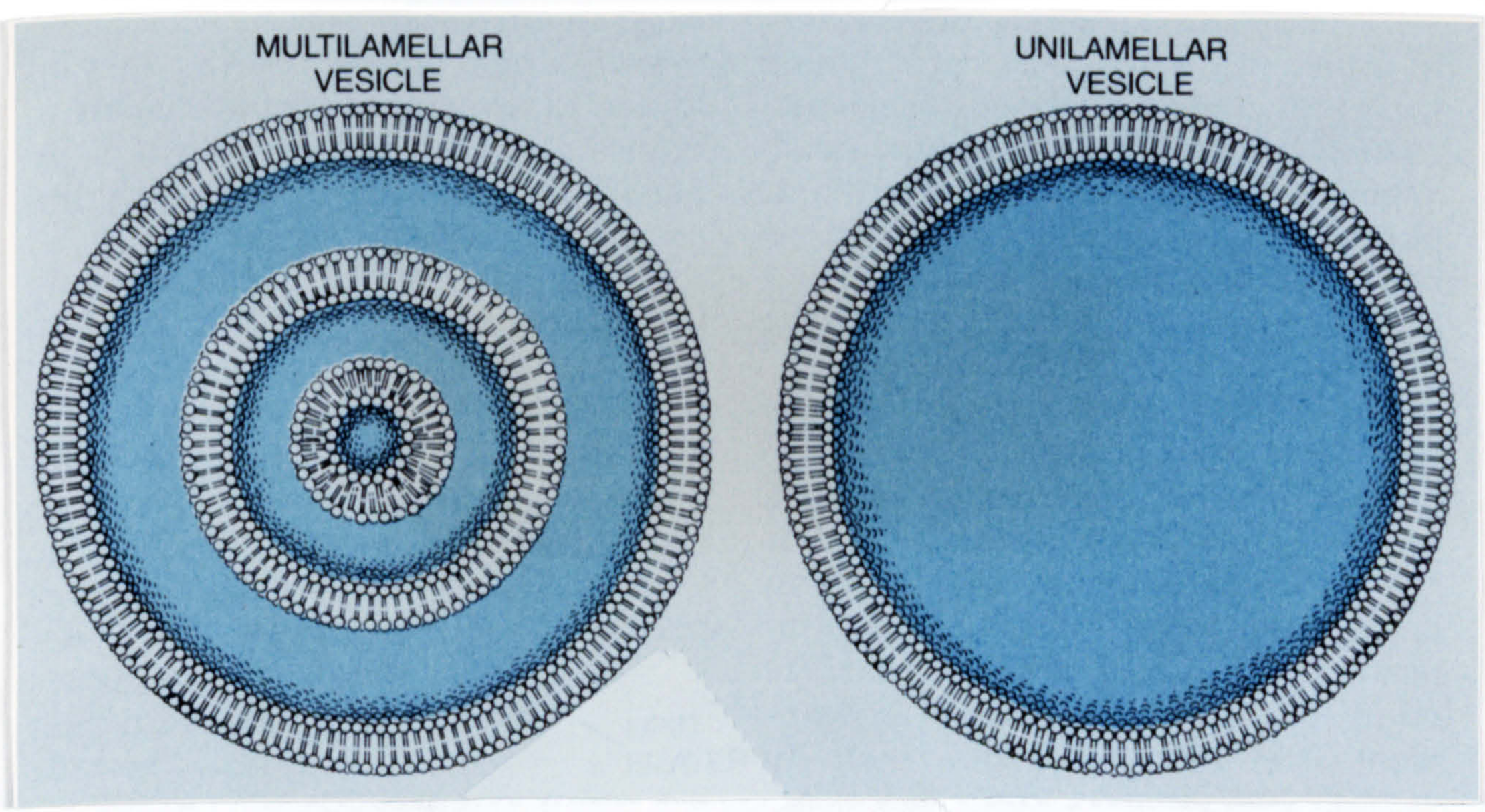


Figure 1: Schematic diagram of liposomes formed by mixing amphipathic lipids and an aqueous solution can be multilamellar (left) or unilamellar (right). Multilamellar vesicles have an "onion skin" structure in which concentric lipid bilayers are separated by aqueous layers (blue areas). Unilamellar vesicles consist of lipid bilayer surrounding an aqueous interior.

(Reproduced from reference, *Ostro,1987*)

3. In vivo free and "liposome-entrapped" drugs are likely to be delivered to different tissues or cell types after administration. While incorporated, liposome-entrapped compounds will follow the fate of the liposome and they will be released only at the site of liposome degradation. This allows newer possibilities in site specific delivery of drugs, or drug targeting.
4. Liposomes can accomodate both hydrophilic and hydrophobic drugs, with little or no need for chemical modifications of the drug. This is important for product registration purposes since existing drugs can be used in these novel formulations.
5. Liposomes can be prepared with a variety of properties, for example, differences in size, composition, surface charge, etc.

Therefore, it would seem possible to design an optimal carrier for different applications by careful selection of these parameters.

In view of their intrinsic properties therefore, liposomes should improve existing drug therapy by increasing efficacy and/or reducing side effects. Considerable research efforts have been devoted to investigations on the use of liposomes as drug carriers. Nearly 6500 articles and 139 patents have been published since 1971 and these numbers are increasing.

1.3.1. PREPARATION OF LIPOSOMES

The literature hosts a large number of preparation procedures ranging from the simple through the novel to the complex. Below are summarized a few of the standard techniques.

Multilamellar Vesicles

The original preparation *(Bangham et.al.1965)* of multilamellar vesicles (MLV) was achieved by simple mechanical shaking of a thin dry film of egg phosphatidyl-choline (PC) with an aqueous solution in a round-bottomed flask. This method produces a heterogeneous mixture of closed multilamellar vesicles of varying size. Most phospholipids studied so far have been found to produce such vesicles, including purified preparations or lipid extracts from tissues. The major advantage of MLV preparation is the simplicity of the procedure and the fact that it is applicable to a wide variety of different lipid mixtures. The encapsulated volume of MLV in terms of litres of aqueous space per mole of lipid is limited and several-fold less than that of unilamellar vesicles of comparable size. A simple procedure optimising entrapment levels for MLV has been described by Kirby and Gregoriadis *(1984)*. Preformed liposomes containing drug are flash-frozen as a thin film in a round bottomed flask, freeze-dried under vacuum and rehydrated under controlled conditions. The ability to freeze or rehydrate vesicles offers the added advantage of long term storage.

Small Unilamellar Vesicles

In addition to the simple procedure used by Bangham, numerous alternative methods of preparation have been described. Small unilamellar vesicles (SUV) can be obtained by sonication of MLV preparations *(Huang and Charlton,1971)*, by

injecting an ethanol solution of the phospholipids in an aqueous phase *(Batzri and Korn,1973)*. The particle size of MLV can also be reduced by extrusion at high pressure through a French Press *(Barenholz et.al, 1979;Hamilton et.al,1980)*. The yield of unilamellar vesicles and the vesicle size are dependent on the pressure . Barenholz et.al *(1979)* reported egg PC vesicles to be somewhat larger than that of sonicated SUV (31.5-52.5 nm) but Hamilton et.al. *(1980)* found the size of French Press SUV containing no cholesterol to be similar (15-30 nm) to that of sonicated SUV.

Large Unilamellar Vesicles

Large unilamellar vesicles (LUV) are produced by dialysing a detergent containing phospholipid solution *(Milsmann et.al,1978)* by injecting phospholipids dissolved in diethylether *(Deamer and Bangham,1976)* or petroleum ether *(Schieren, 1978)* into a warmed aqueous phase. Extrusion of MLV through polycarbonate membranes (0.1μm pore size) also results in the production of LUV with an average diameter, 0.9μm *(Hope et.al,1985)*. However, most of these methods show fairly poor entrapment yields for water soluble drugs, usually below ten percent.

The reversed phase evaporation technique *(Szoka and Papahadjopoulos,1978)* produces large unilamellar vesicles (0.1-0.9μm diameter), abbreviated to REV, which exhibit a high capture volume and have a high encapsulation efficiency. In this method, the drug containing aqueous phase is emulsified in the presence of phospholipids in diethylether. Evaporation of the solvent under vacuum forms large unilamellar vesicles. Recently the Double Emulsion Technique was developed *(Schneider,1977)*. In this method the drug-containing aqueous solution is dispersed and sonicated in a non water-miscible organic solvent ,for example, dibutylether, cyclohexane, etc. containing the phospholipids. The resulting water-in-oil emulsion is then introduced under agitation into a second aqueous medium thereby forming

a double emulsion. Evaporation of the organic solvent(s) under a stream of nitrogen, forms unilamellar vesicles with an average diameter of 0.15μm. These LUV possess an encapsulation efficiency in the range thirty to eighty percent.

1.3.2. SEPARATION OF FREE DRUG

When hydrophobic drugs of appropriate structure are associated with liposomes by inclusion in the bilayer phase, the degree of "encapsulation" is dependent upon the saturation of the lipid phase. Under these circumstances, it is possible to achieve degrees of encapsulation of over ninety percent, making it unneccesary to remove the unbound drug. However in the case of water soluble drugs, the encapsulated drug is only a fraction of the total drug used. An additional step is required to remove the unbound drug from the drug-loaded liposomes. Dialysis, centrifugation and gel filtration are some of the procedures used routinely for this purpose.

Dialysis

Dialysis is routinely used *(Olson et.al,1979)* for the removal of unentrapped drug, except when macromolecular compounds are involved. This technique requires uncomplicated and inexpensive equipment and is conveniently scaled up. Dialysis is effective in removing greater than ninety-five percent of all free drug with sufficient number of changes of the dialysing medium. However this process is slow. Typically, removal of this percentage of free drug in a liposomal dispersion might require a minimum of three changes of the external medium over a ten to twenty-four hour period at room temperature or in a cold room (4°C). Care must be taken to balance the osmotic strengths on either side of the membrane or the volume of the liposomal dispersion will alter during dialysis. It is also possible

that the presence of the external dialysing medium in equilibrium with the vesicles might induce leakage of the encapsulated drug.

Centrifugation

Centrifugation at various speeds is an effective means of isolating liposomes from the free drug in the suspending medium. Two or more resuspensions and spin stages are usually included to effect a complete removal of the free drug. The centrifugal force required to pull liposomes down into a pellet is dependent on the size of the liposomes. High speeds (>100,000*g*) and refridgerated conditions are required for liposomes in the small to medium size ranges. The use of refrid-gerated centrifuges operating at high speeds with large volumes (for large scale preparations) of liposomal dispersions is energy intensive and expensive. Therefore, it may not be suitable for the isolation of small liposomes . For relatively large liposomes, low centrifugation speeds around 1000*g* offers the advantages of a short time scale of operation. However, the osmotic strength of the resuspending medium must be equal to that of the original liposomal dispersion to minimise osmotic shock and rupture of vesicles.

Gel Filtration

Gel permeation chromatography is used extensively both to separate liposomes from unbound drug and to fractionate liposomal dispersions containing a mixture of various sizes of vesicles. The technique is very effective and rapid under laboratory conditions. Although gel filtration is used in the purification of biological materials such as insulin on a large scale, the technique is slow and laborious. Additionally, dilution of the liposomal suspension with the eluting medium may necessitate another concentration step which could damage the vesicles.

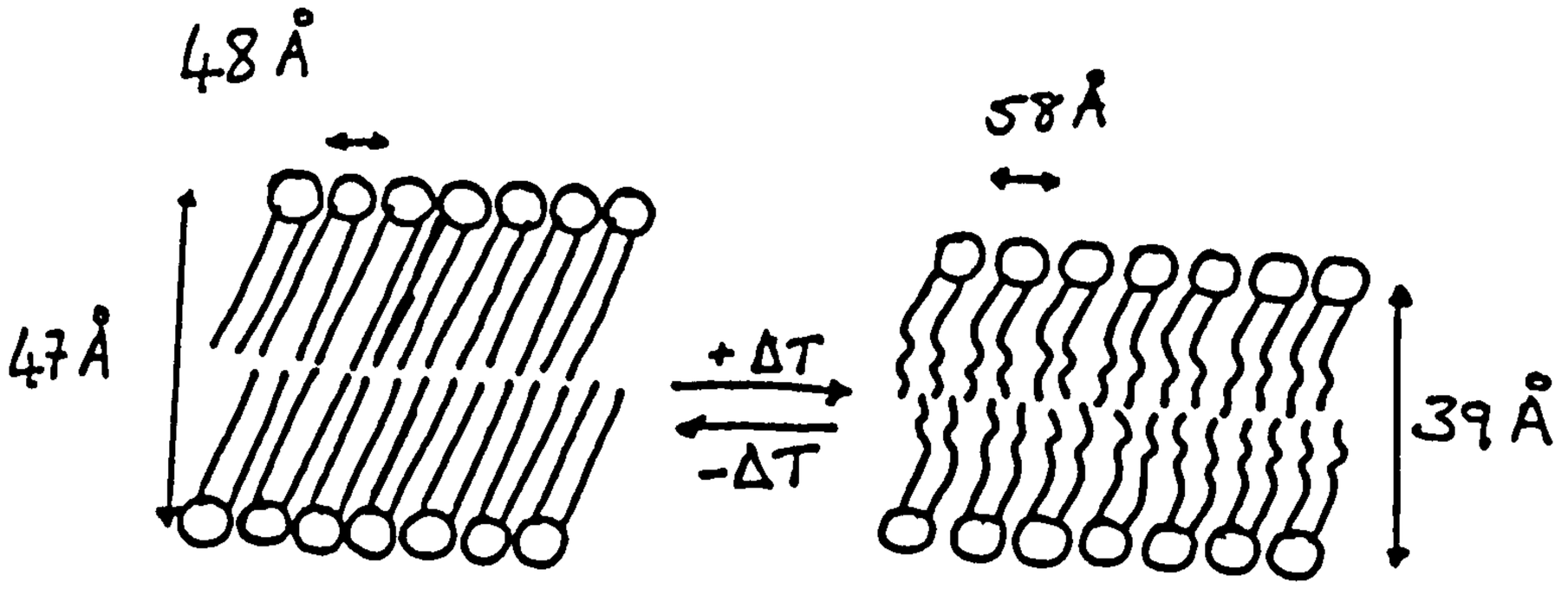
Ultrafiltration

Ultrafiltration has been used *(Rao,1984)* for the separation of non-associated solutes from liposomes, but its use is not recommended since considerable amounts of non-entrapped material often remains attached to the filtration membrane after long periods of concentration and dilution. It has more potential for concentrating liposomes which have been diluted during column chromatography to separate non-associated material.

1.3.3. COMPOSITIONS AND TISSUE DISTRIBUTION OF LIPOSOMES

Besides the size and the structure, there are other parameters which can be conveniently altered, most notably, the lipid composition, the surface charge and the membrane fluidity. The most generally used phospholipid components include egg phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG) and sphingomyelin (SM). Synthetic lecithins such as dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC) and dimyristoylphosphatidylcholine (DMPC) are also often used. In addition to phospholipids, large amounts of other lipids can be incorporated into the liposome membrane. Cholesterol, which by itself does not form vesicles, can be incorporated (upto 50 mol%) into the phospholipid lamellae. Cholesterol has been extensively used to reduce the leakage of entrapped water-soluble drugs and to increase the stability of liposomes in plasma (see discussion, page 97). The net surface charge of liposomes can be modified by addition of other long-chain amphiphatic molecules such as, stearylamine (for positively charged liposomes) or compounds such as phosphatidylserine, phosphatidic acid or dicetylphosphate for negatively charged liposomes.

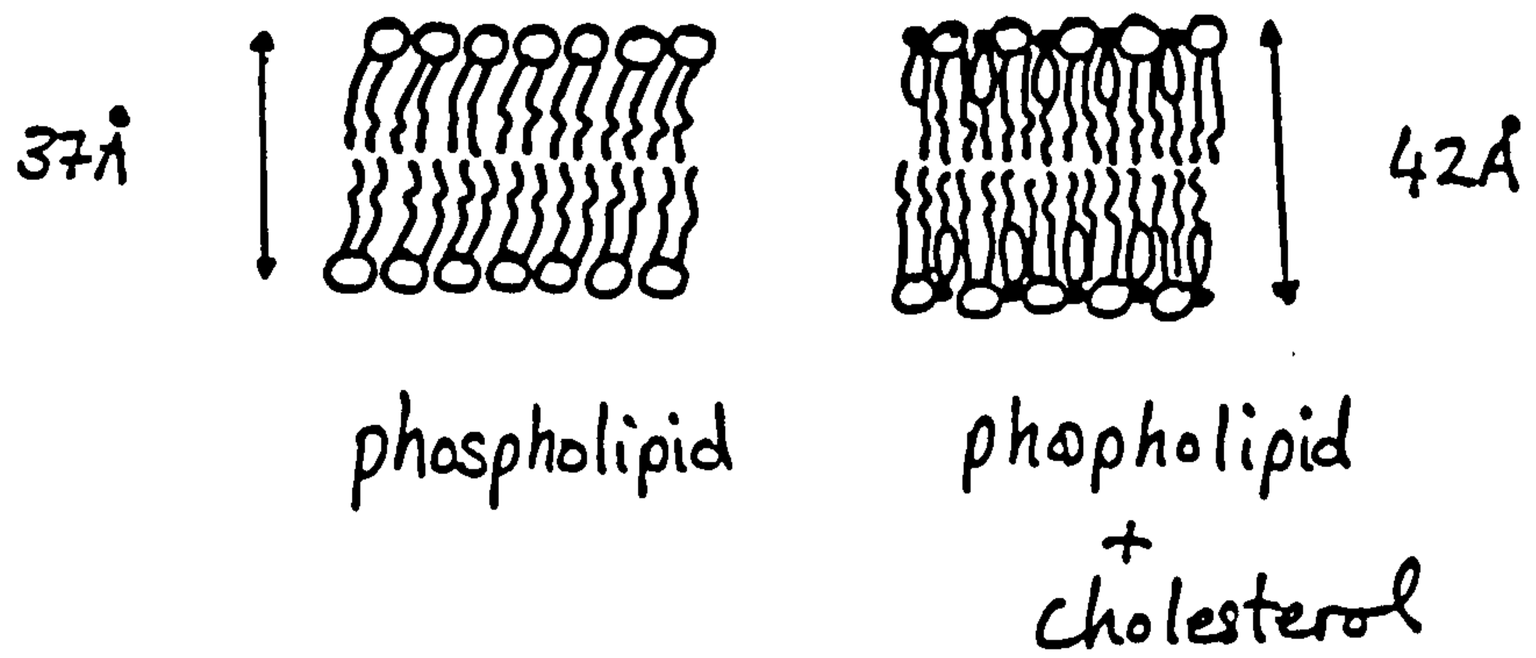
Another important parameter, the phase transition temperature (T_c) of the fatty acyl groups of phospholipids has been shown to influence the composition and permeability of liposomes and alter their interactions with isolated cells. At temperatures above the T_c, liposomes are designated as "fluid" whereas below this temperature they are "solid". In the fluid form, the permeability is appreciably higher than in the solid form. This T_c is characteristic for each phospholipid species, is defined mostly by the configuration of the acyl chain and also the degree of hydration and chemistry of the head groups *(Williams and Chapman,1970)*. Thus, the presence of cis-double bonds, branching and decreasing chain length tend to reduce the T_c. Shown below is a schematic representation of molecular orientation in phospholipid bilayers below and above the phase transition.



The head groups of the phospholipid molecules are represented by open circles and fatty acids by solid lines. Tightly packed "solid" acyl chains showing inhibited motion by NMR are represented by straight lines. Mobile (liquid) acyl chains are represented by curved lines. The dimensions shown in the above scheme relate to those reported with DPPC bilayers by Lesslauer et.al.*(1972)*. Permeability of liposomes for various solutes increases markedly at T_c of the lipids *(Fukuzawa et.al;1979)*. An interesting practical implication of this phenomenon has been suggested by Yatvin et.al *(1978)* for selected drug delivery. Local hyperthermia may be artificially induced to increase the specific release of an entrapped drug

at defined body areas, for example, tumours heated to a few degrees above body temperature by microwave irradiation.

Incorporation of cholesterol into the phospholipid membranes produces considerable restiction of molecular motion in the region of the first eight to ten carbon atoms of the acyl chain from the lipid-water interface. This effect is accompanied by a condensation of the area per phospholipid molecule, a more perpendicular orientation and a thicker membrane *(Darke et.al.1971)*.



Above is a schematic representation of the molecular architecture of a phospholipid bilayer with and without cholesterol. The graphic representation for phospholipid molecules are as previous, page 18. Cholesterol is represented by a small circle (hydroxyl), a larger circular area (the four-ring steroid skeleton) and a line (hydrocarbon tail). The dimensions are from X-ray data of Levine and Wilkins *(1971)* with egg PC-Cholesterol multilayers. Addition of cholesterol (50 mol%) abolishes the T_c *(Op den Kamp et.al;1975)* and diminishes liposomal suseptibility to protein interactions *(Tall and Lange,1978;De Kruyff et.al,1974)*. Values for T_c of the lipids and surfactant used in this study are included in Appendix 1, page 160.

The tissue distribution and clearance kinetics of liposomes containing drugs are affected by phospholipid composition, size and charge *(Juliano and Stamp,1975;*

Rahman et.al,1982). Smaller liposomes were shown to be better than larger liposomes for specific delivery of their contents to calf thymus cells in vitro *(Machy and Leserman,1983).* Sucesses have been demonstrated for liposomes including in vitro targeting through covalent attachment of proteins onto the surface of liposomes *(Leserman et.al,1981;Heath et.al,1983)* and more recently in vivo targeting for hepatic parenchymal has been achieved using liposome bearing galactose residues (*Van Berkel et.al,1986*) The interactions of liposomes with cells in vivo occur in a complex biological milieu and the binding of proteins from this milieu may also have important effects on liposome behaviour *(Juliano and Lin,1980).*

By using different lipids and modified preparations, a variety of liposomes can be designed, varying in size, structure, surface charge, composition and membrane fluidity. However further changes in these various parameters are obtained after in vivo administration, resulting in anomalous distribution patterns and behaviour, than originally predicted.

1.3.4. STABILITY OF LIPOSOMES

A given liposomal formulation must have adequate stability over the time period between its preparation and ultimate use. This includes the physical stability of the liposomes in terms of the integrity of the encapsulated material and the size parameters and the chemical stability of the component materials (lipids and drugs). Thus a liposomal drug product must show an adequate "shelf life" under normal conditions of storage. In addition, the liposomes should maintain their integrity in vivo until entry into the target tissue or until their function of sustained release is completed.

Studies on the stability and effective storage of liposome preparations are necessary for their development as a drug delivery system. "Shelf life" stability encompasses a number of parameters and the following aspects should be examined:

a. maintenance of the vesicle size, including an examination for the aggregation of units with time;

b. maintenance of the structure of the lipids;

c. chemical stability of the lipids; and

d. influence of biological fluids on the integrity and permeability properties of the liposome.

Liposomes can become physically unstable on storage either due to leakage of the encapsulated drug into the suspending medium and/or due to aggregation or fusion of the liposomes to form larger entities. It is important that a liposomal drug product remains stable over a period of time, preferably under ambient conditions of storage. The increase in size and drug leakage on storage of small unilamellar liposomes were found to be dependent upon the nature both of the lipid components and of the encapsulated marker compounds *(Frokjaer et.al.,1982)*. Drug retention behaviour of a range of liposomal compositions under various conditions of storage has been cited *(Szoka and Papahadjopoulos,1980)*.

The chemical stability of drugs incorporated in liposomes is outside the scope of this discussion. However, the lipid components of a liposomal system are subject to various chemical degradations on storage. The oxidative decomposition of egg PC in liposomes can be retarded considerably by addition of alpha-tocopherol to the lipid mixture *(Hunt, 1982)*. Hence particular care is required when handling unsaturated phospholipids subject to this type of oxidative degradation.

Unsaturated and saturated phospholipids are subject to hydrolysis in aqueous media, resulting initially in the formation of the corresponding lysophospholipid and fatty acid *(Frokjaer et.al,1982)*. These reactions have implications both for the integrity of the liposomes and the leakage of encapsulated drugs.

To optimise the potential of liposomal carriers, it is important to characterise their stability in terms of controlled release of their contents in vitro, simulating physiological conditions in vivo. The stability of various types of liposomes in the presence of blood components has been reviewed *(Gregoriadis et.al,1981 ; Scherphof et.al,1981)*. The chemical nature of the phospholipid, the proportions of cholesterol and the size (and lamellae) of the liposomes all appear to influence their in vivo stability. It is therefore necessary to design and test liposomal systems for adequate stability in vivo.

It is possible to conceive a liposomal drug carrier system for storage either as a normal dispersion or in a form to be reconstituted before use. If the problem of drug leakage can be resolved presentation as a ready to use dispersion has obvious advantages due to simpler preparation techniques. A reconstitutable form or kit, for example, a freeze-dried preparation will avoid both chemical and physical stability problems in storage. Inclusion of trehalose or other such sugars, has been shown to be effective in preventing fusion and leakage from liposomes during freeze drying. Retention of upto 100% of an entrapped water soluble marker and prevention of fusion has been reported (*Madden et.al,1985;Strauss et.al,1986)*.

With increasing therapeutic application and progression into human clinical trials, it will be necessary to routinely prepare sterile batches of vesicles. Aseptic techniques can be applied to most stages of these preparations. Antibiotics may be added to the aqueous phase together with the therapeutic agent in liposome

preparation if necessary. Sterility of smaller vesicles can be achieved by passage through 0.22μm sterile membrane filter, or alternatively, radiation sterilisation may be employed *(Gregoriadis,1976)*. However it must be emphasised that sterility must be maintained throughout the preparation if the liposomes are to pass rigorous pyrogen testing protocols *(Tyrrell and Ryman et al,1976)*.

Measurement of Stability in Biological Fluids

The stability of the vesicles is defined in this context as their capability to retain the structural integrity of the closed lipid bilayer and to prevent leakage of their aqueous contents. The original method used to study permeability of lipid vesicles by Bangham et.al *(1965)*, involved the use of isotope tracers and has been used extensively for the study of efflux rates for ^{22}sodium, ^{42}potassium, ^{36}chloride, ^{86}rubidium and ^{14}glucose. A modification of this method used by Kinsky et al. *(1966)* monitored the spectrophotometric reduction of nicotinamide dinucleotide. Other, non-isotopic markers such as chromate ions have been used *(Weissmann et.al,1966)* to study the permeability of liposomes.

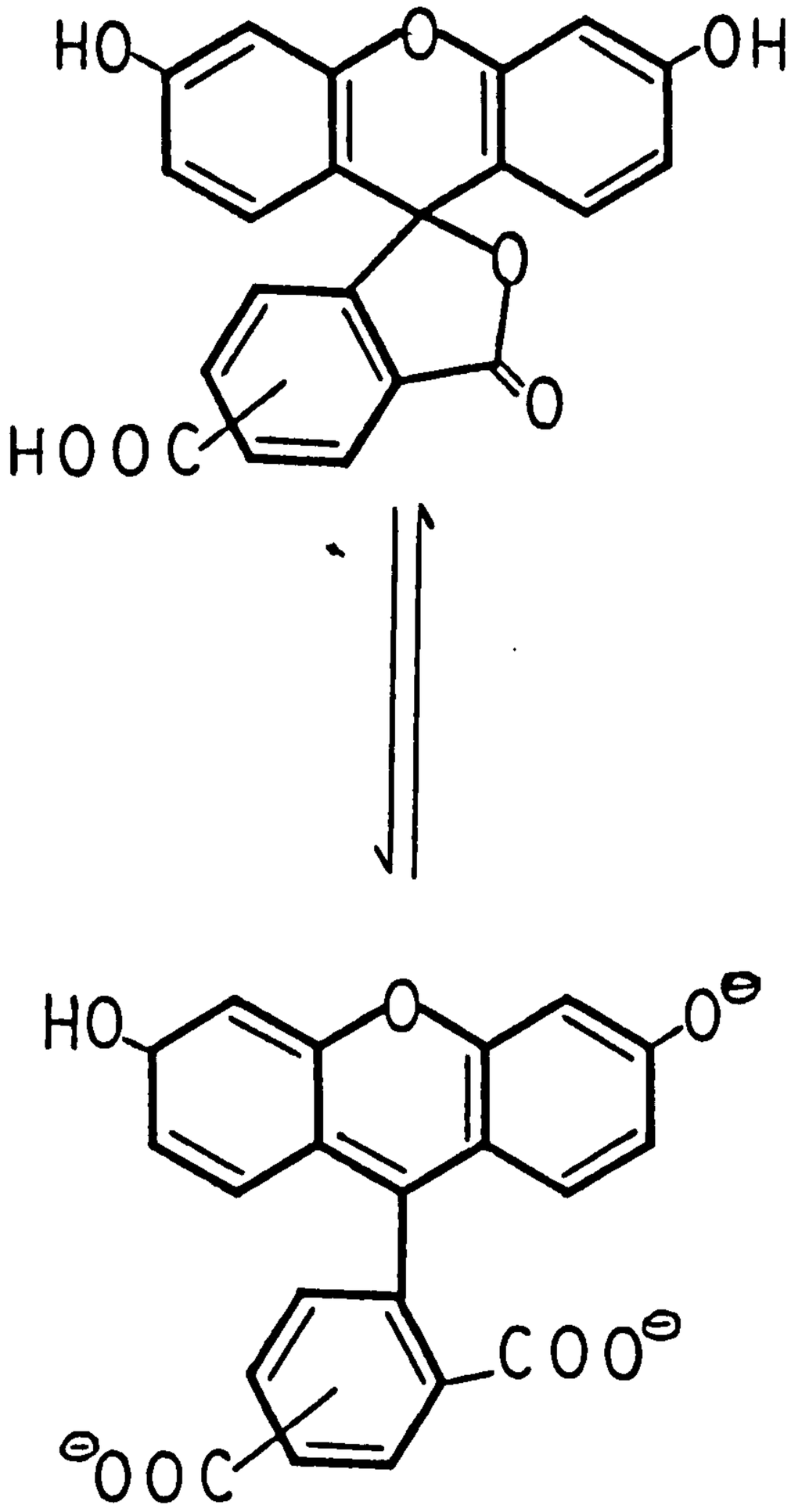
Fluorescent Markers

A useful method to study cell-liposome interactions employs the use of water-soluble markers *(Pagano and Weinstein,1978)*. The fluorescent dye 5,6-carboxy-fluorescein (CF) was specially prepared *(Weinstein et.al,1977)* as such a marker. This highly water-soluble dye has since been used by a large number of laboratories for many studies. CF has been widely used for studying liposome-cell *(Weinstein et.al,1977;Blumenthal et.al,1977;Leung,1980; Van Reswoude and Hoekstra,1980)*, cell-cell *(Dahl et.al,1981)* and liposome-liposome *(Wilschut and Papahadjopoulos,1979)* interactions. It has also been employed for monitoring enzymatic attack on intact liposomes *(Chen,1977)* or perturbation of the lipid

bilayer structure by serum proteins *(Yatvin et.al.1978;Guo et.al.1980)*. The concentration dependent self quenching of CF fluorescence permits leakage from liposomes to be monitored continuously and hence a distinction can be made between endocytosis of liposomes, binding of liposomes to the cell surface and direct transfer of liposome contents to the cytoplasm *(Weinstein et.al.1977)*. However this technique has its own limitations.

Structurally, CF resembles fluorescein but has an extra carboxyl group located at the 5- or 6- position. It has an excitation maximum at approximately 492nm and an emission maximum about 520nm, similar to fluorescein. The latency of CF entrapped in liposomes is much greater than fluorescein due to the additional carboxyl group which decreases the butanol-water partition coefficient ,that is, it's lipophilicity is decreased, by about three orders of magnitude over a broad range of concentrations and pH's *(Grimes et.al.1982)*. This makes CF a better alternative than fluorescein. The most-water soluble and fluorescent form of CF is the trivalent anion predominant at neutral and alkaline pH's *(Weinstein et.al.1986)*, as shown in figure 2. In practice, it is important to calibrate the fluorescent data for the ambient pH, usually pH 7.4, and to be aware that the less soluble forms present in acid medium have a greater tendency to bind to proteins, detergent molecules and other organic materials *(Lelkes and Tandeter.1982)*. Hence when experiments with protein solutions are conducted, the buffer should be at pH 7.0 or higher.

Typical of most aromatic fluorophores *(Parker.1968)*, the fluorescence of CF decreases slightly as the temperature is raised. This decrease results primarily from an increased non-radiative transfer of energy from the excited singlet states of oxygen by intermolecular collision *(Weinstein et.al.1984)*. CF is not a single component dye but is a mixture of different ingredients that show at least six



(at low pH)

(at pH >6.5)

Figure 2: The structure of Carboxyfluorescein (CF); molecular weight, 376.

spots on silica gel thin layer chromatography and exhibits at least six different absorption peaks in the range 440-500nm.

CF can be purified by a method described by Ralston et.al *(1981)*. The fluorescence emission of CF is sensitive to the presence of biological fluids. Experiments have indicated that in a variety of sera, for example, mouse, horse, bovine, foetal calf and human, the fluorescence emission is partially quenched with a decrease in the excitation spectrum *(Lelkes and Tandeter,1982)*. However, despite these drawbacks, experiments can be suitably controlled and CF has proved to be a useful marker in many studies.

As an alternative marker, the fluorophore calcein has also been used *(Allen and Cleland,1980)* which is more water soluble at low pH than CF. Calcein is chemically a combination of fluorescein and ethylenediaminetetraacetic acid and it shares properties of both these compounds *(Diehl and Ellingboe,1956)*. It was first prepared by the interaction of fluorescein, formaldehyde and iminodiacetic acid and it's structure determined *(Wallach et.al,1959)*, as shown in figure 3. Calcein carries a higher net negative charge at physiological pH and possesses a higher molecular weight. It's rate of efflux, therefore, from liposomes is slower because the overall negative charge reduces it's permeability through the membrane. Also, calcein does not show a pH-dependent quenching in the physiological pH range *(Allen,1984)*.

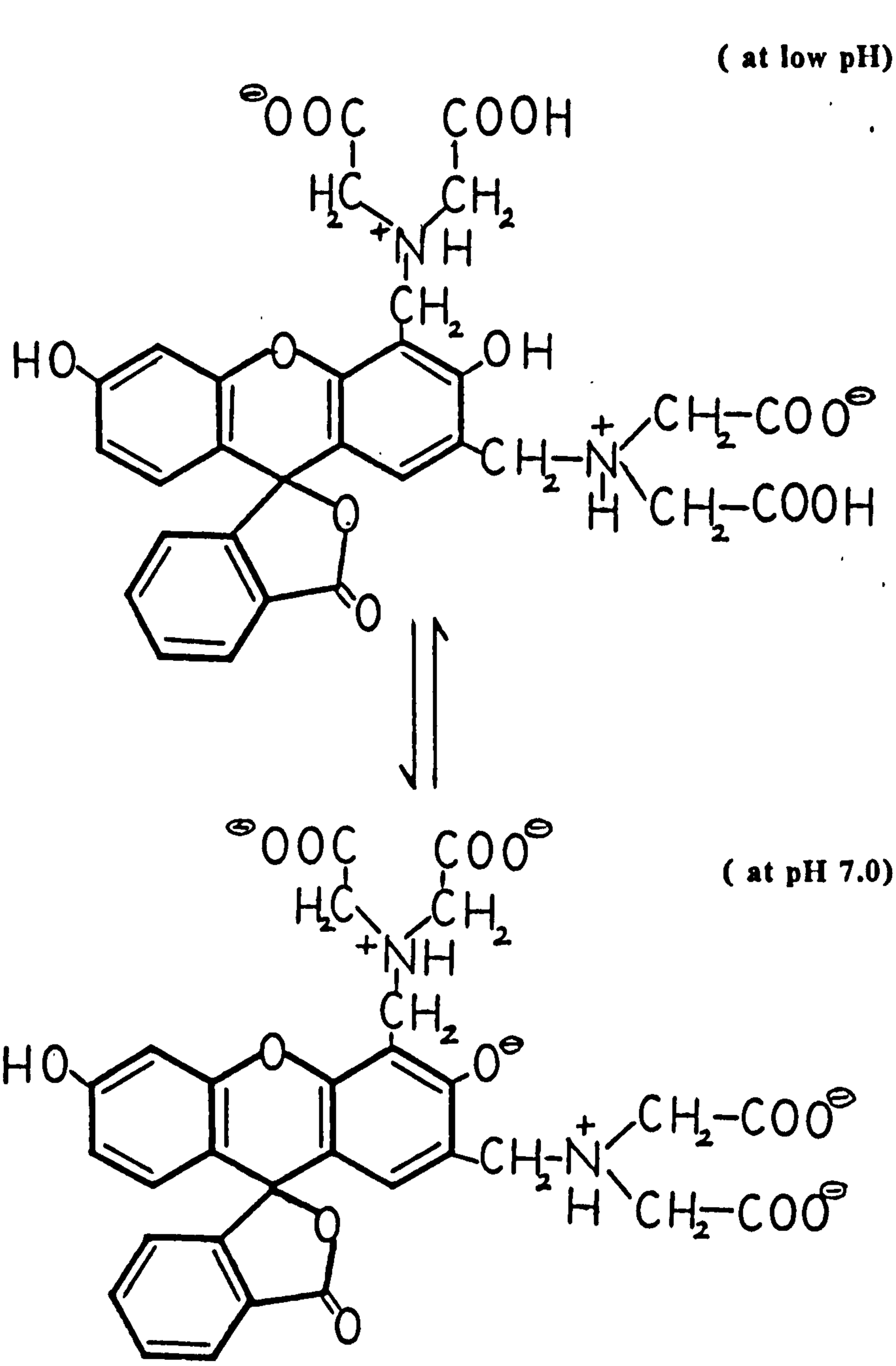


Figure 3: The structure of Calcein; molecular weight, 623.

1.3.5. LIPOSOME INTERACTIONS WITH BLOOD PROTEINS

There have been a number of studies of the interaction of liposomes with proteins and complex mixtures of proteins such as serum. The intent of these studies has been :

1. To examine the proteins capable of binding to liposomes.
2. To assess the stability of liposomes in the biological environment.

The initial event, in vivo, to "foreign" materials and surfaces after contact of blood is the adsorption of a protein layer which "conditions" the surface of the material for further interactions *(Baier and Dutton,1969)*. This layer is believed to consist of proteins originating from plasma.

Proteins constitute the major part of the soluble material in blood, where their concentration is normally 70-80 grams per litre. The number of different proteins totals more than one hundred and fifty.

The most abundant protein found is serum albumin (70%) which has a relatively low molecular weight, approximately 66,000-69,000 daltons and a plasma concentration of 40 grams per litre. It acts as an important plasma expander and as a carrier for substances such as hormones, fatty acids and vitamins *(Spector and Fletcher,1977)*. It has a strong binding affinity for a number of other substances, for example, certain drugs such as acetylsalicylic acid *(Hawkins et.al,1968)*. Other compounds such as fluorescein also bind loosely *(Andersson et.al,1971)* to albumin mainly through hydrophobic and electrostatic interactions. The electrostatic contribution is much less than would be expected; this is probably related to the fact that fluorescein is a non-physiological compound and is "non-specifically" bound to the albumin.

Immunoglobulin G (IgG) also binds to many surfaces exposed to blood or plasma *(Horbett and Weathersby,1981)*. IgG has a concentration of 8-17 grams per litre in normal adults and a molecular weight approximating 150,000 daltons. The molecule comprises of two "heavy" chains (m.wt. 50,000 daltons) making up sixty-seven percent of the molecule and two "light" chains (m.wt. 25,000 daltons) which account for the remaining thirty-three percent of the molecule.

Fibronectin is a high molecular weight (220,000-250,000 daltons) glycoprotein found in a soluble form in blood and other body fluids and in an insoluble form in tissues. The concentration of fibronectin in plasma is around 300μg/ml. Recent reviews demonstrate the role of fibronectin *(Mosher,1984;Ouaissi and Cabron,1985)* but there is only little information about it's interaction with membrane phospholipid components. Rossi and Wallace *(1983)* has shown the binding of fibronectin to phospholipid vesicles of a number of compositions. This binding of fibronectin to liposomes can modulate liposome interaction with cells. Hsu and Juliano *(1982)* showed that fibronectin could enhance liposome uptake by macrophages, presumably by interacting with cell surface fibronectin receptor *(Brown and Juliano,1985)*.

Fibrinogen absorption from plasma or blood to a wide variety of surfaces has been observed by a number of investigators *(Horbett and Weathersby,1981;Horbett,1981; Inlenfeld and Cooper,1979;Vroman et.al,1980)*. Fibrinogen is a high molecular weight plasma protein (m.wt. 340,000 daltons) and is necessary for the formation of blood clots. Fibrinogen has been shown to adsorb to glass *(Brash et.al,1984)*, after contact with blood and appears to be an important constituent of the adsorbed protein, even though it is known that the absolute surface concentration is low. Previous data *(Horbett and Weathersby,1981)* suggest that relative to other blood proteins such as albumin, IgG and haemoglobin, fibrinogen is enriched on

the surface compared to the plasma phase by a factor of two or three depending on the nature of the surface.

The use of liposomes for drug delivery necessitates the examination of the effects of various biological fluids on the stability of liposomes. Liposomes recovered from plasma were found to have altered electrophoretic mobilities *(Black and Gregoriadis,1976)*, indicating possible adsorption of plasma proteins. The same study showed that alpha-2 macroglobulin bound strongly to the surfaces of liposomes, which had a composition of:

a. egg PC and cholesterol, molar ratio 7:2 (neutral),

b. inclusion of 10% phosphatidic acid, molar ratio 7:2:1 (negative) and

c. with 10% stearylamine, molar ratio 7:2:1 (positive).

This was the only protein found specifically bound to liposomes, irrespective of size or charge on the vesicle. Alpha-2 macroglobulin was positively identified by immunoelectrophoresis technique *(Clarke and Freeman,1968)* against anti-(human alpha-2 macroglobulin). Serum components such as IgG *(Weissmann et.al,1975)* and lipoproteins *(Morrisett et.al,1977)* also bind strongly to various types of liposomes. Both neutral and positively charged liposomes have been shown *(Juliano and Lin, 1980)* to bind a group of high molecular weight (>200,000 daltons) polypeptides which did not appear to be bound by negatively charged vesicles. Some of these bound proteins could readily be identified as major blood components, such as, albumin, IgG subunits, apoprotein A_1, alpha-2 macroglobulin, whereas other bound proteins were not readily identified and probably represented minor serum components. The pattern of bound proteins was highly dependent on the chemical and physical properties of the liposomes. For example, while negatively charged liposomes bound an apparently random sample of serum proteins, positive and neutral liposomes tended to bind selectively to a group of high molecular weight (>200,000 daltons) protein components. Binding of these protein components was

very rapid (one minute at 37°C), essentially irreversible, and the components remained at the outer surface of the liposome and did not disrupt the vesicle structure.

These workers *(Juliano and Lin,1980)* also reported marked changes on the clotting ability of plasma after exposure to either positive or negative liposomes. This effect may be a result of the depletion of one or more clotting factor(s) by its binding to the liposomes. Further studies, by the same group, showed that clotting factor VII binds to liposomes irrespective of charge, where as factor VIII and fibrinogen bind to liposomes, which carry a positive and negative charge.

Albumin can also interact with small unilamellar vesicles (SUV), bringing about a release of entrapped markers and mediating transfer of phospholipid from the vesicle to itself *(Zborowski et.al,1977)*. The injection of liposomes in vivo or their incubation with plasma in vitro leads to a major alteration in their size and physical charecteristics *(Krupp et.al,1976)*.

Serum proteins also promote the efflux of the fluorescent dye calcein from SUV *(Allen and Cleland,1980)*. As found by others, these vesicles could be stabilised against protein disruption by the inclusion of cholesterol.

The stability of liposomes in vivo (blood circulation) and in vitro (in the presence of serum, plasma or whole blood) is affected by the liposome cholesterol content *(Kirby et.al,1980a)*. Incorporation of cholesterol into liposomes condenses the phospholipid molecules above their T_c and reduces permeability to solutes *(Demel and DeKruft,1976)*. This additional "compact packing" of phospholipids by cholesterol can help prevent their removal by high density lipoproteins found in plasma and preserve liposomal stability in serum *(Gregoriadis et.al,1981)*.

One implication of these studies is that, upon injection into the blood, liposomes rapidly become irreversibly coated with a layer of serum proteins which alters their surface properties. The nature of this layer (which differs for different types of liposomes) is a main determinant of the clearance kinetics and tissue distribution of injected liposomes. Active research is still being pursued in this important area.

Surface Potential and its Determination

Natural membranes bear net surface charges due to the presence of ionized groups in their lipids and proteins *(McLaughlin,1977)*. The association between fixed surface charges and the free ions in the surrounding aqueous medium, gives rise to an interfacial electric field which determines surface potential *(Avegard and Haydon,1973)*. A large number of biological phenomena, from cell adhesiveness to membrane permeability are influenced by surface potential *(McLaughlin,1977; Avegard and Haydon,1973)*. As a consequence, many attempts have been made to measure this parameter *(Gaffney and Mich,1976;Haydon and Myers,1973)*. The surface potential is an important parameter influencing liposomal behaviour. In vivo, surface charge density has been found to influence the distribution of liposomes *(Rahman et.al,1980)* and in vitro, a high potential might contribute to their physical stability by reducing aggregation and fusion *(Frokjaer et.al,1982)*.

The charge that develops at the surface of a colloidal particle may arise from any of several mechanisms, depending on the nature of the particle and its surrounding medium *(Hunter,1981)*. For particles dispersed in liquids, two of the most important factors are the ionisation of groups on the particle surface and the differential adsorption of ions of opposite charge from solution. The development of a net charge at the particle surface affects the distribution of ions in the surrounding interfacial region, resulting in an incresed concentration of counter

ions (that is, ions of charge opposite to that of the particle), close to the surface. Thus, an electrical double layer is formed around each particle. The double layer may be considered to consist of two parts: an inner region that includes ions bound relatively strongly to the surface by adsorption, and an outer or diffuse region in which ion distribution is determined by a balance of electrostatic forces and random thermal motion. In the region of the particle, the potential decays with distance from the surface eventually reaching zero in the bulk solution. An individual particle and its most closely associated ions move through the solution as a unit and the potential at the boundary of this unit, that is, at the surface of shear between the particle with it's ion atmosphere and the surrounding medium, is known as the **zeta potential**.

In simplest terms, a vesicle with a net negatively charged surface, suspended in an electrolyte solution in an electrophoresis apparatus, will attract ions of opposite sign to form the so-called Stern layer around itself (see Figure 4). The relatively fixed ions of the Stern layer will in turn attract counter ions, which form the diffuse electrical double-layer. The concentrations of anions will be higher than cations near the surface of the vesicle. At greater distances these concentrations tend to become equal due to Brownian motion. The diffuse double-layer of ions is therefore considered an ionic mixture, rather than in terms of a number of concentric shells composed of distinct ionic species. As the vesicle moves through its suspending fluid in the electrophoresis apparatus, part of its diffuse double-layer moves with it and part stays behind with the bulk phase. The plane between that part of the diffuse electrical double-layer moving with the cell and that part remaining with the bulk phase of the suspending fluid, is the hydrodynamic slip-plane.

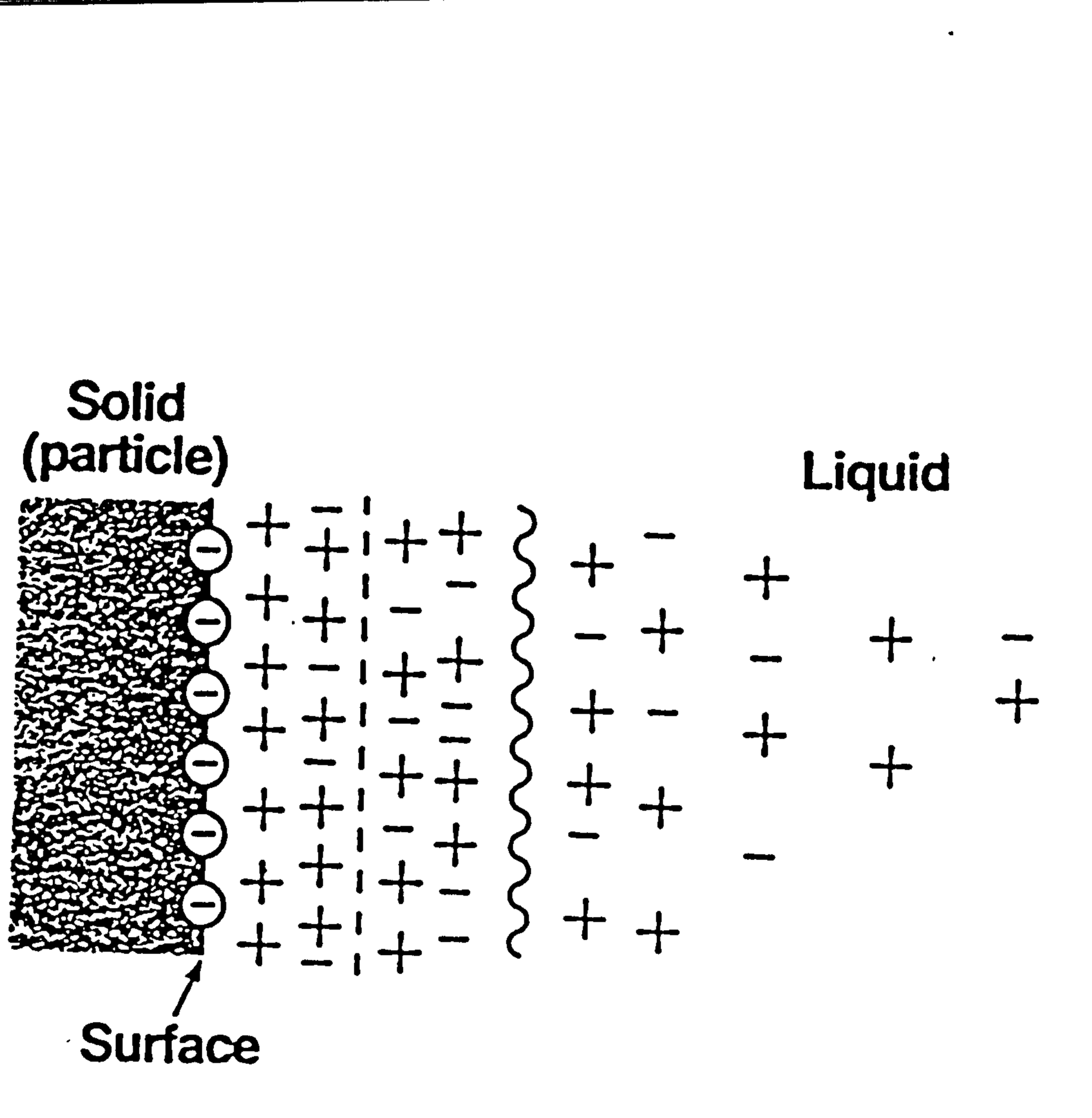


Figure 4: The electrical double layer in the interfacial region to a charged surface and the potentials in the surface region.

Electrophoresis is, essentially, a measurement of the electrical potential between an imaginary electrode located at the hydrodynamic slip-plane and one placed an infinite distance away in the medium. This potential, the zeta potential,ξ, is less than the true surface potential,ψ_o, but the difference between the two is a matter of conjecture (see later, page 38).

Electrophoretic mobilities allow rough approximates, within limits, of zeta-potential to be made and even rougher estimates of surface potential,(ψ_o). However, for vesicles of similar surface configuration, measured under similar, nontraumatic environmental conditions, comparisons of average surface charge density may be made from electrophoretic data, but absolute values of average surface charge density are not exact.

Measurement of the Zeta Potential from Electrophoretic Mobility

Several phenomena, for example, electrophoresis, electroosmosis, streaming potential and sedimentation potential can be exploited as a measure of zeta potential and can be grouped under the heading of electrokinetic effects. They involve the movement of a charged surface relative to its surrounding fluid, with an electric potential either causing or resulting from the movement. The most widely studied of these phenomena is electrophoresis, the movement of charged particles suspended in a liquid medium under the influence of an applied electric field *(Shaw,1968)*.

When an electric field is applied across an electrolyte, charged particles suspended in the electrolyte are attracted toward the electrode of opposite charge. Viscous forces acting on the particle tend to oppose this movement and, when an equilibrium is reached between electrical attraction and viscous drag, the particle moves with a constant velocity. The velocity is dependent on the strength of the

electric field or voltage gradient, the dielectric constant and the viscosity of the medium, and the zeta potential. The velocity of a particle under unit electric field is referred to as it's electrophoretic mobility. Zeta potential is related to electrophoretic mobility by the Henry equation:

$$\mu = \frac{\epsilon \xi f(K_a)}{6 \pi \eta} \qquad \text{.....[1]}$$

where μ= mobility; ϵ= dielectric constant; η= viscosity; a = particle radius and K is the Debye Huckel parameter, which depends on the electrolyte concentration. The units of K are reciprocal length, and 1/K is often taken as a measure of the "thickness" of the electrical double layer surrounding the particle.

Electrophoretic determinations of zeta potential are commonly made in high electrolyte concentration, which results in a large value for K_a. Under these conditions, $f(K_a)$= 1.5 and the Smoluchowski form of the Henry equation becomes operative:

$$\mu = \frac{\epsilon \xi}{4 \pi \eta} \qquad \text{.....[2]}$$

which, in water at 25^{0}C, reduces to:

$$\xi = 12.85\ \mu \ \text{(mV)} \qquad \text{.....[3]}$$

Thus, measurement of electrophoretic mobility leads to a simple calculation of zeta potential. At low values of K_a (for instance, sub-micron particles in a low ionic strength medium), $f(K_a)$= 1.0, and an equally simple relationship results in which the $4\pi\eta$ factor of equation [2] is replaced by $6\pi\eta$.

Various techniques exist for measuring electrophoretic mobility. Since the most practical systems exhibit a range of electrophoretic mobilities for the several distinctly different particles, for example, as is found in blood, the technique of microelectrophoresis is generally preferred. The essence of a microelectrophoresis system is a cell, flat or cylindrical, at the ends of which are the two electrodes between which the potential gradient is applied. The particles move toward the appropriate electrode and their velocity is measured and expressed per unit field strength as their mobility. Early methods of microelectrophoresis involved the direct observation of individual particles using high resolution microscope techniques with manual timing of their progress over a fixed measured distance. This technique is particularly difficult in the case of poorly visible particles such as those of small size or unfavourable refractive index. Problems in measurement also arise with large particles where sedimentation occurs. A further limitation of manual methods of microelectrophoresis is that particle concentration has to be sufficiently reduced to allow an individual particle to be observed for the duration of the measurement.

Alternatively, microelectrophoresis is performed using a low power laser and modern day instruments display the mobilities of a number of particles in a given sample in the form of a spectrum, from which zeta potentials are calculated and displayed automatically, such as the Malvern Zetasizer IIc^{R*}. The complete measurement takes only a few minutes and the concentration of the sample does not affect the results. The Zetasizer offers the added capability of submicron particle size analysis to complement the electrophoretic measurement capability.

Calculation of Surface Charge

The net charge on the vesicles can be derived from the electrophoretic mobility, μ, using the Helmholtz-Smoluchowski equation, as shown overleaf:

$$\xi = \frac{\eta \mu}{\epsilon_o \epsilon_r} \qquad \text{.....[4]}$$

The zeta potentials are obtained by substituting the individual values of μ and ϵ_o, the permittivity of free space, η and ϵ_r, the viscosity and the real part of the relative permittivity of the suspending electrolyte, respectively. For aqueous solutions of low ionic strengths, the physical constants for pure water can be used without serious error. For water at 25^0C (298K), these values are as follows: $\eta = 8.3 \times 10^{-4}$ N.s.m^{-2}, $\epsilon_r = 78.54$ *(Weast,1984)*. The potential, ξ, derived from equation [4] corresponds to the potential at the hydrodynamic plane of shear, a small distance beyond the outer membrane of the vesicle. No satisfactory theory appears to have been developed which accurately relates ξ to the actual surface potential, ψ_o, of a cell or colloidal particle. Measurements *(Pething et.al,1984)* using anionic free radicals to probe the surface charge of Ehrlich ascites cells have provided a value of -5.7mV for the difference between the zeta potential and the actual potential at the cell membrane surface. For the purposes of the work described here, several assumptions are made.

Assumption 1 : ψ_o is equal to ξ.

Assumption 2 : the vesicle wall is planar, and

Assumption 3 : the charges are distributed uniformly over the surface.

Then, for an electrolyte composed of mixed valency ions, the surface potential, ψ_o, can be related to the surface charge density, σ, by:

$$\sigma^2 = 2\epsilon_o\epsilon_r\, kT \sum_j n_j b \left[\exp\left(\frac{-Z_j q \psi_o}{kT}\right) - 1 \right] \qquad \text{.....[5]}$$

In equation [5], which is summed over all ionic species, j, of valency Z_j , k is the Boltzmann constant, q is the absolute magnitude of the charge on an electron (1.602×10^{-19} coulombs), T, is the absolute temperature and $n_j b$ is the ionic

concentration (ions dm^{-3}) in the bulk electrolyte away from the surface of the vesicle.

If the electrolyte is not of mixed valency, but is a Z-Z electrolyte, then equation [5] simplifies to:

$$\sigma = (8n_b\epsilon_o\epsilon_r kT)^{\frac{1}{2}} . \sinh\left(\frac{Zq\psi_o}{2kT}\right) \quad \text{.....[6]}$$

Since $n_b = NC_b$, where N is the Avogadro constant and C_b is the bulk molar concentration of the electrolyte, then numerical substitution into equation [6], with $\epsilon_r = 78.54$ and $T = 298K$ gives :

$$\sigma = 11.77\ C_b^{\frac{1}{2}} . \sinh\left(\frac{Zq\psi_o}{2kT}\right) \quad \text{.....[7]}$$

In this equation, σ, is in C/cm^2, C_b is in mol. l^{-1} and ψ_o is in volts. The convention used in equation [7] is that the surface charge density is taken to be negative for negative values of surface potential, ψ_o . Grahame *(1947)* adopted the opposite convention, that is, σ is positive for negative ψ_o, and vice versa, and so a negative sign appears in his equivalent formula.

Gel Electrophoresis

Protein adsorption to vesicles is known to affect their surface charge *(Alving et.al,1978)* and is an important parameter in their clearance rate from the circulation *(New et.al,1978)*. It has been shown that liposomes of various compositions have different surface charge characteristics and acquire different arrays of bound protein upon exposure to blood; thus while all types of vesicle acquire a net negative surface charge in the circulation, the identity of the bound protein, rather than the surface charge, probably modulates the interaction of liposomes with reticuloendothelial cells and thus their clearance from blood.

Since these bound proteins carry a net charge at any pH, other than their iso-electric point, a useful method of studying their nature and composition makes use of this charge. Proteins migrate at a rate which depends on the charge density (the ratio of charge to mass); the higher the charge mass ratio, the faster the molecule migrate. In theory, separation of different proteins as discrete zones is readily achieved provided their relative mobilities are sufficiently different and the distance allowed for migration is sufficiently large. Therefore, separation of proteins using polyacrylamide gel electrophoresis (PAGE) depends on the charge density of the proteins at the pH selected. Further, the use of gels prevents convection currents, minimises diffusion and may also actively participate in the separation process by interacting with the migrating particles. These gels can be considered as porous media in which pore size is of the same order as that of the protein molecule so that molecular sieving occurs and separation depends on charge density and size. Polyacrylamide gel has become the medium of choice for zone electrophoresis of most proteins since a wide range of pore size is readily available. In addition, polyacrylamide gels have the advantages chemical inertness, stability over a wide range of pH, temperature and ionic strength and trans-parency which makes recording easy.

1.4. INTERACTION OF LIPOSOMES WITH CELLS

The interaction of phospholipid vesicles with cells is a promising area in cell biology since they have a potential for introducing new material into the cytoplasm or the membrane of the cell. To date, growth-regulating nucleotides *(Papahadjopoulos et.al.1974)*, drugs *(Mayhew et.al.1976)*, proteins *(Weissmann et.al. 1977)*, nucleic acids *(Mayhew et.al.1977)* and intact viruses *(Wilson et.al.1977)* have been introduced into cultured cells after encapsulation in vesicles. Lipid molecules

(Martin and McDonald,1976) have been introduced into the red cell membrane and water-soluble fluorescent molecules into cytoplasm of lymphocytes *(Weinstein et.al.,1977;Blumenthal et.al.,1977)* by interaction of vesicles containing these compounds and the recipient cell. These studies have promoted the potential of lipid vesicles as pharmacological tools. However, although the subject has been the focus of considerable study *(Pagano and Weinstein,1978;Kimelberg and Mayhew,1978)*, the mechanism of vesicle uptake by cells appears to be complex and remains poorly understood. Various investigators have shown that adsorption, phospholipid exchange, endocytosis and fusion are all possible pathways for the interaction of vesicles with cells, as shown in figure 5. Adsorption would appear to be the common initial interaction which precedes the subsequent mechanisms.

After adsorption to the cell (figure 5a), a liposome is likely to slowly release its contents, some of which may enter the cell, depending on the nature of the vesicle contents and the type of cell involved. An endocytosed liposome may be processed (figure 5b), by a lysosome, an intracellular digestive organelle, after which the lipid components of the liposome may be incorporated into the membrane of the cell, whereas the aqueous solutes that escape lysosomal degradation may be incorporated into the cytoplasm. A liposome that undergoes lipid exchange (figure 5c) may take up lipid from the membrane of the cell and in return may give up some lipid to the cell. When a liposome fuses with a cell (figure 5d), the liposomal membrane merges with the cell membrane and the liposomal contents become integrated with the cytoplasm of the cell.

A model for some of these interactions of liposomes with an in vitro cell system is provided by cells of Tetrahymena elliotti. T.elliotti is a free-living ciliated protozoan, approximately seventy microns by thirty microns in size, as shown in figure 6, as shown on page 43.

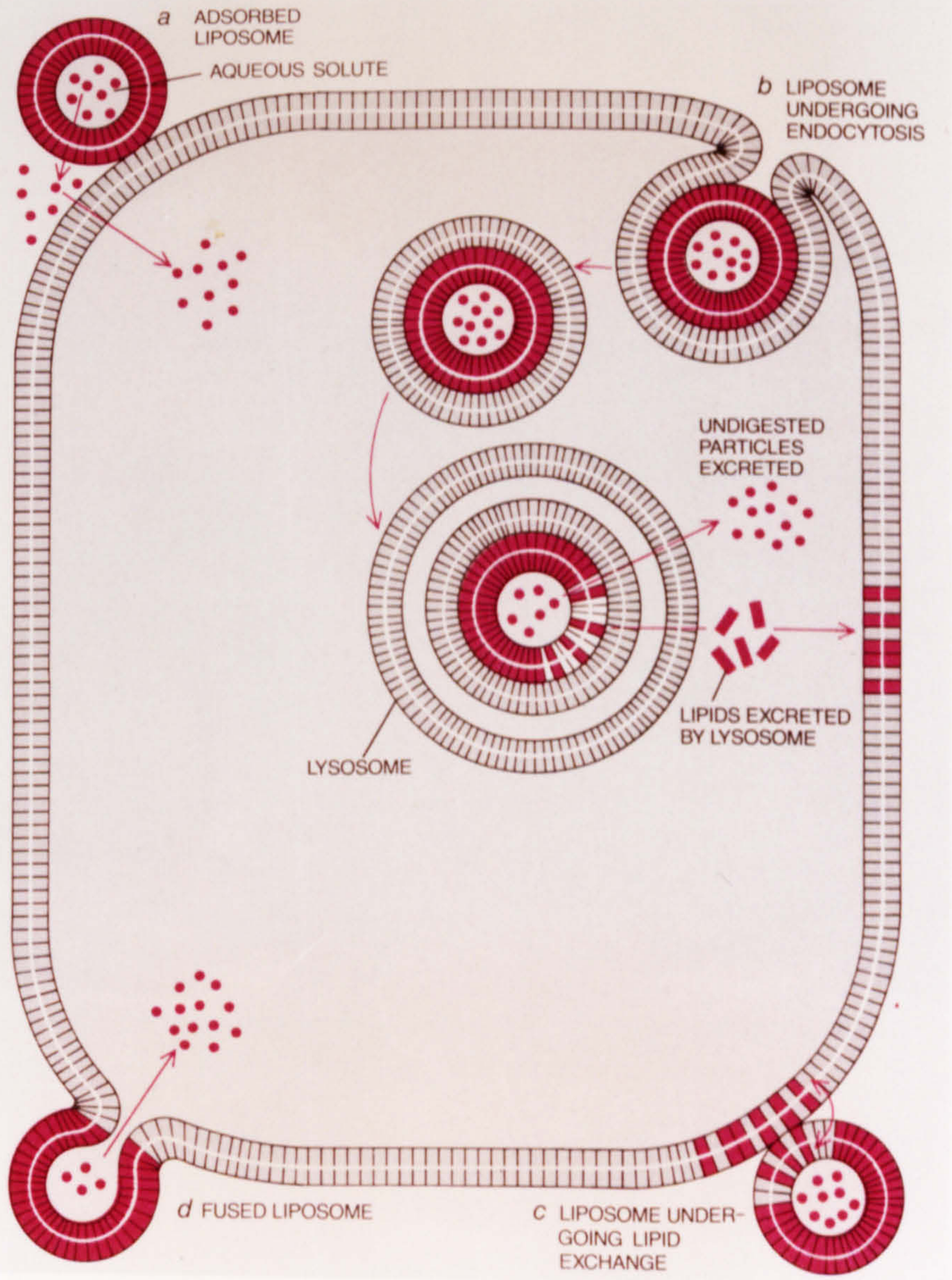


Figure 5: A schematic diagram showing the various points of attachment of liposomes with a cell. (see text for explanation).

(Reproduced from *Ostro (1987)*.

The ciliate protozoon, *Tetrahymena*. (a) A general view, showing external appearance. (b) Diagrammatic cross section, showing main structural features of the cell.

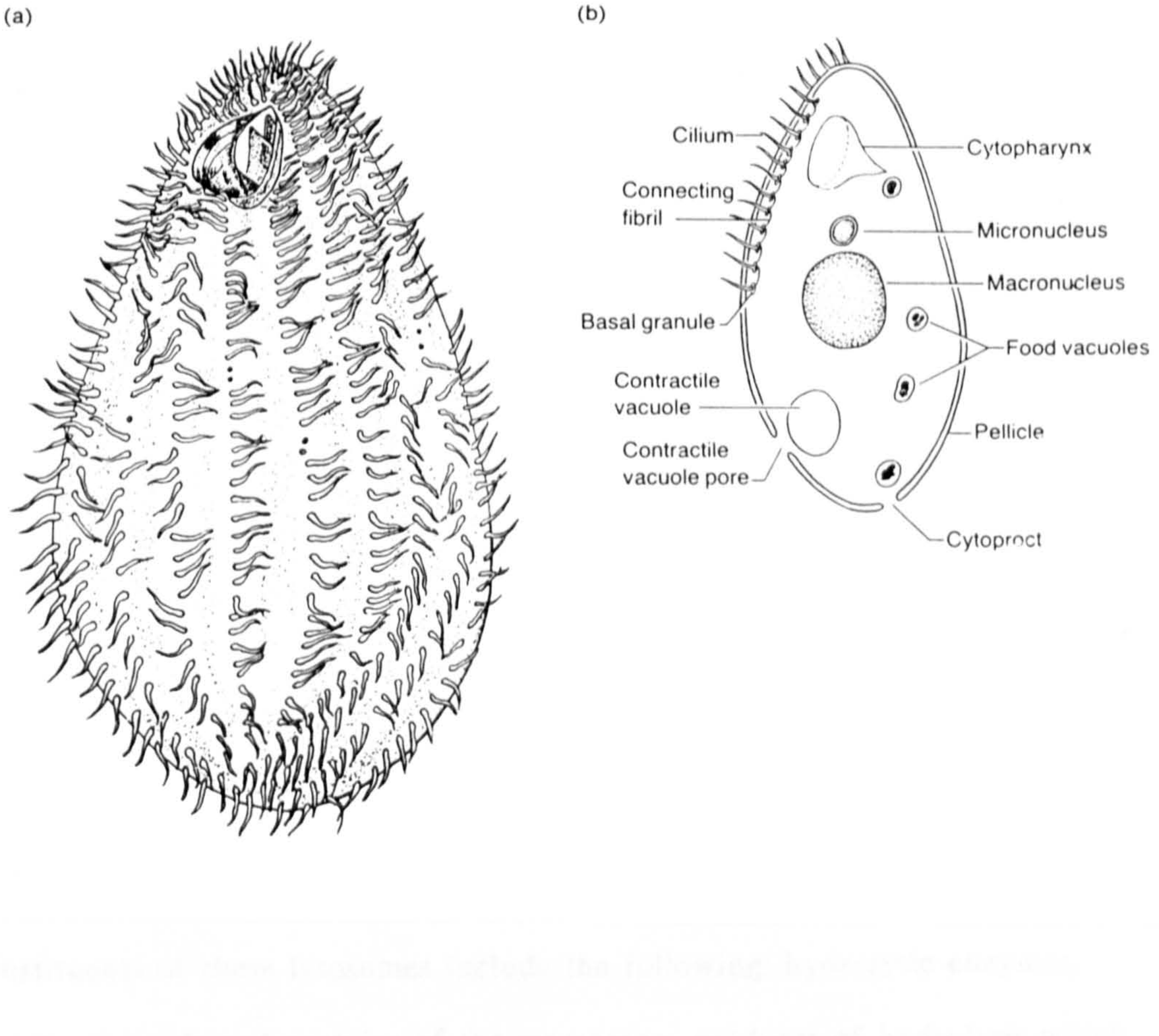


Figure 6: A diagramatic representation of Tetrahymena elliotti.

Microscopic studies of ingestion in Tetrahymena *(Levy and Elliott,1968)* have shown that after entrapment, particulate material is invested with a membrane within the oral apparatus and enters the cytoplasm as a food vacuole or phagosome. Autophagic vacuoles (cytolysosomes) are also formed upon exposure to non-nutrient media *(Nilsson,1970a)* as well as during the stationary phase of growth in nutrient media.*(Elliott and Clemmens,1966)*.

Nutrients in solution may also be taken up by the formation of pinocytic vacuoles at specific sites along the pellicle, several of which coalesce to form the equivalent of a food vacuole *(Elliott and Clemmens,1966)*. Degradation of engulfed material begins when the phagosome or cytolysosome fuses with a primary lysosome to form a phagolysosome *(Elliott and Clemmens,1966)*. After fusion with primary lysosomes both food vacuoles and autophagic vacuoles appear to be processed in the same way, that is, digestion of the vacuolar contents with permeation of the nutrient products into the cytosol and eventual egestion of the indigestible residue via the cytoproct or cell anus.

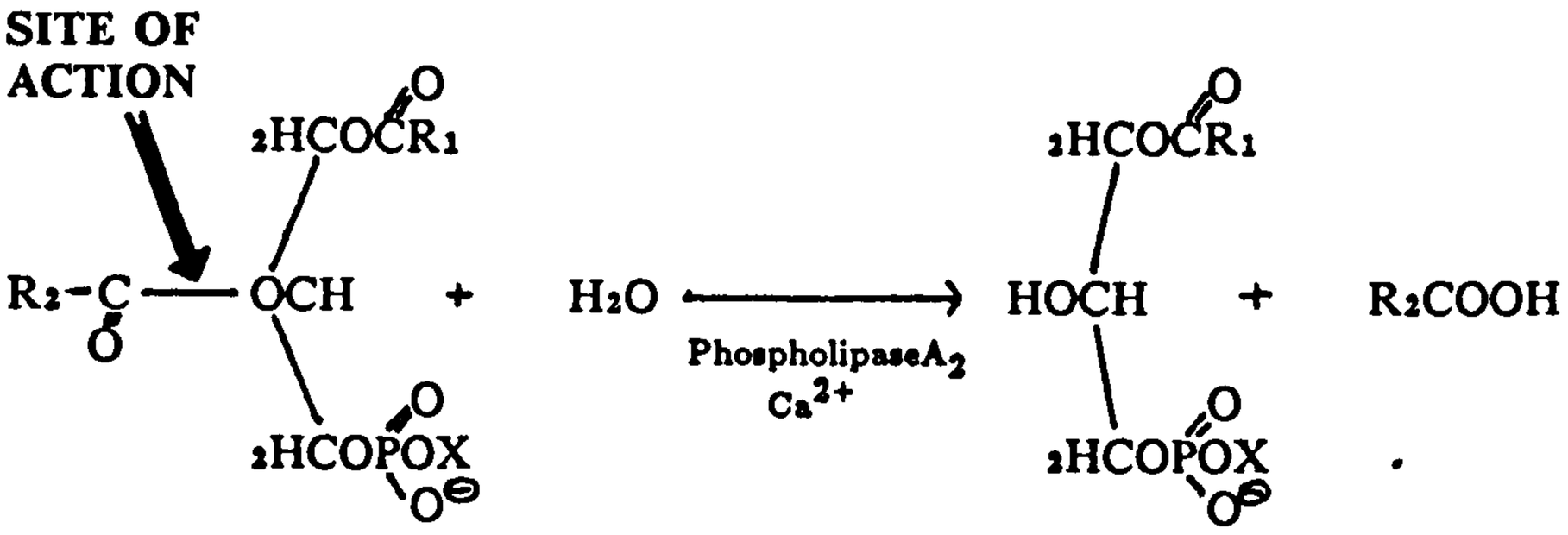
Lysosomes are membrane-delimited structures containing characteristic hydrolytic enzymes, most of which have acid pH optima. The lysosomes of Tetrahymena, first seen in electron micrographs *(Elliott,1965)* and sedimented from homogenates *(Muller et.al,1966)*, are similar to those of many other cell types and contain a number of hydrolases with acidic pH optima *(Muller et.al,1966)*. The known constituents of these lysosomes include the following: hydrolytic enzymes, structural lipids and proteins of the membrane, products of hydrolysis which accumulate in the lysosomes and cellular parts and macromolecules which have yet to be digested or which resist digestion.

Since pure lysosomes from T.elliotti cannot be obtained in sufficient quantities the enzymes used in this work were all purchased commercially. However, the hydrolytic enzymes within these lysosomes fall into the usual classification. A number of lysosomal enzymes exist (for a detailed review, see *Dingle,1972*) but the action of two that are relevant to the intracellular processing of liposomes and other related vesicles will be described here.

Phospholipase A_2

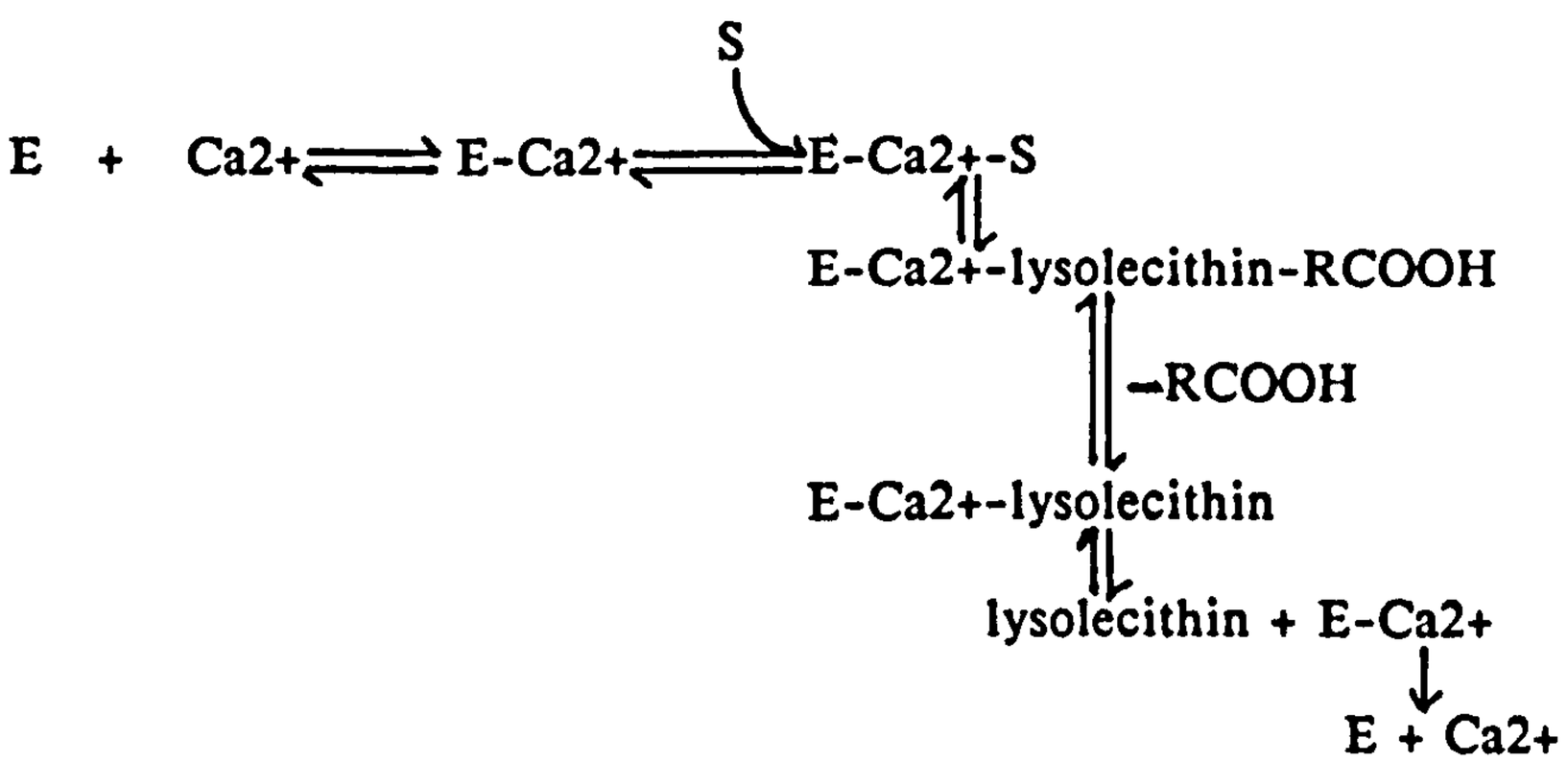
Phospholipase A_2 (EC 3.1.1.4.) belongs to the class of lipolytic enzymes that are characterised by their ability to hydrolyse lipid substrates. The phospholipases are classified according to the particular ester bond of the phosphoglyceride substrate hydrolysed by the enzyme. Phospholipase A_2, specifically, catalyses the hydrolysis of the 2-acyl ester bonds of naturally occuring and synthetic phosphoglycerides *(van Deenan and de Haas,1964)*, producing a 1-acyl-lysophosphoglyceride and fatty acid.

Mode of Action



X represents any of the polar head group moieties found in 3-sn-phosphoglycerides, for example, H, choline, etc., R_1 and R_2 are alkyl chains.

The enzyme is highly stereospecific and has an absolute requirement for calcium ions that bind in a 1:1 molar ratio to the enzyme. The Ca^{2+} adds to the enzyme before the substrate attaches to this complex *(Nilsson, 1970b)*. This attachment induces a conformational change in the enzyme-Ca^{2+}-substrate complex. The fatty acid is released from the enzyme, followed by the other product, lysophosphatidylcholine (lysolecithin) *(Nilsson, 1970b)*. The stepwise reactions are summarized below, where E represents phospholipase A_2, S is the substrate, for example, phosphatidylcholine and RCOOH is the released fatty acid.



An important and characteristic feature of most lipolytic enzymes is the strong dependence of the catalytic properties of these enzymes on the physical state of their substrates.

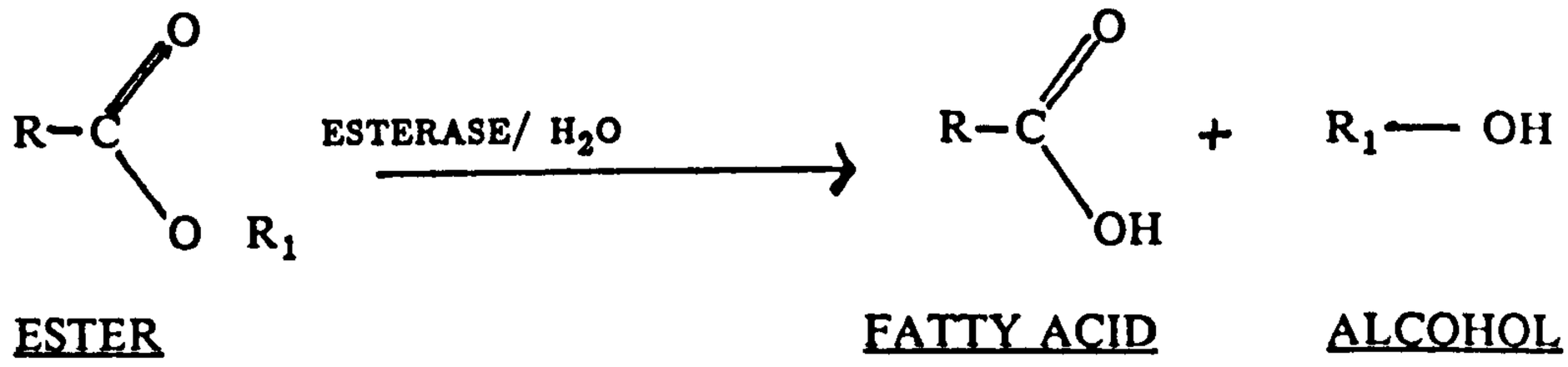
Liposomes are known to be poor substrates for pure pancreatic phospholipase A_2 *(de Haas et.al.1968)*, although, saturated long chain lecithins become more susceptible to hydrolysis at their thermotropic phase transition *(Op den Kamp et.al.1975)*. Both below and above these temperatures (T_c), the more regular and tighter packing of the phospholipid molecules may prevent formation of the enzyme-interface complex. At the T_c, domains of molecules in the gel state exist together with domains where the lipids are in the liquid-crystalline state, and surface defects at the borders between these domains would allow the enzyme to

penetrate the interface and hydrolyse substrate molecules *(Op den Kamp et.al. 1975)*. Investigations with snake venom phospholipases show these enzymes are more active on bilayer substrate systems, which are their natural substrates *(Slotboom et.al.1982)*. However, crude snake venom also includes a number of other enzymes, as shown in Appendix 2, page 161.

Carboxylic Ester Hydrolase

Carboxylic ester hydrolase (EC 3.1.1.1.) catalyses the hydrolysis of a large number of uncharged carboxylic esters. In contrast to lipases their action is generally restricted to short chain fatty acid esters.

Mode of Action



ESTER FATTY ACID ALCOHOL

Unlike phospholipase A_2, this enzyme does not require specific co-factors and is thought to hydrolyse most, commonly encountered, ester linkages.

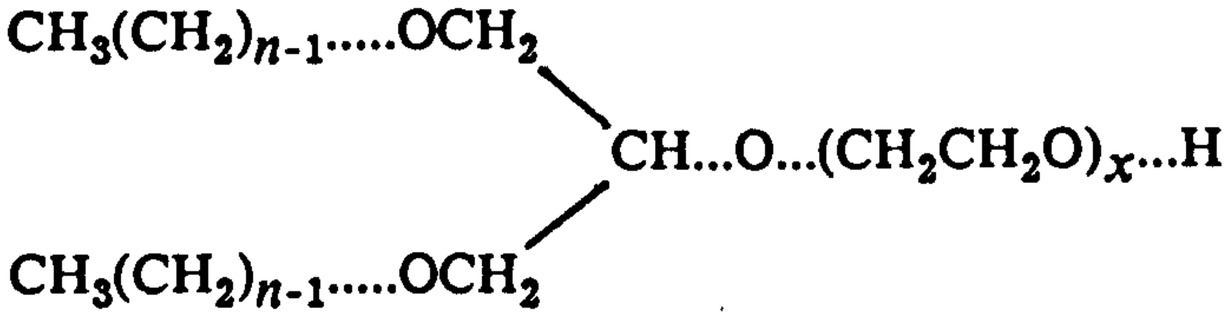
1.5. NON-IONIC SURFACTANT VESICLES

The formation of surfactant vesicles was first detected following the sonication of a didodecyldimethyl bromide solution *(Kunitake and Okahata,1977)*. Since then a large variety of synthetic amphiphiles have been shown to form membrane-like molecular aggregates *(Kunitake,1986)*, such as bilayers, monolayers and vesicles, as

shown in figure 7, page 49. It is the hydrophobic effect *(Tanford,1980)* which provides the driving force for such aggregation, when surfactant molecules are placed in an aqueous environment.

The type of aggregate formed and its properties, is defined by the structure of the amphiphile. Ionic *(Kunitake and Okahata,1978)*, non-ionic and zwitter-ionic dialkyl amphiphiles *(Handjani-Vila et.al,1979;Okahata et.al,1981)* are capable of forming vesicles. Although the length of the chain is important for bilayer formation, it is not essential for the hydrophobic part of the molecule to be a double chain *(Kunitake and Okahata,1980)*. Many single chain ionic *(Kunitake and Okahata,1980;Hargreaves,1978)* and non-ionic *(Baillie et.al,1985)* amphiphiles form vesicles. In general, non-ionic surfactant molecules contain polyethoxy chains which, together with the alkyl chain length, determine the aggregate morphology.

General Structure:



hydrophobic **hydrophilic**

n= 12,14,16 and 18 units

x= 6-30

Some non-ionic surfactants have been shown to be relatively non toxic and to enhance absorption of drugs across membranes *(Kaneda et.al,1974)* by increasing membrane permeability *(Attwood and Florence,1983)* and in some cases, by solubilising biomembrane components *(Whitmore et.al,1979)*. This property of amphiphiles, that is, their capacity to solubilise and hence alter the characteristics of many drug molecules, potentiates their use in the field of drug delivery.

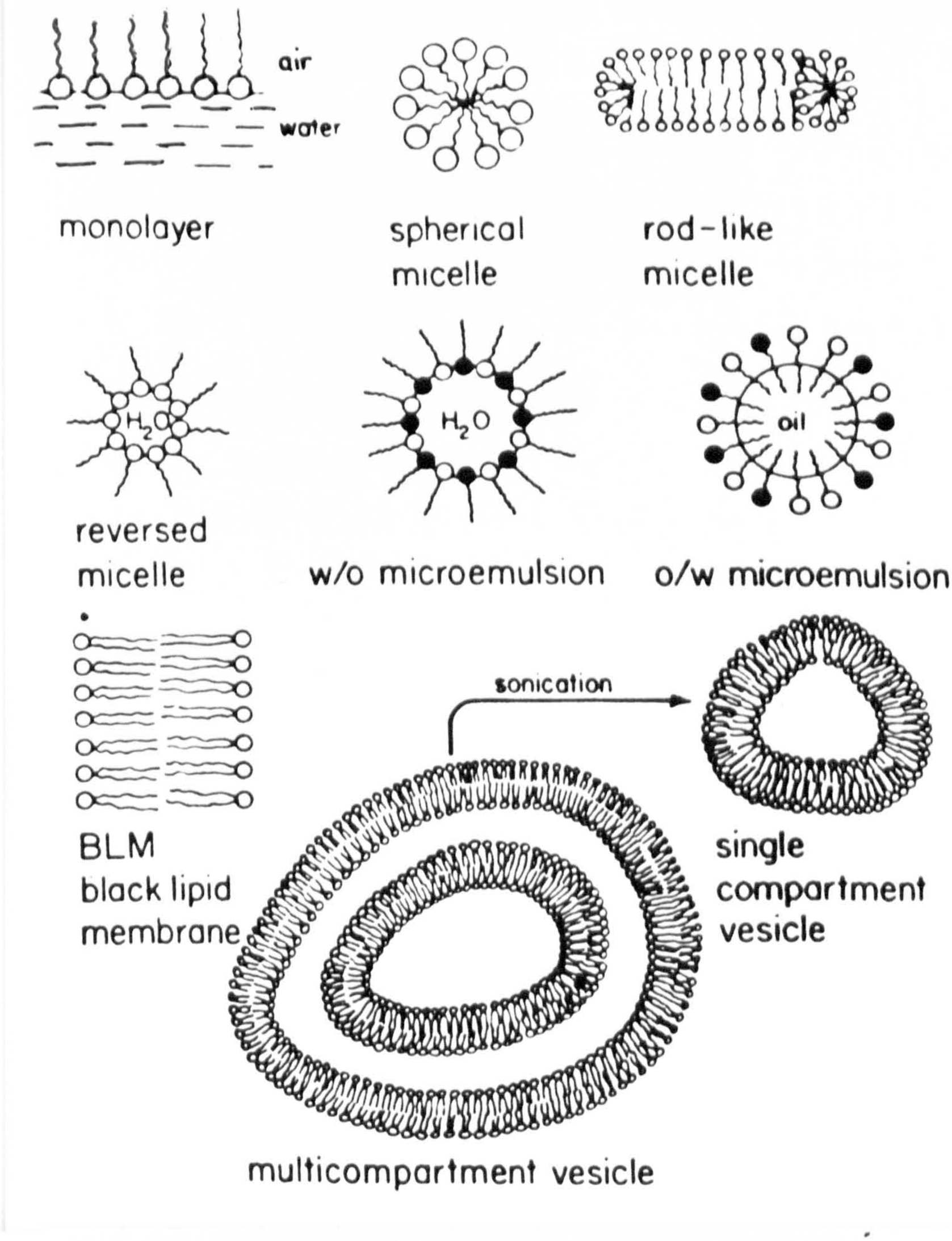


Figure 7: A representation of organized structures formed from surfactants.

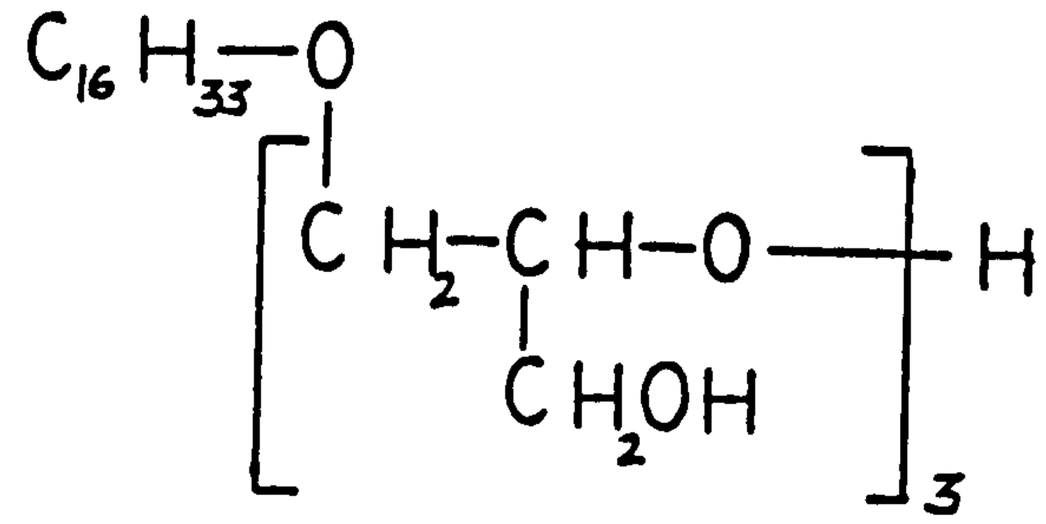
(photograph from, **Fendler, J.H and Tundo, P (1984)**, "figure 1").

1.5.1. NIOSOMES: POTENTIAL DRUG CARRIERS

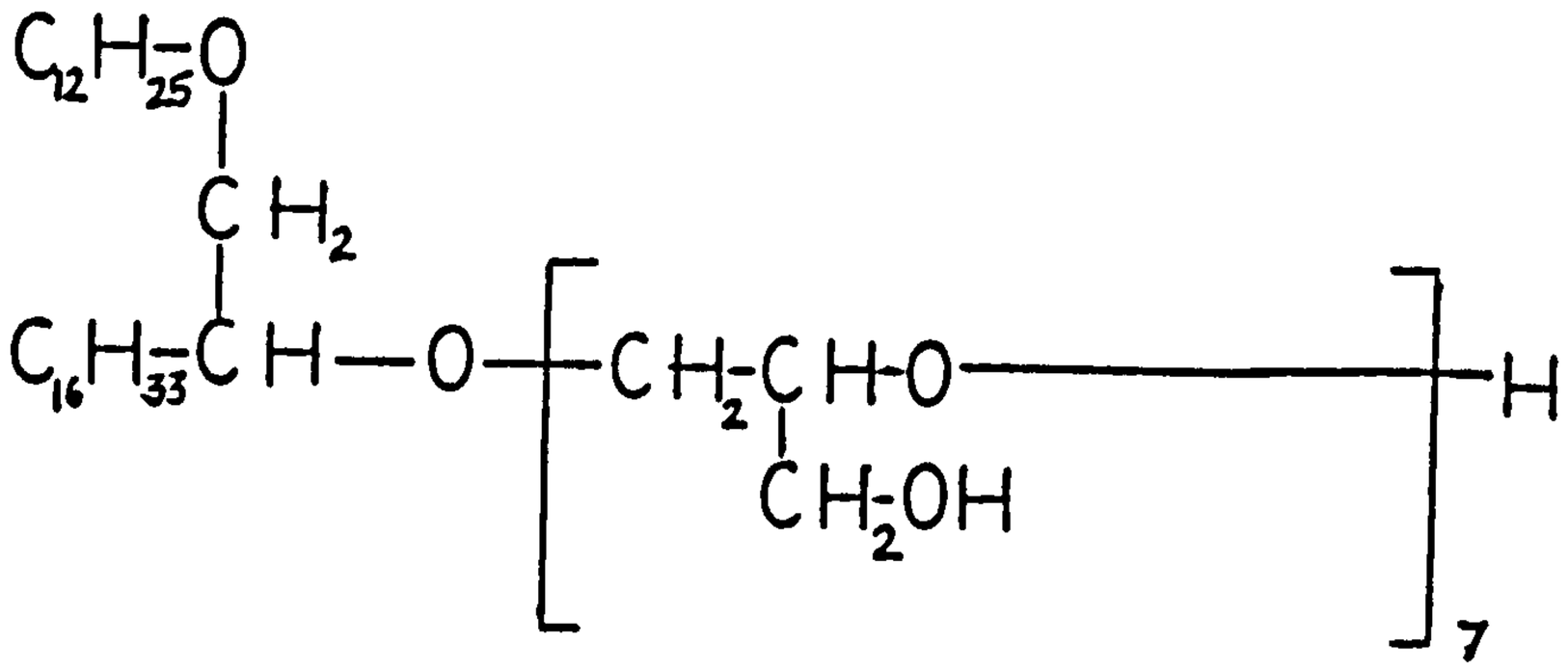
The discovery *(Handjani-Vila et.al,1979)* that non-ionic surfactant molecules, such as surfactant 1, as shown in figure 8, are capable of forming vesicles, niosomes, entrapping an aqueous solution, was a lead to their potential use as drug carriers.

A system which could combine the advantages of liposomes with the ability to increase membrane permeability displayed by the non-ionic surfactants would be of great interest. An investigation to compare and contrast some of their relevant properties with the apparently similar and well characterised liposome system *(Gregoriadis and Allison,1980)* is vital to the development of a niosomal drug carrier system. A major prerequisite to the use of niosomes and other vesicles as drug carriers is their integrity in biological fluids. These are, for example, interstitial fluid (sub-cutaneous administration), synovial fluid (intra-articular injection), contents of the stomach and intestine (oral route) and peritoneal cavity (intra-peritoneal administration). However, the great majority of potential in vivo applications involves intravenous administration so that stability in blood, especially plasma has been studied extensively. In a carrier role, niosomes must be able to both circulate in the body and retain drugs for significant periods of time to optimise access to, and interaction with, target tissue and in appropriate circumstances delivery of their contents to the interior of cells. An understanding of these processes which may affect niosome integrity in vivo is essential to a study of "niosome-encapsulated drugs".

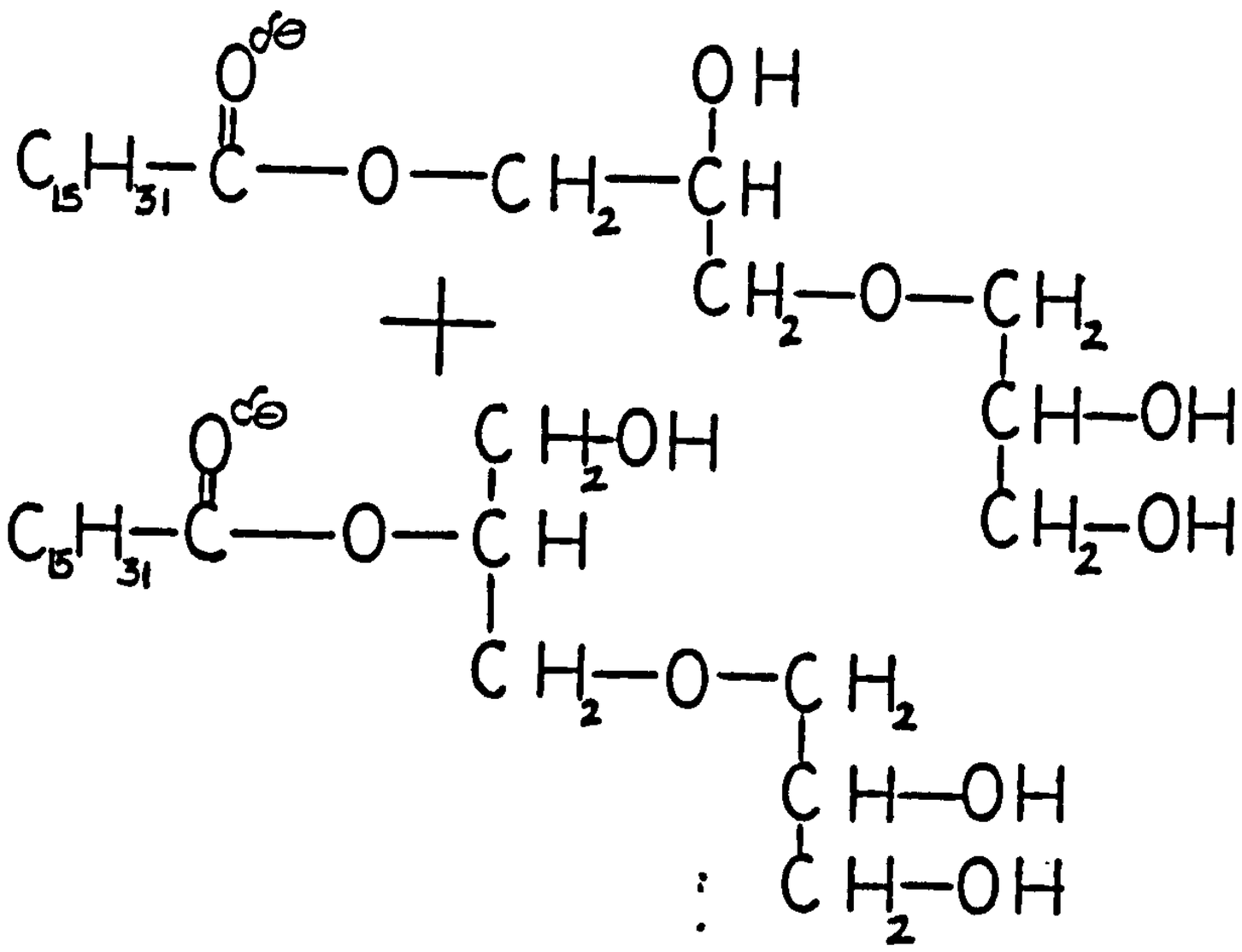
Preliminary work within these laboratories has shown *(Baillie et.al,1984,1985)*, that niosomes appear to be similar, in terms of their physical properties to liposomes. Studies, in mice, have shown modified tissue distribution and excretion of methotrexate entrapped in niosomes *(Azmin et.al,1985&1986)*. Niosome formulation caused methotrexate accumulation in the liver and enhanced level of the drug in



Surfactant I
m.wt.,473



Surfactant II
m.wt., 972



Surfactant III
m.wt., 404

A (92%)

B (8%)

Figure 8: Three vesicle forming non-ionic surfactants synthesised by *Vanlerberghe et.al (1978).* **3** and **7** are number average values. Surfactant **I** and **II**; the hydrophobic alkyl chain ether-linked to the hydrophilic polyglycerol head group. Surfactant **III** is ester-linked and is a mixture of 2 isomers, **A** and **B.**

the brain after intravenous administration. Similar reports of brain accumulation have been reported for methotrexate entrapped in liposomes after intravenous injection into rats *(Freise et.al,1981)*. Niosomally-entrapped doxorubicin has also been shown to have altered tissue distribution . Intravenous injection of hand shaken niosomes containing doxorubicin into mice *(Rogerson 1986;Rogerson et.al,1988)* showed no apparent liver or spleen loading with drug but some evidence of accumulation of doxorubicin in the lungs. These results may be a direct consequence of the size distribution of the vesicles used, although the lung-loaded perhaps indicates intravenous aggregation of the niosomes. Increased anti-leishmanial activity after passive targeting of sodium stibogluconate to the liver using niosomally-entrapped drug *(Baillie et.al,1986)* is further evidence of the potential of drug-carrier role for niosomes. As part of an approach to the optimisation of this drug-carrying potential of niosomes, it is important to characterise their stability in terms of release of entrapped solute. The result of a series of in vitro experiments, simulating physiological conditions in vivo, are presented here to define the effects of blood proteins in niosome stability and in addition the influence of pH and temperature on the integrity of various types of non-ionic vesicle.

Cosmetic Application

Niosome^{R*} has recently been marketed commercially as a skin anti-aging system by Lancôme Laboratories, France. The "niosome system" is a complex of niosomes believed to transport active elements within the skin. These active elements are transported enclosed in the niosome sphere and are fully protected, so they can be released intact where the skin needs them most. These niosomes are thought to have an added integral reconstructive action because of their "biomimitism" phenomenon. This gives them the potential to become incorporated into the skin's

intercellular support organisation to reconstruct it where it has become disorganised.

Liposomes have set the standard in the field of drug carrier systems for invetigating these new surfactants with a variety of composition effects. Niosomes may provide an alternative type of carrier system.

This thesis reports part of the work from ongoing research in our laboratories.

AIMS OF PROJECT

1. Preparation of niosomes from three non-ionic surfactants provided and investigation of physical properties in direct comparison with liposomes.

2. Stability of vesicles in human plasma and serum, electrophoretic mobility measurements and surface charge calculations.

3. Identification of proteins adsorbed to niosomes; a comparative study with liposomes.

4. *In vivo* stability of vesicles in an eukaryotic cell, *Tetrahymena elliotti*; uptake and degradation studies.

SECTION 2

EXPERIMENTAL

2.1. MATERIALS

Non-ionic surfactants, I,II and III, were a gift from L'Oréal, France.

5,6-carboxyfluorscein (CF; Eastman, Kodak,Liverpool,England) was partially purified (see page 56) over activated charcoal before use.

Cuprophan dialysis tubing (size 15mm diameter) was purchased from Medicell International Ltd, London, England.

Acrylamide, ammonium persulphate (APS), amidoblack (napthol blue black), bis-acrylamide, bovine serum albumin (BSA), bromophenol blue, calcein, carboxylic ester hydrolase, cholesterol, 4-chloro-1-napthol, Coomassie blue R250, dicetyl-phosphate (DCP), dipalmitoylphosphatidylcholine (DPPC), dimyristalphosphatidyl-choline (DMPC), egg phosphatidylcholine (egg PC), glutaraldehyde, glycerol, glycine, horse radish peroxidase, mercaptoethanol, phospholipase A_2, sodium dodecyl sulphate (SDS), snake venom from Vipera russelli (Russell's Viper), stearylamine, thrombin and trypsin were purchased from Sigma Chemical Company, Poole, England.

EDTA, N,N,N',N'-tetramethylethylenediamine (TEMED) and urea were purchased from BDH Chemicals, Poole, England.

Rabbit antihuman IgG was purchased from Miles Laboratories, Slough, England.

Plasma, pooled citrated human, was obtained from the Royal Infirmary Hospital, Glasgow, Scotland. Serum was prepared from citrated plasma by the addition of thrombin and heat-inactivated.

Tetrahymena elliotti strain 1630/IC (T. elliotti) was obtained from the Culture Centre of Algae and Protozoa, Cambridge, England.

Proteose peptone and liver extract were purchased from Oxoid, Basingstoke, England.

All other reagents were of analytical grade.

2.2. METHODS

2.2.1. PRODUCTION OF VESICLES

Ether Injection (E.I.) Niosomes

Surfactant or surfactant/cholesterol mixture (1.50 X 10^{-4}M) was dissolved in diethylether (20ml) and injected slowly (0.25ml min^{-1}) through a needle (14 gauge) into an aqueous solution of CF (5ml, 0.2M, pH7.4) maintained at 60oC.

Hand Shaken (H.S.) Niosomes

Surfactant or surfactant/cholesterol mixture (1.50 X 10^{-4}M) was dissolved in chloroform (10ml) in a round bottomed flask. The chloroform was removed at room temperature (22^0C) under reduced pressure on a rotary evaporator to form a thin film on the wall of the flask which was then hydrated with CF (5ml, 0.2M) at 50^0C. Gentle agitation for 1 hour completed the formation of niosomes.

H.S. Liposomes

Phospholipid/cholesterol mixture (1.50 X 10^{-4}M) was weighed into a round bottomed flask and dissolved in chloroform (10ml). The chloroform was removed at room temperature as above and the resulting thin film hydrated with CF solution (5ml, 0.2M) at 50^{0}C under an atmosphere of nitrogen and agitated gently for 1 hour.

"Negatively-Charged" Vesicles

Surfactant/cholesterol or phospholipid/cholesterol admixed with dicetylphosphate (DCP, 10mol%, total molarity 1.50 X 10^{-4}M) were dissolved in chloroform (10ml) in a round bottomed flask. The chloroform was removed by evaporation at 22^{0}C under reduced pressure (within a nitrogen atmosphere), leaving a thin layer on the wall of the flask. Hydration with CF (5ml,0.2M) for 1 hour at 50^{0}C with gentle agitation resulted in the production of vesicles.

"Positively-Charged" Vesicles

Surfactant/cholesterol admixed with stearylamine (2 mol%, total molarity, 1.50 X 10^{-4}M) was dissolved in chloroform and vesicles formed as before, using NaCl solution (2.0 X 10^{-3}M) instead of CF.

Purification of CF

CF was purified by modifying the method of Ralston et.al *(1981)*. Commercially available CF (25g) was treated with activated charcoal (10g) in boiling ethanol (300ml) contained in a round-bottomed flask (1 litre). After refluxing for 30 minutes the mixture was cooled and filtered through Whatman No.50 filter paper.

Cold distilled water (600ml) was added and solids allowed to precipitate overnight at 0^0C. The water was removed by filtration and the orange-coloured precipitate was washed thoroughly (4 times, 50ml distilled water), dried at 50^0C in an airtight container and stored in the dark at room temperature.

CF Solution

Aliquots of this orange powder (0.2M) were weighed, made up to volume with the medium (distilled water or phosphate buffered saline [PBS]) and adjusted to pH 7.4 with sodium hydroxide (NaOH, 4N).

Buffered CF

CF is usually made up in distilled water to the required molarity at pH 7.4 by adding sodium hydroxide solution (NaOH, 4N). In "buffered CF", the distilled water was replaced with phosphate buffered saline (PBS) and the requisite amount of NaOH, to the final pH 7.4.

Separation of Free and Entrapped CF

Volumes of aqueous surfactant or phospholipid dispersions (5ml) from ether injection or hand shaken techniques were exhaustively dialysed against PBS, (1.30×10^{-3}M, 0.9%w/v NaCl solution, pH 7.4). In experiments with T. elliotti, NaCl solution was omitted from the buffer.

2.2.2. STABILITY OF VESICLES

Leakage of CF

The CF entrapped within the vesicles is self-quenched at the working concentration of 0.2M at the wavelenghts of measurement. Leakage and ensuing dilution into the extra-vesicular bulk volume, increases the fluorescence of CF markedly, which was measured (486nm excitation, 514nm analyser wavelengths) using a Perkin Elmer^{R*} 203 spectrofluorimeter.

Samples (2.5 X 10^{-2}ml) were added to the test media at "time zero" to give a final volume of 5ml. The fluorescence measured at these times were taken as zero percent, although in practice 15 seconds elapsed before these readings could be recorded. This amounted to 2-6% intensity of the total fluorescence. The samples were gently agitated throughout the experiments and further readings obtained at various time intervals. Maximum fluorescence (100%) for all niosomes and liposome suspensions, F_{tot}, was measured after vesicle disruption by addition of propan-1-ol (0.1ml), or Triton X-100 (0.1ml). The leakage of CF was corrected for background fluorescence at "time zero", F_o. The percentage of CF released in each sample was calculated as follows:

$$\% \text{ Release} = \frac{100\ (F_t - F_o)}{F_{tot}}$$

in which F_t = intensity at time "t".

Effect of pH

The leakage of CF was measured as described above, by challenging the vesicle suspensions (2.5 X 10^{-2}ml) with a variety of different buffers (McIlvaines citric phosphate buffer, pH 2.0 to 8.0) and incubating at 37^{0}C. All the solutions used

were of equal ionic strength (1.24) to that of the CF solution within the vesicle, thus preventing osmotically driven leakage of the CF solution from the vesicles.

Effect of temperature

The efflux of CF from the vesicles was measured after incubation of a suspension (2.5×10^{-2}ml) in buffer (5ml, PBS at pH 7.4) at various temperatures (4^0C, 22^0C, 37^0C and 50^0C) for various time intervals.

Preparation of Human Serum

Serum was prepared from plasma by treatment with thrombin (20 NIH units ml^{-1}) at 37^0C for 10 minutes with gentle stirring, after which period the clot formed was removed. Serum was heat-inactivated by incubation at 51^0C for 30 minutes. Any dilutions of plasma or serum were made by addition of PBS (pH 7.4) to the correct volume.

Effect of Plasma

Leakage of CF from the vesicles was measured as above (page 58) after incubation of a suspension (2.5×10^{-2}ml) of vesicles in human plasma (100%) at 37^0C. Total fluorescence (100%) was evaluated by disrupting all the vesicles using Triton X-100 (0.1ml). Leakage of CF was also monitored in the same way in the presence of serum and heat-inactivated serum.

Effect of 10% BSA

BSA (400mg) was dissolved in PBS (100ml, pH 7.4) at 37^0C. Leakage of vesicles was measured as above.

2.2.3. MEASUREMENT OF SURFACE POTENTIAL

The electrophoretic mobility (μ) was measured as a function of pH in a laterally placed flat cell micro-electrophoresis apparatus (Rank Brothers, Bottisham, Cambridge, England) with an optical assembly and constant temperature bath at 25°C. The mobility was determined by measuring the time taken in seconds for the vesicles to travel a pre-determined distance (usually 2cm) under the influence of a constant known electric field (80 Volts). The vesicles were suspended in solution (NaCl, 2 X 10^{-3}M), the pH of which was varied by the addition of dilute hydrochloric acid (HCl) or sodium hydroxide (NaOH). The mobilities of at least 40 vesicles were measured at each pH and an average mobility obtained.

To measure the effect of plasma (human, 50%) on electrophoretic mobility, vesicles were prepared entrapping NaCl (2 X 10^{-3}M) in glucose solution (0.2M). These vesicles were incubated in plasma for 2 hours, centrifuged at 210*g* for 5 minutes, washed twice and their electrophoretic mobility measured as described above. These measurements were used to calculate surface potentials.

2.2.4. IDENTIFICATION OF ADSORBED PROTEINS

Gel Electrophoresis

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate (SDS; 0.1%) was carried out according to the method of Laemmli *(1970)* with minor modifications, using a Shandon^{R*} vertical slab unit 200 (Bio-rad, Hertfordshire, England).

Sample Preparation

Vesicle suspension (1ml) was incubated with plasma (2ml;10% or 100%) for 1 hour, 2 hours or 24 hours. Samples were then diluted with PBS (10ml) and the vesicles centrifuged at 1000*g* for 5 minutes. The pellet was resuspended, washed 3 times and suspended in PBS (1ml). Measured quantities (2.5×10^{-2}mg-7.0×10^{-2}mg protein per 2.0×10^{-2}ml-5.0×10^{-2}ml sample buffer) were dissolved in sample buffer (6.25×10^{-2}M Tris-HCl, plus 2%SDS, 10%glycerol, 5%mercaptoethanol, 0.001%bromothymol blue, pH 6.8), heated (37°C for 15 minutes) and cooled.

Cleavage by Trypsin, EDTA, Urea and 1M NaOH

Vesicles were incubated with plasma (1 hour, 37°C). These preparations were centrifuged and the resulting pellets washed in buffer (PBS, 1.3×10^{-3}M, pH7.4) and resuspended. Samples were reincubated with freshly prepared trypsin (1mM), EDTA (5mM), urea (4M) and NaCl (1M) for 30 minutes. These samples were then processed by centrifugation, and resuspended for analysis on polyacrylamide gel as previously described above.

Slab Gel Electrophoresis

Samples were carefully loaded onto a 7.5% acrylamide gel and run normally at 200V until the dye entered the separating gel. The voltage was then reduced to 100V and left to run for 5 to 6 hours. The run was terminated when the dye front reached 2cm from the bottom of the gel.

Staining and Destaining of Gels

Gel slabs were placed in a plastic tray containing Coomassie blue R250 (0.1 % w/v in water:methanol:glacial acetic acid, 9:9:2) and agitated slowly. The gels were fully stained within 4 to 6 hours at room temperature (22^{0}C). After staining was completed, excess stain was removed to allow protein bands to be seen clearly. Gels were destained in several washes of water:methanol:glacial acetic acid (9:9:2).

Drying Gels

Gels were dried using a Bio-rad^{R*} drier (model 224) apparatus. Two sheets of filter paper (3mm) were placed on the stainless steel support screen of the dryer and wetted with water. The slab gel was aligned onto this, care being taken not to trap air bubbles under the gel since this would result in cracking during drying. The gel was overlaid with a sheet of pre-wetted Saren Wrap^{R*} (cling film), then a porous plastic sheet, and finally the silicon sheet (attached to the apparatus) forming a leak-proof seal. Vacuum was supplied by a water aspirator or vacuum pump fitted with a cold-finger water-trap and the heating block of the dryer was turned on. The exact time for drying depended on the size and concentration of the gel, generally 2 hours was sufficient. If air enters the assembly before drying is complete the gel will crack. The resulting dried gel was sandwiched between its filter paper and the protective Saren Wrap^{R*}.

Photography of Stained Protein Bands

Wet slab gels were laid directly onto an opal white screen illuminator, avoiding air bubbles, and kept moist during photography by addition of 7% acetic acid. A fine grain, panchromatic film was used and a medium-red filter to increase band contrast.

Electroblotting

This was carried out by the method of Towbin et. al *(1979)*. Proteins were first subjected to PAGE-SDS. Then the proteins were transferred to nitrocellulose sheets. A sheet of nitrocellulose (0.45 μm pore size in roll form, Millipore^{R*}) was wetted with water and laid on a scouring pad (Scotch-Brite^{R*}) which was supported by a stiff plastic grid. The gel to be blotted was placed on the nitro-cellulose sheet and any air bubbles were carefully removed. A second pad and plastic grid were added and secured in position by two strong rubber bands. The assembly was immersed vertically in an electrophoretic Trans-blot (Bio-Rad^{R*}) with the nitrocellulose sheet facing the cathode (-ve). The electrode buffer was 25mM Tris 192mM glycine in 20% methanol (vol/vol), pH 8.3. The electrophoretic run was at 100mA, conveniently overnight.

Staining and Destaining of Blots

The blot was sectioned and stained with freshly prepared amidoblack (0.1% w/v in 45% methanol/10% acetic acid) for 10 minutes. This blot was then immediately destained by washing in several changes of hot acetic acid (2% v/v, 90^{o}C). This manipulation was carried out in a ventilated fume hood.

Immunological Detection of Proteins on Nitrocellulose

Antigens (proteins) transferred from the gels to the nitrocellulose, paper were detected by Enzyme Linked Immuno Sorbent Assay (ELISA) *(Towbin et.al,1979)*. The electrophoretic blots (not stained with amido black) were soaked in 3% BSA/5% GS (goat serum) in buffer (20mM Tris-HCl,140mM NaCl solution,pH 7.4) at room temperature, for 1 hour to block non-specific protein binding sites. They were then washed in buffer containing Tween 20 (0.2% buffer/Tween) and

incubated with the antibody, horseradish peroxidase-conjugated rabbit antihuman IgG for 2 hours at 4°C. The antibody used was diluted 1 in 500 in BSA/GS/buffer before use. To detect the antibody reaction the nitocellulose paper was incubated in the dark with 4-chloro-1-napthol (6mg/ml in methanol) and buffer containing 0.01% H_2O_2 (6ml). The reaction was terminated within 10 minutes (indicated by the developed dark colour) by washing the paper in a large volume of buffer or deionised water. The blots were dried between filter papers (background staining reduced on drying). These were stored protected from light.

2.2.5. *IN VIVO* UPTAKE AND DEGRADATION BY CELLS

The ciliate, T.elliotti strain 1630/IC, was cultured axenically in proteose peptone (2% w/v) and liver extract (0.1% w/v) at room temperature (22°C). The cells were harvested from these cultures after 4 days growth by centrifugation at 110*g* for 15 minutes and the cell pellet was resuspended in phosphate buffer (100ml, pH 7.4). The cells were resedimented at 110*g* for 15 minutes and the supernatent decanted. The pellet was made up to volume (100ml) and left to recover from centrifugation at room temperature for 24 hours. Samples (0.1ml) of niosome or liposome suspension were mixed with this cell suspension (1ml) at time zero. At suitable intervals, volumes (1.5×10^{-2}ml) of this mixture were fixed in glutaraldehyde (2.5×10^{-2}ml, 0.1% solution) and examined by epi-fluorescence microscopy (Polyvar^{R*}). Further evidence of vesicular integrity was obtained using a camera mounted on the microscope and time-sequence photography (Kodak^{R*} film,160 ASA). At time intervals, the total fluorescence in each of 100 T.elliotti cells was measured using a spectrophotometer and an average percent of total fluorescence obtained. Photographs at these various time intervals (0,1,2,3,4,5 and

6 hours) were taken and vacuole counts were recorded for over 40 cells at each interval and vesicle type.

Effect of Enzymes on Vesicles (in vitro)

Solutions of phospholipase A_2, snake venom and carboxylic ester hydrolase were all freshly prepared before use.

Phospholipase A_2 activity has an absolute requirement for calcium ions (Ca^{2+}) and stock solutions were prepared by dissolving pure phospholipase (10mg) or crude venom (12.5 mg) in Tris-HCl buffer (2.0×10^{-2} M, 100ml, pH 7.4), containing $CaCl_2.2H_2O$ (3.2g).

Carboxylic ester hydrolase (5mg) was reconstituted before use by dissolving in Tris-HCl buffer (2.0×10^{-2} M, 1000ml, pH 7.4).

The leakage of CF (0.2M) from vesicles varying in their compositions was measured, as described, page 58; and compared, when challenged in vitro with the above enzyme solutions (2.5×10^{-2} ml). Leakage was measured over a time period of 6 hours. These values were corrected for CF leakage into buffer, as follows:

$$\% \text{ Release} = \frac{100\ (F_e - F_b)}{F_{tot}}$$

where F_e = fluorescence intensity in enzyme solution, ,

and F_b = intensity in buffer, both at time T.

F_{tot} = maximum intensity of fluorescence.

SECTION 3

RESULTS AND DISCUSSION

3.1. PRODUCTION OF NIOSOMES

Vesicles of various compositions (table 1) were prepared for use in this study using either the ether injection method *(Deamer and Bangham,1976)* or, the original hand-shaken method *(Bangham et.al.1965)*. The former method results, as reported for liposomes, in the formation of large unilamellar vesicles, with good entrapment efficiencies and greater stability in terms of leakage (see later, figure 9, page 72). However, since not all the components required for vesicle production were soluble in diethyl ether, the hand-shaken method was favoured for this present study. This method produced multilamellar vesicles with comparable entrapment efficiencies (table 2, page 68) but higher rates of leakage (figure 9). The hand-shaken method was preferred for several reasons:

a. these vesicles were easier to produce and handle, with more reproducible entrapment efficiencies between batches;

b. the overall stability of liposomes and niosomes were easily compared due to their higher rates of leakage, a major aim of this work; and

c. the components required to prepare the vesicles can be dissolved in suitable solvents, for example, chloroform, which are easily removed before the hydration process, thus ensuring a quick, simple and non-hazardous procedure in the production of every type of vesicle.

The entrapped volume of CF (ml mol^{-1}) was measured for 10 different types of vesicles and is shown in table 2, page 68. Entrapment is greater for liposomes than in all types of niosomes but does not appear to depend on the surfactant used. These entrapment efficiencies are low but are comparable with previous reports in the literature for liposomes *(Deamer,1984)*. However, these results suggest that manipulation of compositions may increase the entrapment of vesicles, example, addition of lipid (10%) with the original (100%) surfactant composition.

Table 1: NOMENCLATURE OF VESICLES INVESTIGATED

Composition (mol %)	Notation
100% Surfactant I	I100
50% Surfactant I 50% Cholesterol	I50:CHOL50
60% Surfactant I 30% Cholesterol 10% DCP	I60:CHOL30:DCP10
68% Surfactant I 30% Cholesterol 2% Stearylamine	I68:CHOL30:SA2
50% Surfactant II 50% Cholesterol	II50:CHOL50
60% Surfactant II 30% Cholesterol 10% DCP	II60:CHOL30:DCP10
100% Surfactant III	III100
50% Surfactant III 50% Cholesterol	III50:CHOL50
70% Surfactant III 20% Cholesterol 10% DCP	III70:CHOL20:DCP10
100% DPPC	DPPC100
50% DPPC 50% Cholesterol	DPPC50:CHOL50
70% DPPC 20% Cholesterol 10% DCP	DPPC70:CHOL20:DCP10
50% Egg PC 50% Cholesterol	Egg PC50:CHOL50
50% DMPC 50% Cholesterol	DMPC50:CHOL50

Quantification of Entrapment

To avoid the misleading use of "percentage of starting material entrapped" as the criterion for quantifying the substances in liposomes, it is advised that entrapment is expressed as the concentration (moles) or weight (mg) of aqueous medium per weight (mg) of liposomal lipid. The rationale for this has been discussed in detail elsewhere *(Tyrell and Heath et.al.1976)*. The entrapment values reported here (Table 2, page 69) were expressed in volume (ml) of CF per mole of surfactant, or lipid mixture. The efficiencies were calculated as a percentage of the total CF available for entrapment at hydration.

Removal of Unentrapped CF

Free drug or marker, can be removed after preparation of the vesicles in several ways (see introduction, page 15). Gel chromatography has been used in many laboratories and has proved an effective method of separation of free drug from entrapped drug. However, this method takes a considerable period of time and results in the collection of very dilute vesicle samples, therefore a concentration step is usually required, for example, ultrafiltration or centrifugation. After separation of vesicles from unentrapped CF, much of the free marker remains in the column and this is difficult to remove. Hence, cleaning the column after every use is time-consuming and the purchase of large quantities of column material (Sephadex^{R*}) is not economically sound. The process of centrifugation provides a quick, reliable method of separation requiring only a bench top centifuge, for sedimenting medium to large vesicles, or an ultracentrifuge for smaller vesicles.. However, typical of many types of compact cells, centrifugal force causes rupture and leakage of the vesicles. The method of choice in this study to separate free from entrapped drug was dialysis. This was easily performed, conveniently over-night, with several changes of eqi-osmolar buffer (for details see experimental,

TABLE 2

Methods of Production

Vesicle Type	Hand Shaken			Ether Injection		
	Entrapment ($ml.\ mol^{-1}$)	Efficiency (%)	(n^*)	Entrapment ($ml.mol^{-1}$)	Efficiency (%)	(n^*)
I100	1670±260	6	6	1111±200	4.2	3
I50:CHOL50	760±100	3	6	763±100	3.0	6
I60:CHOL30:DCP10	700±99	3	6	601±108	2.3	3
II50:CHOL50	979±129	4	4	1569±120	5.9	6
II60:CHOL50:DCP10	720±86	3	6	1212±99	4.5	3
III100	1590±63	6	6	1487±206	5.6	4
III50:CHOL50	650±52	3	6	503±86	1.9	4
III70:CHOL20:DCP10	320±68	1	6	355±46	1.3	3
DPPC100	2310±321	9	4	not determined		
DPPC50:CHOL50	1140±90	5	2	not determined		

(n^*) = number of determinations.

The values in this table have been rounded to the nearest whole number.

page 57). Occasionally it was necessary to centrifuge the vesicles at low speeds (<1000*g*) in the preparation of samples for polyacrlyamide slab gel electrophoresis (see later, page 99), but here the leakage properties of the vesicles were not of prime concern.

CF, obtained commercially, is a mixture of the 5- and 6- isomers of carboxylic acid in unequal propotions. It also contains several impurities which can affect;

a. leakage of CF from the vesicles,

b. the stability of the vesicles.

The most effective method of purifying CF is already described *(Ralston et.al. 1981)* but alternative short cuts are also employed. In the experiments described in this work, only two batches were purchased. In order to obtain at least 80% yields, the CF was partially purified over activated charcoal and recrystallised as described in the experimental (page 56). The CF was not purified completely since column chromatography gave very poor yields (<10%). Therefore, the CF used in these experiments may contain a small number of impurities which can affect its leakage through membranes. This may explain any discrepancies in the results when compared with the literature values for liposomes. However, the same material was used in all the vesicles studied, and a comparison was possible since it was assumed that the impurities affect all types of vesicles in a similar manner. Also the assays were easy to perform and gave consistent values between different batches of vesicles prepared. Ideally the purified CF should be checked by high pressure liquid chromatography (HPLC) or by thin layer.chromatography (TLC) for other products.

To determine the interference by Triton X-100, propan-1-ol or the vesicles on the fluorescence of CF, various concentrations of each (0.5%-5.0%) were added to CF solutions. The mixtures were incubated for 90 minutes at 37^{0}C and monitored

spectrofluorometrically. The detergent, alcohol or vesicles had no adverse effect on the fluorescence of CF.

3.2. LEAKAGE OF CF

Buffer

The rates of leakage from vesicles is dependent on the method of production, the nature of the entrapped substance, external factors such as, temperature and pH, osmotic pressure *(Baillie et.al.1985)* and their composition. Figure 9 illustrates the different rates of leakage from **I50:CHOL50** vesicles (a representative vesicle preparation) produced by the ether injection (E.I.) technique and the hand-shaken (H.S.) method. The leakage rate was much higher with the latter method. In both cases the entrapped substance was CF (0.2M,pH 7.4) and the external medium was PBS (Sorensens,1.3 X 10^{-2}M,pH 7.4, 22^{0}C). Leakage was measured as described in the experimental section, page 58.

The inclusion of cholesterol in membranes produces restriction of molecular motion in the region of the first 8 to 10 carbon atoms of the acyl chain from the lipid-water interface, leaving the remainder of the chains relatively free. This effect is accompanied by a condensation of the area per molecule, a more perpendicular orientation and a thicker membrane *(Hsia et.al.1971)*. Cholesterol suppresses the gel-to-liquid crystal transition in liposomes; below, the T_c it acts as a fluidising agent, while above the T_c it serves as a condensing agent *(Demel and De Kruyff,1976)*. In vitro studies have shown that incorporation of cholesterol into liposomal phospholipid bilayers reduces the permeability of a variety of substances through the bilayers *(Inoue,1974)* and reduces phospholipid loss to high density lipoproteins *(Kirby et.al.1980b)*.

CF Release from niosomes,I50:CHOL50, produced by **ether injection** and **hand-shaken** methods at 22oC.

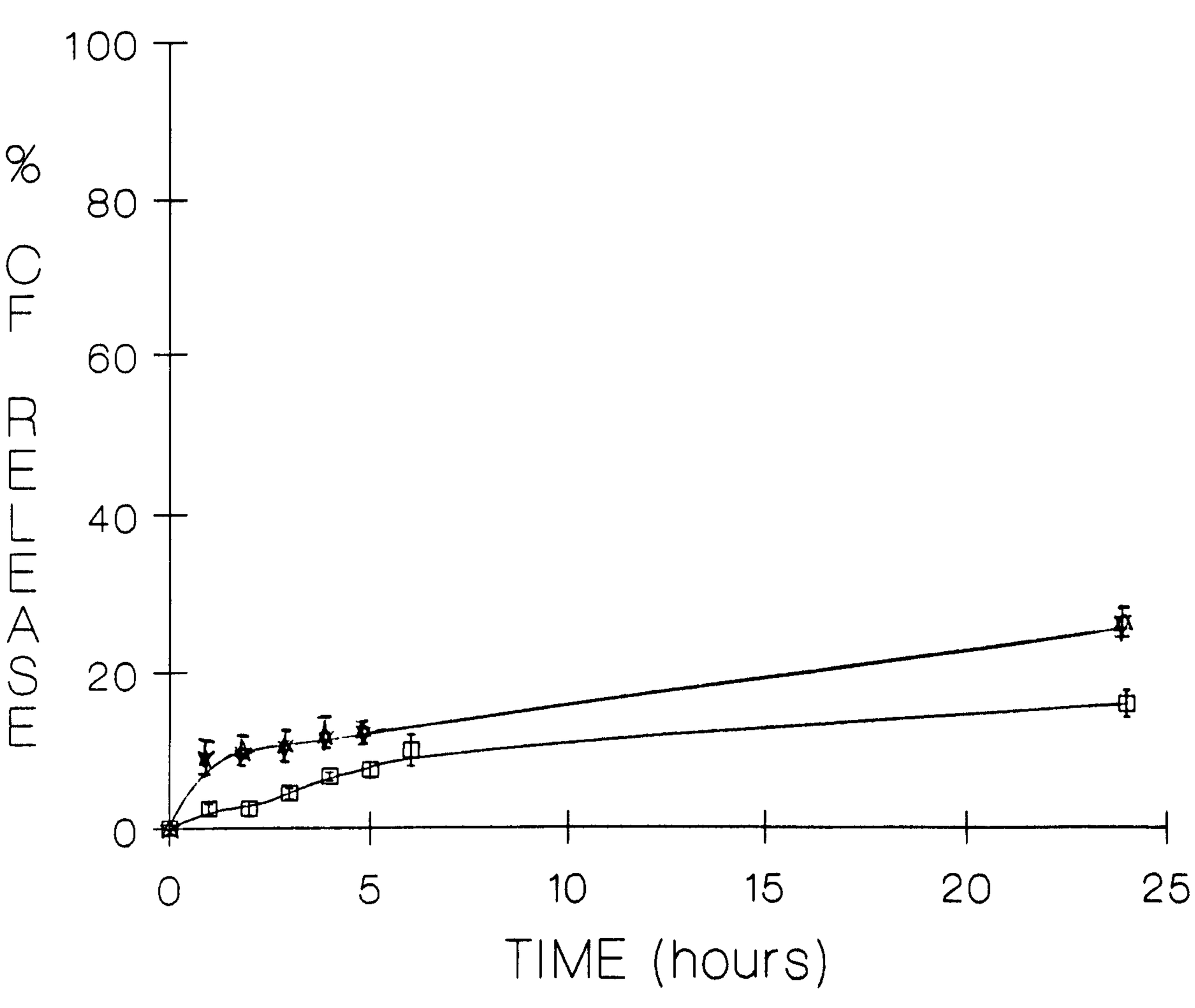


FIGURE 9

ETHER INJECTION HAND SHAKEN

N.B. In the above graph the error bars are standard error of mean (±).

Structural studies on phosphatidylcholine-cholesterol small unilamellar vesicles suggest that the optimum percentage inclusion of cholesterol is 32 mol% for vesicle formation. Beyond this, the vesicle is no longer spherical possessing a small ill-defined asymmetry *(Newman and Huang,1975)*. However vesicle structures are produced incorporating up to 50 mol% cholesterol into niosomes and it has been reported *(Vanlerberghe et.al,1978)* that 50 mol% cholesterol is required to abolish the transition isotherm. Vesicle formation from mono-alkyl non-ionic surfactant I, is an apparently novel observation but can be predicted on theoretical grounds. It is probable that molecular interactions of the surfactants with cholesterol are responsible for many physical characteristics of the resultant vesicles.

Niosomes were produced containing various percentages of cholesterol (0-50 mol%) and their entrapment values measured, as in figure 10. For all surfactants, I,II and III, optimal CF entrapment was found with the inclusion of 40 mol% cholesterol. Addition of DCP (10 mol%) significantly reduced entrapment values. The results indicate, for surfactant I niosomes, that leakage of CF is also greatly reduced with inclusion of 30-40 mol% cholesterol, as in figure 11.

Vesicles containing 100% surfactant have the highest entrapment efficiencies for niosomes but are the least stable and readily release their contents with time. Vesicles containing 50 mol% cholesterol are more stable in terms of CF efflux and inclusion of small amounts of DCP (10 mol%) produces a subsequent increase in leakage of CF. Increasing the mol-fraction of a charged lipid increases the membrane fluidity *(Papahadjopoulos et.al,1973)* resulting in increased membrane permeability.

Vesicles produced using surfactant II, had a minimum requirement of cholesterol (10 mol%) for their formation. This surfactant appeared to have several molecules

Entrapment of CF as a function of **cholesterol content** (mol%) for niosomes from surfactant **I,II** and **III**.

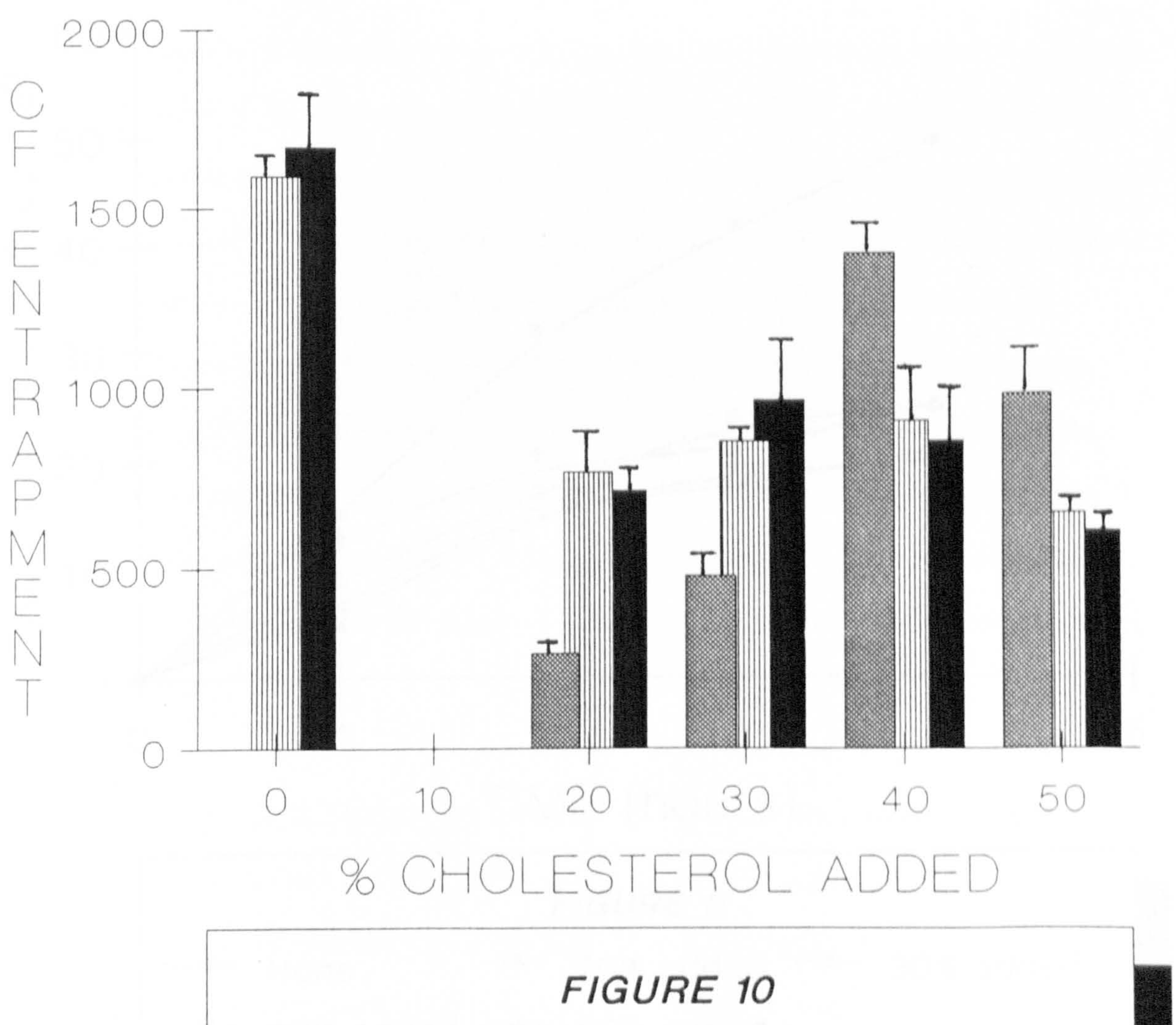


FIGURE 10

The entrapped marker was CF at 200mM and the entrapment is expressed as **litres per mole**.

Leakage of CF from niosomes prepared from Surfactant **I** containing varying amounts of **cholesterol**

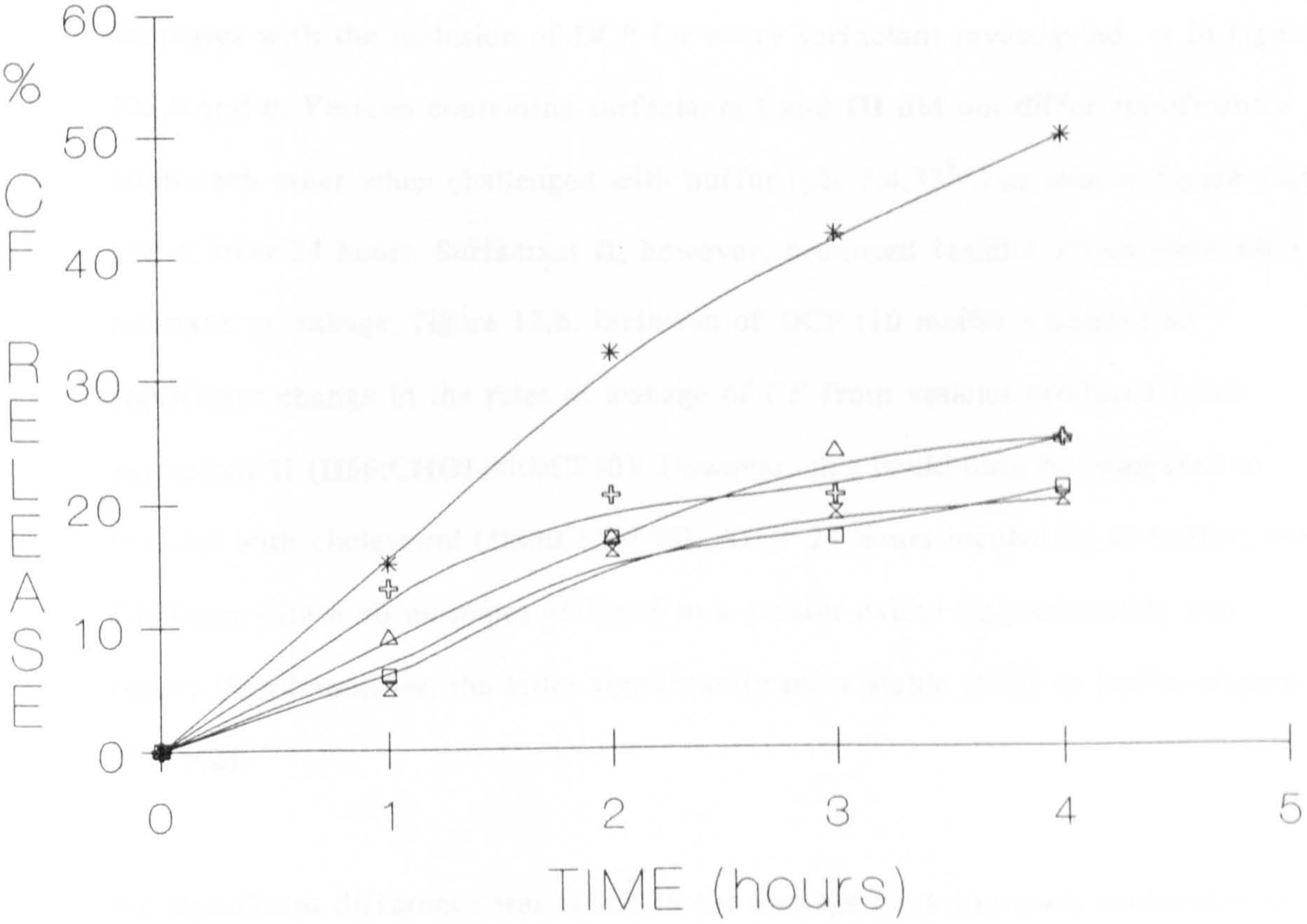


FIGURE 11

The leakage was determined in buffer (PBS, pH7.4 at 22oC) and the cholesterol content is in mol %

of water present with it. This "gummy" material could be dried, at 100^{0}C for several hours, to a paste. However the structural integrity of this dried product was no longer certain. Hence it was used as provided and 100% surfactant vesicles could not be prepared.

Vesicles in buffer (PBS) leak their contents gradually with time and as stated earlier, page 71, the stability increases with the inclusion of cholesterol and decreases with the inclusion of DCP for every surfactant investigated, as in figure 12a,b,and c. Vesicles containing surfactants I and III did not differ significantly from each other when challenged with buffer (pH 7.4,37^{0}C) as seen in figure 12,a and c, over 24 hours. Surfactant II, however, produced vesicles which were more resistant to leakage, figure 12,b. Inclusion of DCP (10 mol%) produced no significant change in the rates of leakage of CF from vesicles produced from surfactant II (II60:CHOL30:DCP10). However, this could only be compared to vesicles with cholesterol (II50:CHOL50). After 24 hours incubation in buffer, the CF from within all niosomes effluxed to a greater extent (approximately two times) than liposomes, the latter significantly more stable (50%) in buffer (figure 12,a,b,c).

No significant difference was found in the leakage from niosomes encapsulating "buffered" or "non-bufferd" CF (figure 13, page 78) suggesting that buffered CF solution is not essential for entrapment. Figure 13 further demonstrates that incorporating 50 mol% cholesterol into the bilayer of vesicles, reduces leakage.

Since the fluorescence of CF is dependent on changes in temperature and pH *(Allen and Cleland,1980)*, these were carefully controlled in all experiments. For each measurement, at least 2 controls were used; a 0% standard, containing "empty" vesicles (i.e. vesicles containing only PBS as entrapped substance) suspended in PBS; and a 100% standard containing PBS and a sample of vesicles

CF Release in Buffer(PBS, pH7.4)
for niosomes **I100, I50:CHOL50,**
and **I60:CHOL30:DCP10.**

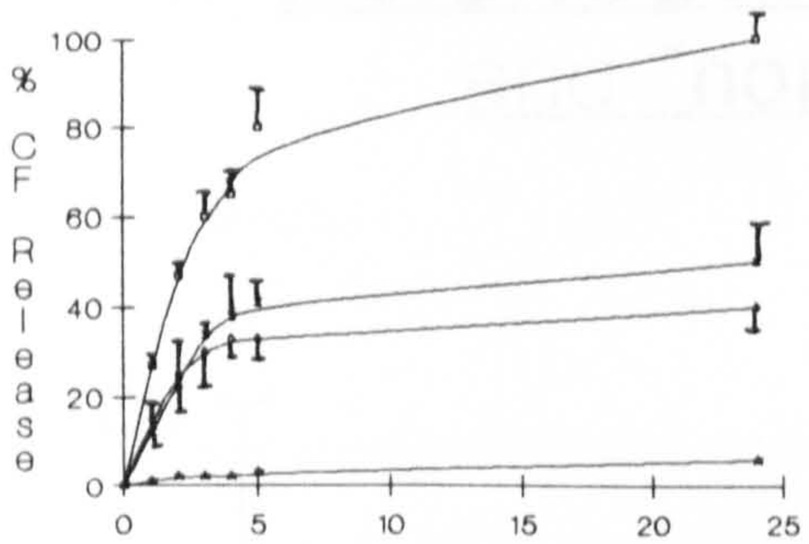


FIGURE 12a

I100 — I50:CHOL50
I60:CHOL30:DCP10 — DPPC50:CHOL50

Please refer to text for more details on surfactants, composition and conditions.

CF Release in Buffer(PBS,pH7.4)
with **I150:CHOL50, I160:CHOL30:DCP10**
and liposomes **DPPC50:CHOL50.**

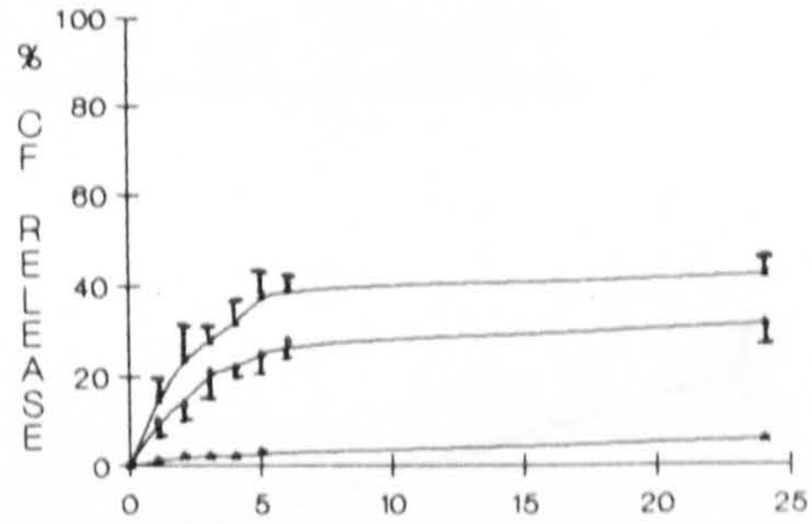


FIGURE 12b

I150:CHOL50 — I160:CHOL30:DCP10
DPPC50:CHOL50

Please refer to text for more details on surfactants, composition and conditions.

CF Release in Buffer (PBS,pH7.4)
with **III100, III50:CHOL50** and
III60:CHOL30:DCP10.

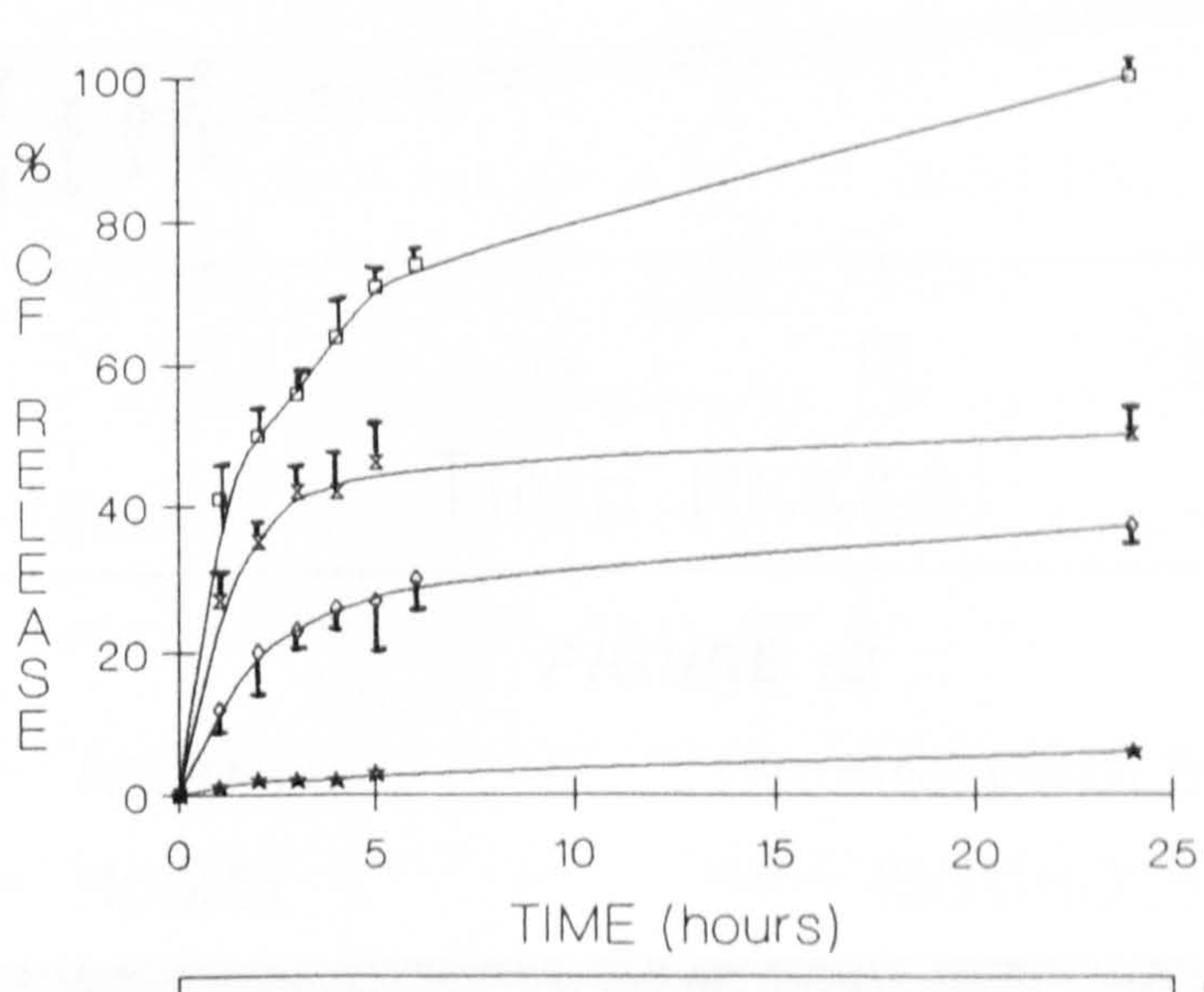


FIGURE 12c

III100 — III50:CHOL50
III60:CHOL30:DCP10 — DPPC50:CHOL50

The liposome preparation has been added for comparison.

CF Release from niosomes, **I100** and **I50:CHOL50** containing **"buffered"(B)** and **"normal"(N)** CF at 22oC.

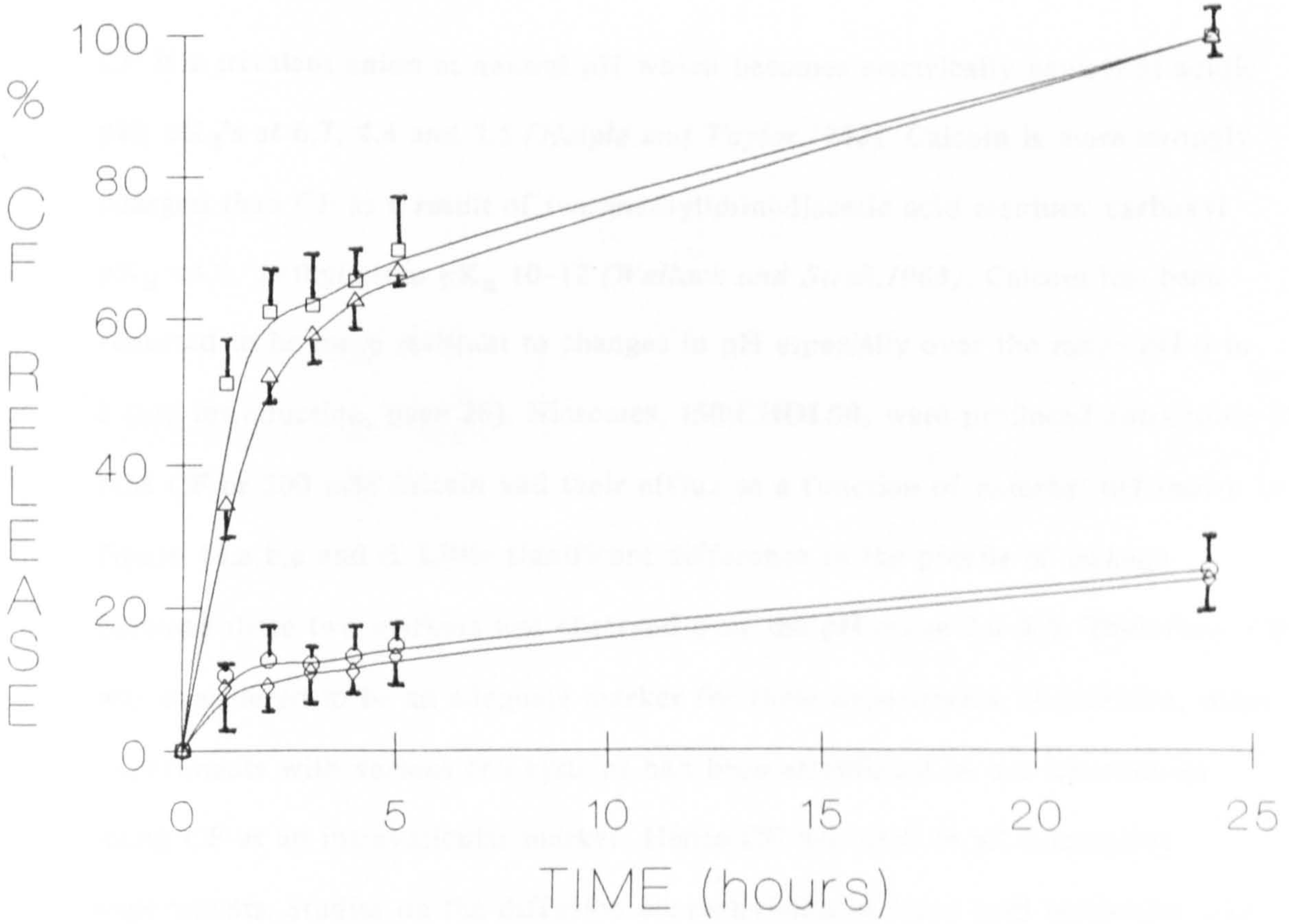


FIGURE 13

Note that there is little difference between the two preparations irrespective of the **enclosed marker**.

completely disrupted using propan-1-ol or Triton X-100, resulting in a suitable dilution of CF and maximum fluorescence for these conditions. Low levels of fluorescence can be detected by increasing the sensitivity of the fluorimeter. Readings at this very high sensitivity settings were discarded.

pH Effects

CF is a trivalent anion at neutral pH which becomes electrically neutral at acidic pH; pK_a's at 6.7, 4.4 and 3.5 *(Heiple and Taylor,1982)*. Calcein is more strongly charged than CF as a result of two methyliminodiacetic acid residues; carboxyl pK_a <4.0; methylimino pK_a 10-12 *(Wallach and Steck,1963)*. Calcein has been reported to be more resistant to changes in pH especially over the range pH 6 to 8 (see introduction, page 26). Niosomes, **I50;CHOL50**, were produced entrapping 200 mM CF or 200 mM calcein and their efflux as a function of external pH shown in figure 14,a,b,c and d. Little significant difference in the profile of leakage between these two markers was observed over the pH range 2.0-8.0. Therefore, CF was considered to be an adequate marker for these experiments. In addition, other experiments with various cell systems had been established in our laboratories using CF as an intravesicular marker. Hence CF was used in all subsequent experiments. Studies on the diffusion characteristics of weak acid molecules, like CF, has implications for drug encapsulation and delivery in biological systems, for example, methotrexate, used in chemotherapy, is a weak acid and is expected to possess similar properties in terms of leakage at various pH values.

The effect of pH on CF leakage from 100% surfactant vesicles of all types is shown in figure 15,a,b,c and d. For all vesicles examined, CF efflux was most rapid at pH 4.0 and below. Rapid efflux at low pH (4.0 and below) is probably a

Release from niosomes at **pH 2.0** containing **CF** and **calcein** as entrapped markers.

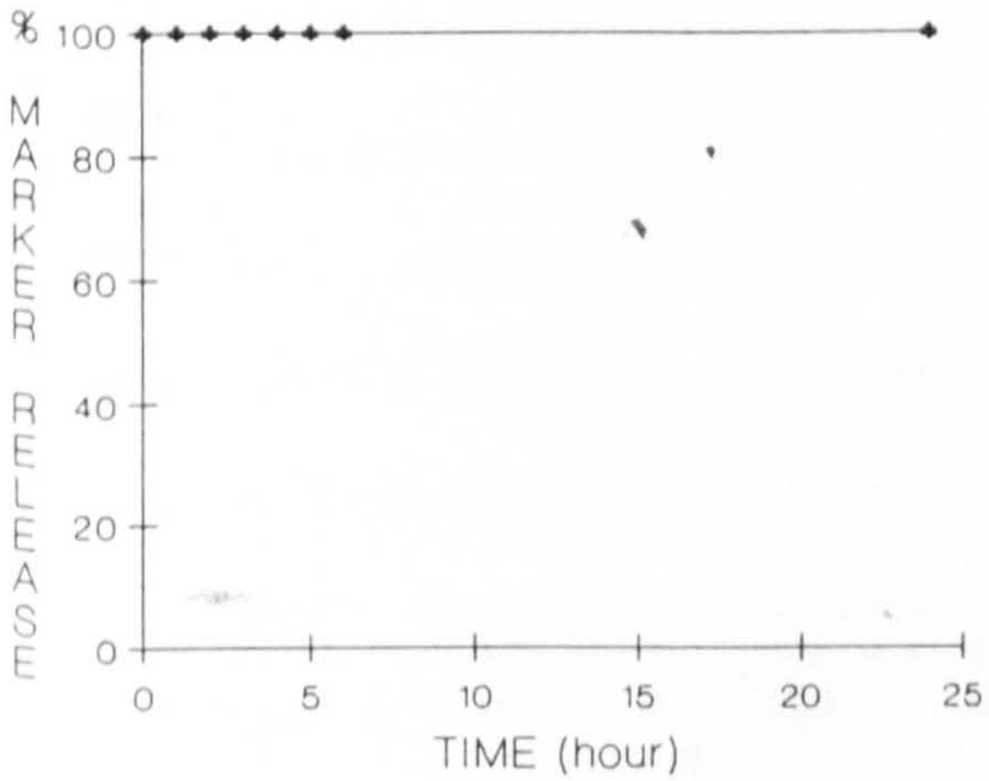


FIGURE 14a

The niosome used was **I50:CHOL50** and the temperature kept at 37oC. The niosomes contained cholesterol at 50 mol%.

Release of **CF** and **Calcein** from niosomes prepared from surfactant I with added cholesterol at a **pH** of **4.0**.

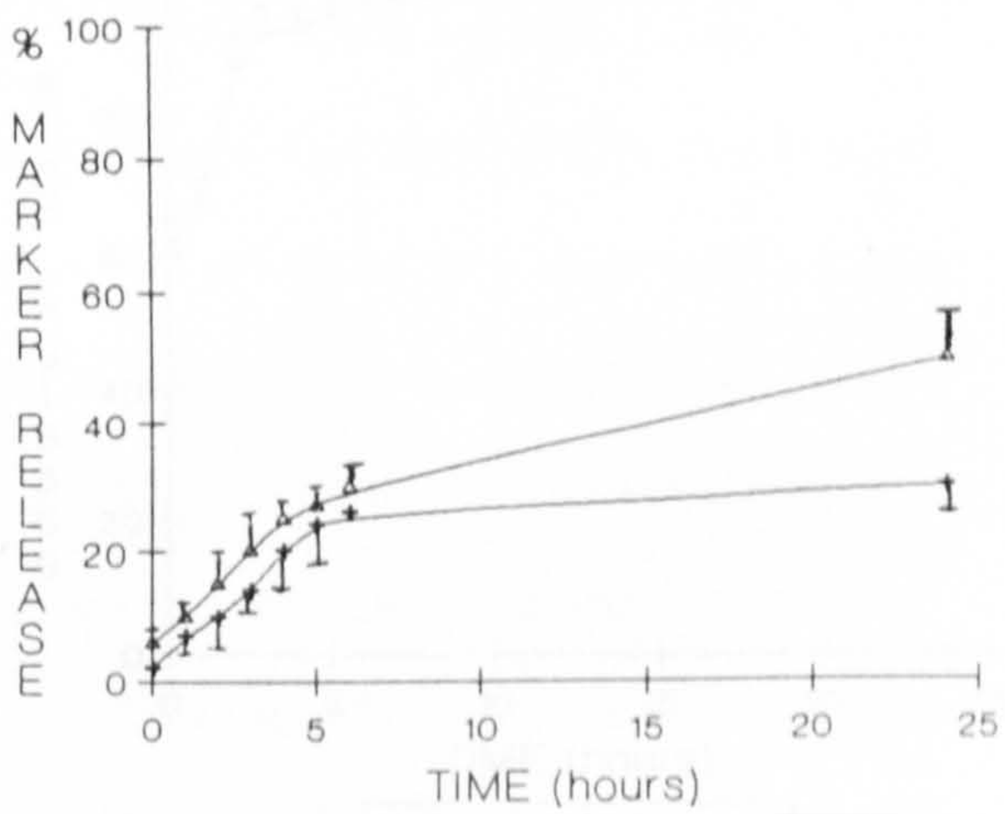


FIGURE 14b

Temperature was maintained at 37oC and equi-osmolar markers used

Release of marker from niosomes entrapped with **CF** and **Calcein** at 200mM at a **pH** of **6.0**.

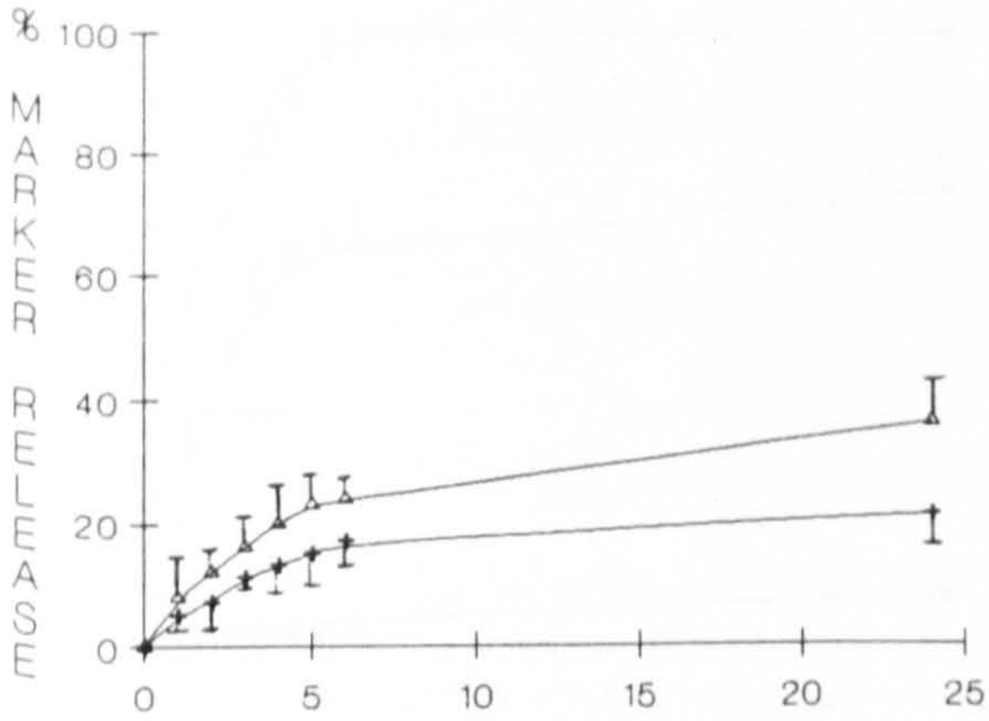


FIGURE 14c

Cholesterol was used, 50 mol% to prepare the niosomes and the temperature of the experiment maintained at 37oC.

Release at **pH 8.0** from within niosomes of entrapped **CF** and **Calcein** used as markers

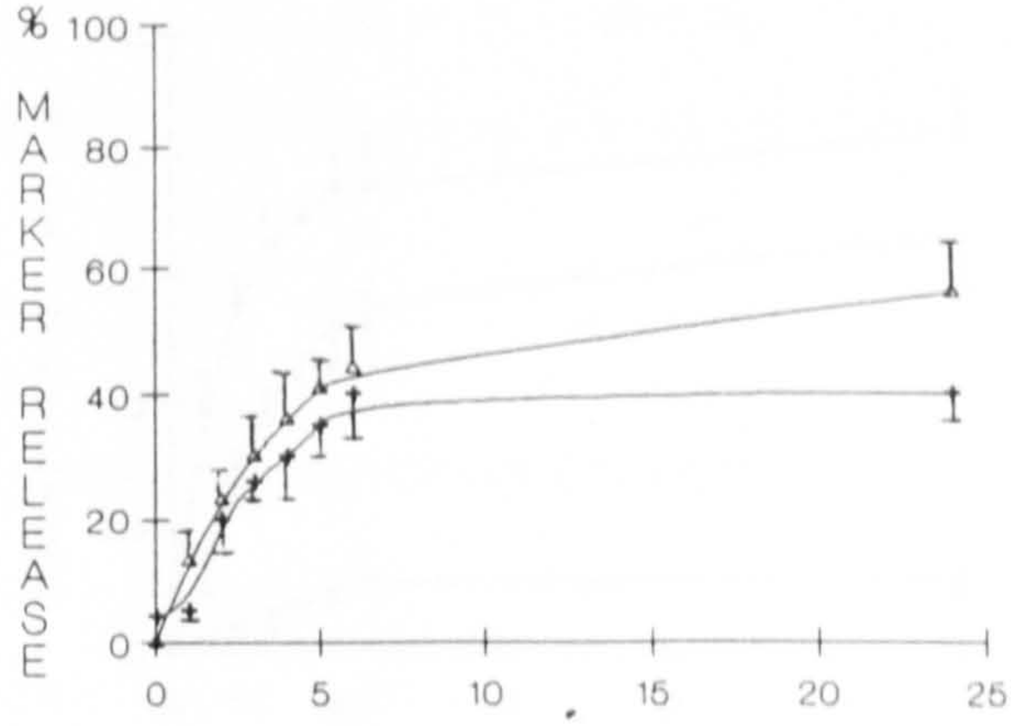


FIGURE 14d

Niosome, **I50:CHOL50**, was prepared by the addition of cholesterol 50 mol%.

CF Release from niosomes, **I100** and **III100** at **pH 2.0**

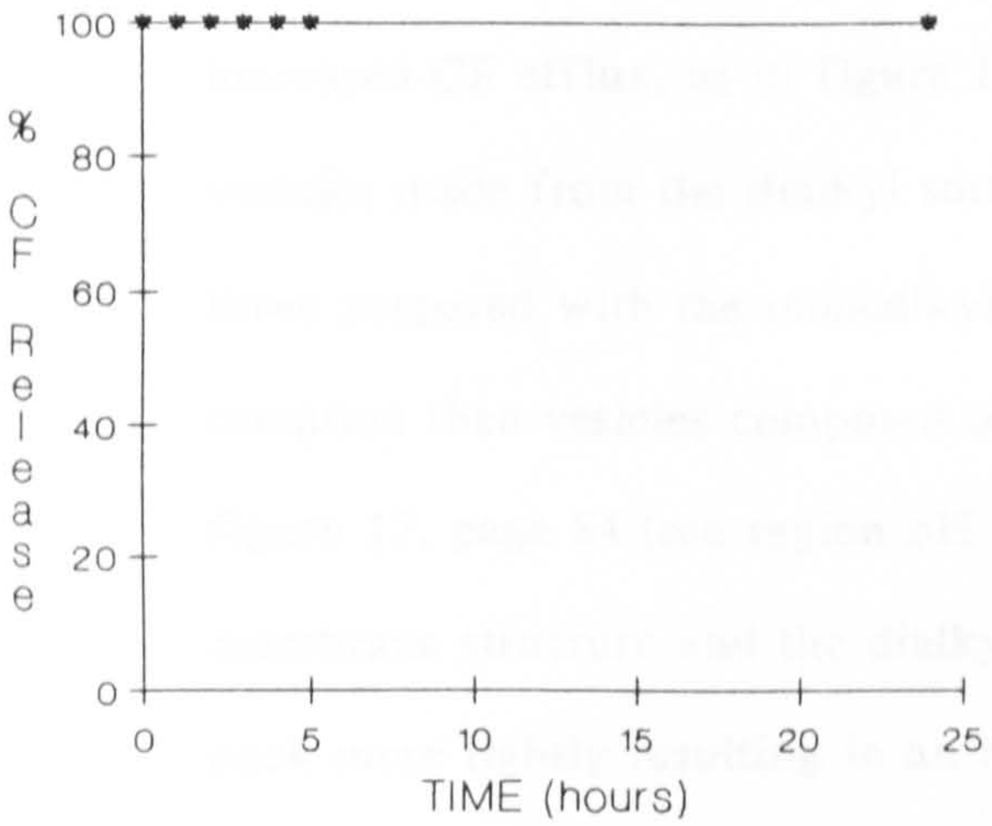


FIGURE 15a

There was total leakage at this low pH from both surfactant **I** and **III** vesicle types.

CF Release from niosomes, **I100** and **III100** at **pH 4.0**

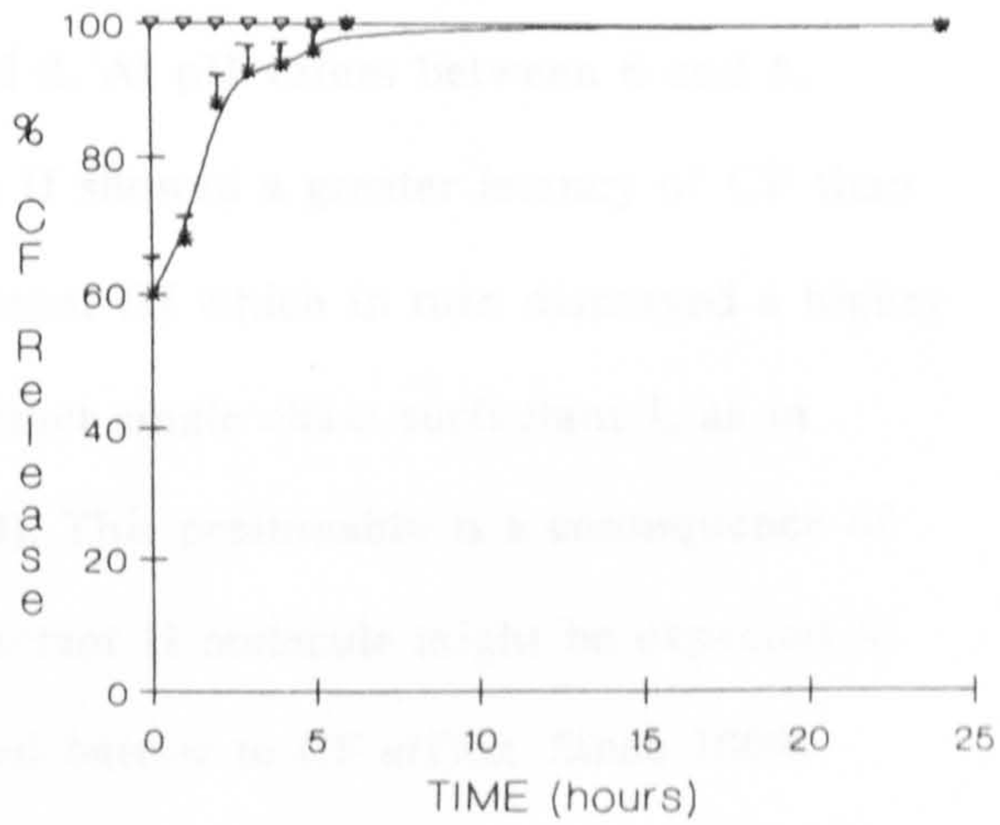


FIGURE 15b

Niosomes prepared from surfactant **III** are more susceptible to the effect of pH than from **I**.

CF Release from niosomes, **I100** and **III100** and from liosomes, **DPPC100** at **pH 6.0**

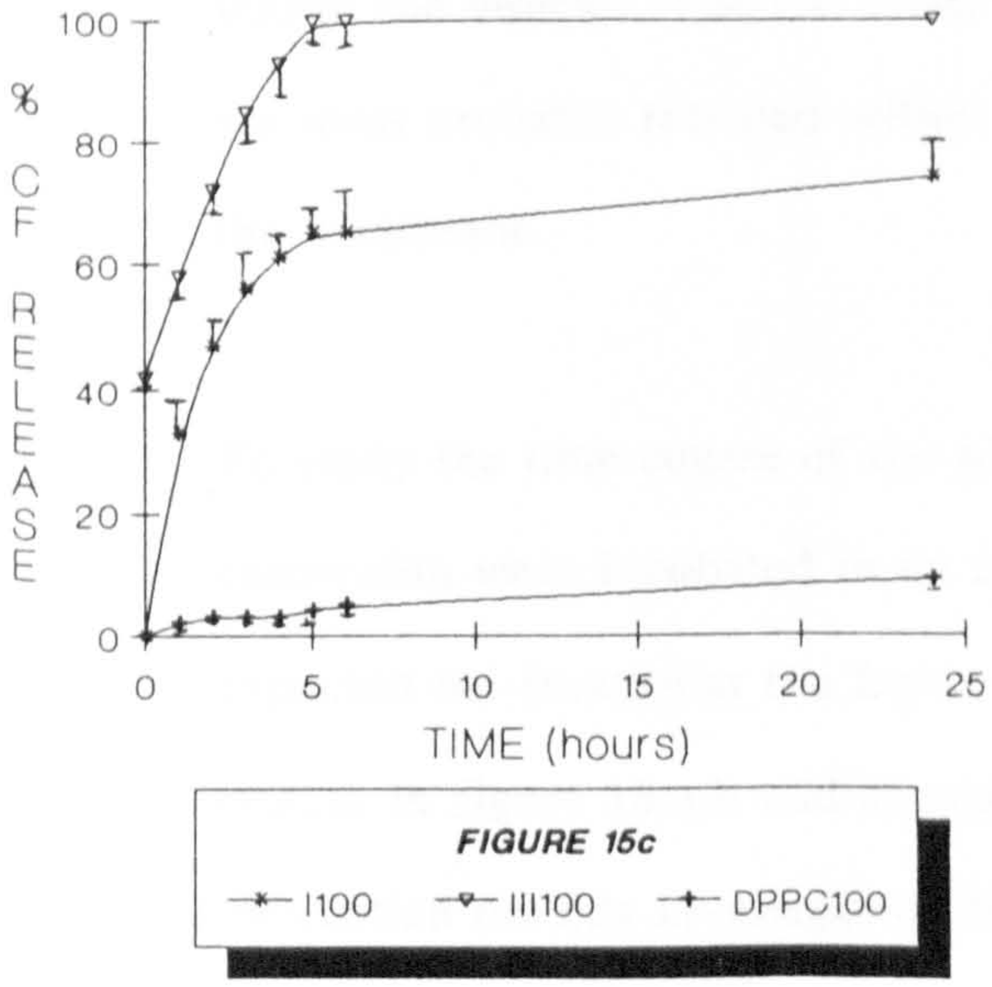


FIGURE 15c

The liposomal preparation is the most stable at this pH when compared to the two niosome preparations.

CF Release from niosomes, **I100** and **III100** and from liposomes, **DPPC100** at **pH 8.0**

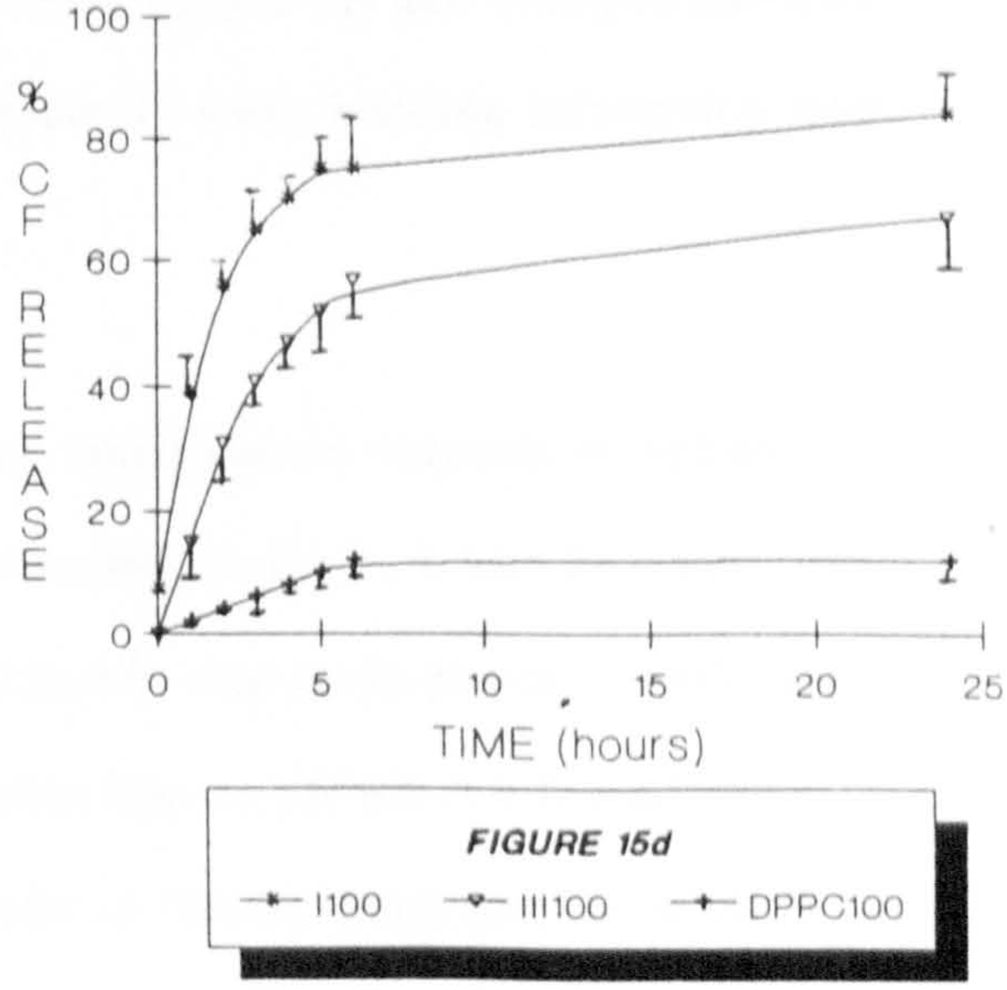


FIGURE 15d

Niosomes from surfactant II (100%) was not possible. Liposomes have been added for comparison .

result of protonation of the carboxyl moiety of CF (at high H^+ concentration) which enhances CF diffusion across the bilayer to the external media. However, inclusion of cholesterol in the bilayer appeared to confer a stabilising effect on the vesicle at pH 4.0 and 6.0, as in figure 16,a and b, and inclusion of DCP increased CF efflux, as in figure 16,c and d. At pH values between 6 and 8, vesicles made from the dialkyl surfactant II showed a greater latency of CF than those prepared with the monoalkyl surfactant III which in turn displayed a higher retention than vesicles composed of the other single chain surfactant I, as in figure 17, page 84 (see region pH 6.0-8.0). This presumably is a consequence of membrane structure and the dialkyl surfactant II molecule might be expected to pack more tightly resulting in an increased barrier to CF efflux. Since 100% surfactant II vesicles could not be prepared, this comparison was only made with vesicles containing 50 mol% cholesterol, as shown in figure 17 legend, page 84. However, the highest latency for all vesicles studied was found near pH 7.4., minima of all graphs in figure 17. The pH of the CF marker at the beginning of the experiment was measured and set at 7.4 and it is envisaged that when the pH of the external media is at 7.4, an equilibrium exists reducing loss of CF from within the vesicles. The CF exists as the anion at this pH and charged particles are most probably retained within the vesicle by weak bonding interaction with the membrane.

To study the time course of the pH effect, small known volumes of vesicle suspension were incubated in an appropriate buffer for 1, 6 and 24 hours. As expected the leakage at the lower pH's, 2.0-4.0, was much greater (maxima % CF release in figure 18,a,b and c, page 85) than that at pH 6.0-8.0 for all the incubation periods investigated (the minima in figure 18,a,b and c). After 6 hours incubation, figure 18,b, leakage at all pH's was greater (approximately 30%, as in figure 18,a versus 18,b) and this was more evident after 24 hours, as in

CF Release in niosomes from surfactant **I, II** and **III** with **added cholesterol** (50 mol%) at **pH 4.0**.

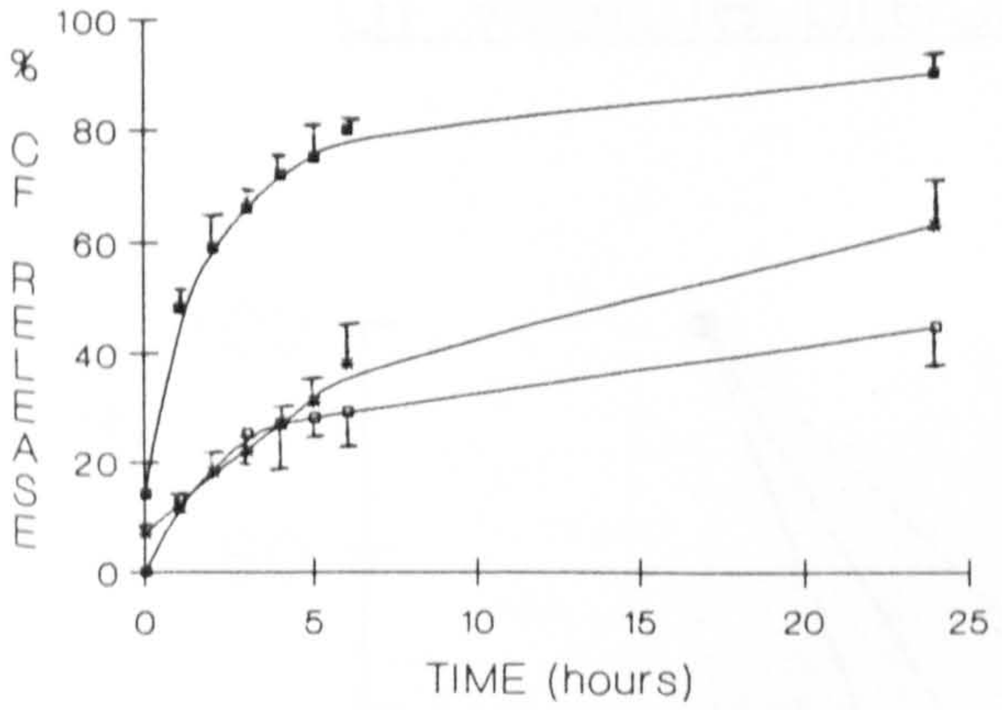


FIGURE 16a

Cholesterol stabilises these vesicles from the effect of pH as explained in the text.

CF Release from niosomes containing surfactant **I,II** and **III** with **added cholesterol** at **pH 6.0**.

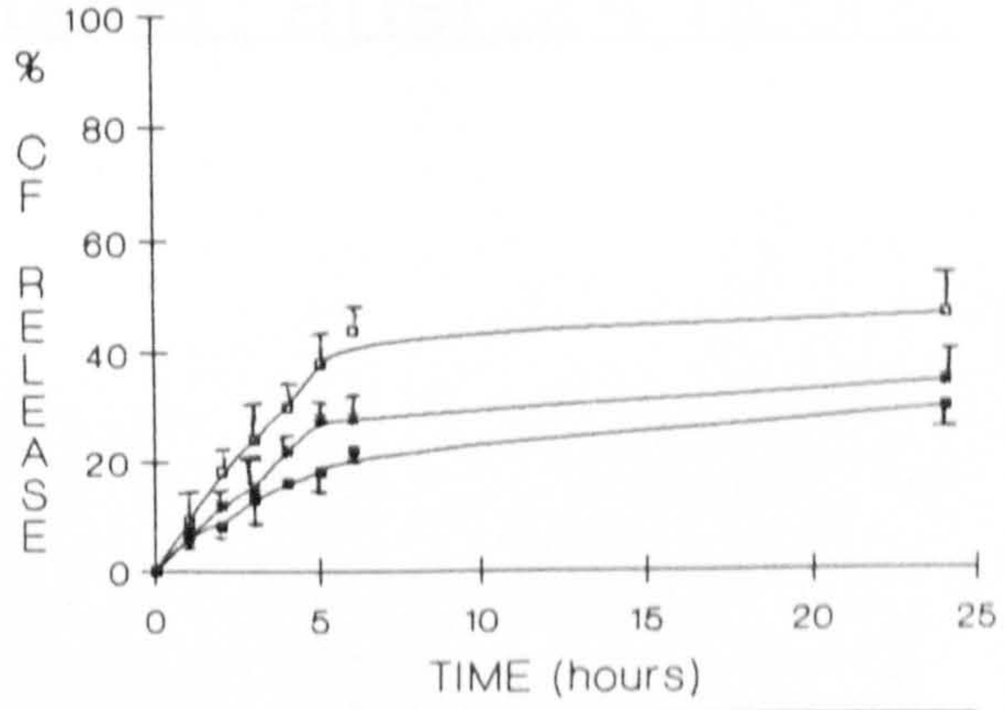


FIGURE 16b

At pH 6.0 the vesicle are nearly all less leakier than at pH 4.0 as mentioned in the text.

CF Release from niosome with surfactant **I,II** and **III** with **added DCP** at **pH 4.0**

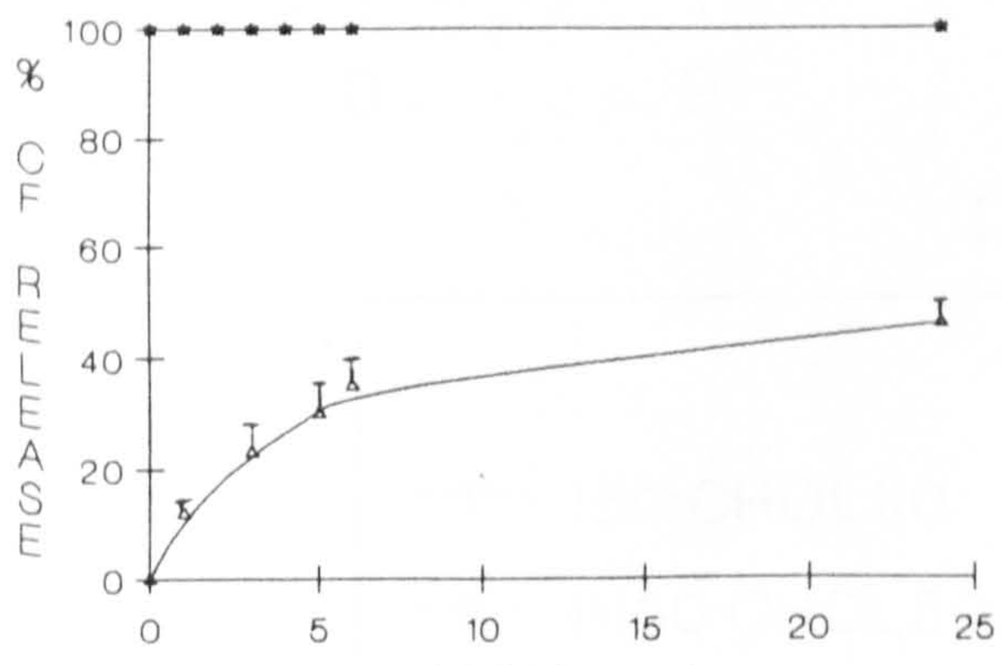


FIGURE 16c

The temperature of the experiment was set at 37oC and the vesicles had 10 mol% DCP added to them.

CF Release from niosomes prepared from surfactant **I,II** and **III** with **added DCP** at **pH 6.0**.

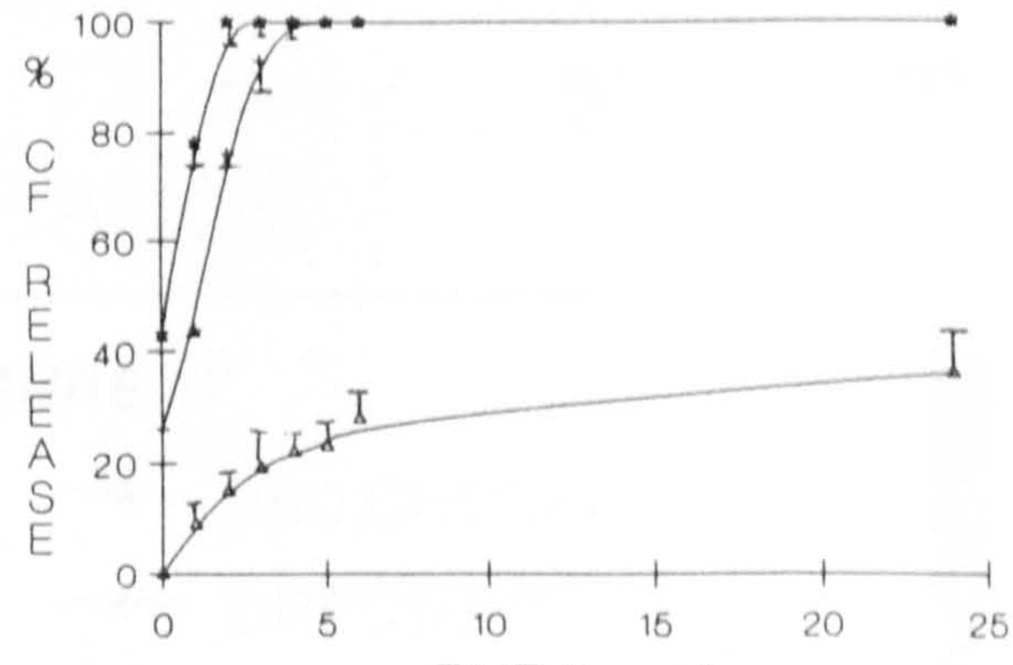


FIGURE 16d

The addition of DCP markedly affects the release kinetics. The vesicles are less leakier at pH 6.0 than pH 4.0.

CF Release from **niosomes** and **liposomes** over a **pH range 2.0 to 8.0** for a variety of vesicle preparations after 24 hours.

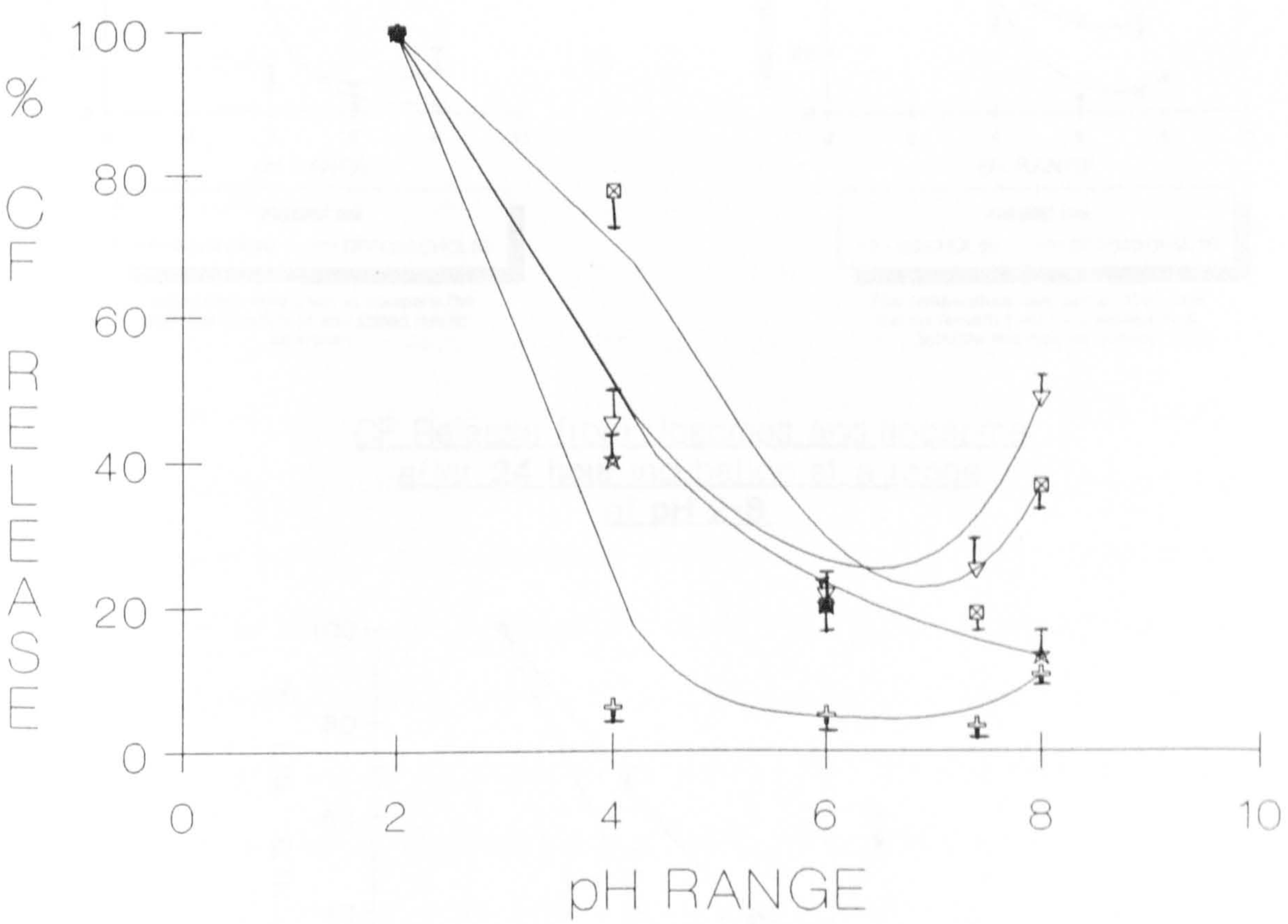


FIGURE 17

The temperature was 37oC and a comparison of vesicles with cholesterol is shown as a typical example.

CF Release from niosome prepared from surfactant I compared with liposome after **1 hour** over **pH 2-8**.

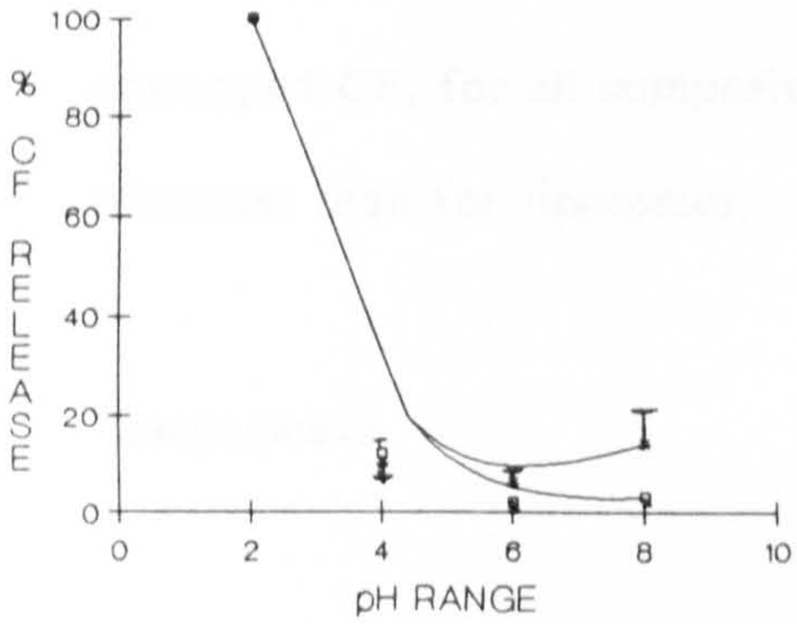


FIGURE 18a

Liposomes were used to compare the leakage kinetics of entrapped marker as shown.

CF Release from niosome, **I50:CHOL50** and liposome,**DPPC50:CHOL50** after **6 hr** incubation over a **pH range of 2-8**.

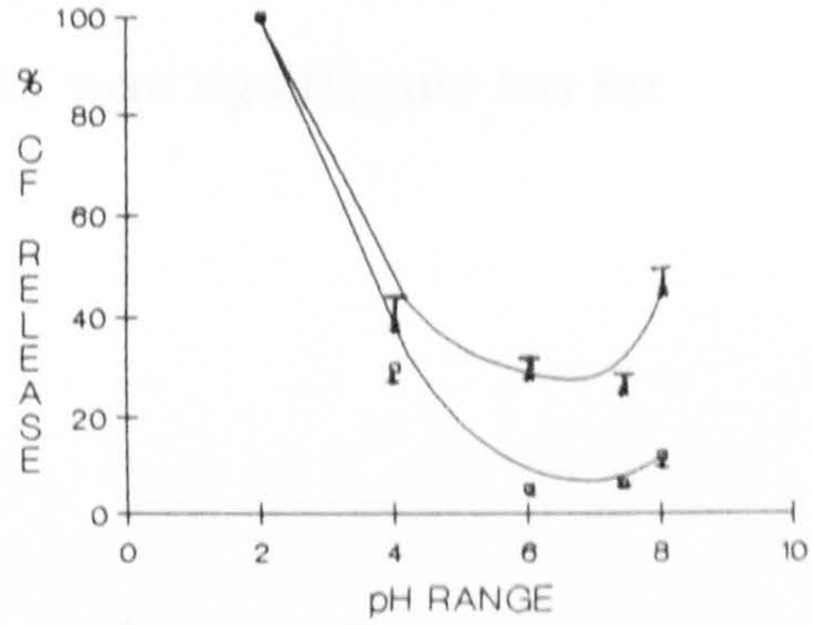


FIGURE 18b

The temperature was set at 37oC and the surfactant **I** vesicles served as a suitable example as shown.

CF Release from niosomes and liposome after **24 hour** incubation at a range of **pH 2-8**.

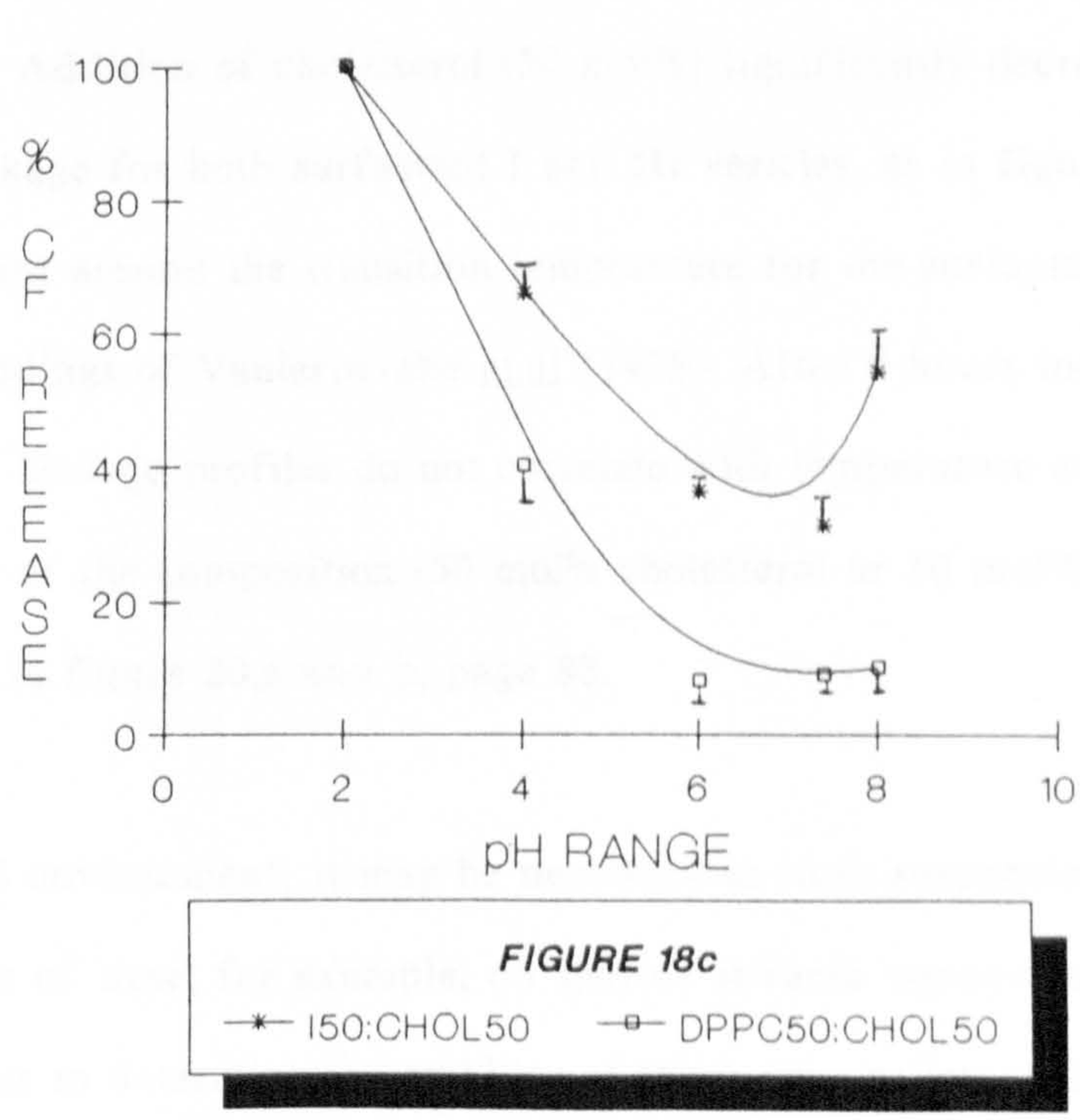


FIGURE 18c

For this experiment the temperature was set at 37oC and niosome containing cholesterol was **compared to liposomes**

figure 18,c. Liposomes follow a similar trend but are more stable, as seen in figure 18.a,b and c, page 85. These above experiments were all performed at 37°C and were measured at appropriate time intervals together with a control for CF to account for any changes in the fluorescence characteristics. The retention of entrapped CF, for all compositions investigated, were significantly less for niosomes than for liposomes.

Temperature

The effects of various temperatures, 4°C, 22°C, 37°C and 50° were investigated using CF as the entrapped marker at pH 7.4 in eqi-osmolar PBS. All vesicles examined showed a similar trend; that is, increased CF leakage with increased temperature. In all cases although leakage at 4°C and 22°C (room temperature) were similar, a significant increase was apparent at 37°C and 50°C and this was most pronounced at lower incubation periods, for example, after 1 hour, as in figure 19,a. Addition of cholesterol (50 mol%) significantly decreased temperature induced leakage for both surfactant I and III vesicles, as in figure 19,b. This was most apparent around the transition temperature for the surfactants, in agreement with the findings of Vanlerberghe et.al *(1978)*. After 6 hours incubation or greater, the leakage profiles do not correlate with temperature and are erratic, irrespective of the composition (50 mol% cholesterol or 10 mol% DCP) of the vesicles, as in figure 20,a and b, page 88.

In a clinical environment, it may be necessary to store suspensions of vesicles over periods of time, for example, on shelves at room temperature (22°C). It is advantageous to determine the stability of these suspensions under these conditions. It is also important to estimate the leakage, before administering these suspensions in vivo. In some cases the composition of the vesicles may require modification to optimise latency of the entrapped drug.

CF Release from niosomes at
various temperatures after **1 hour**
for surfactant **I** and **III (100%)**

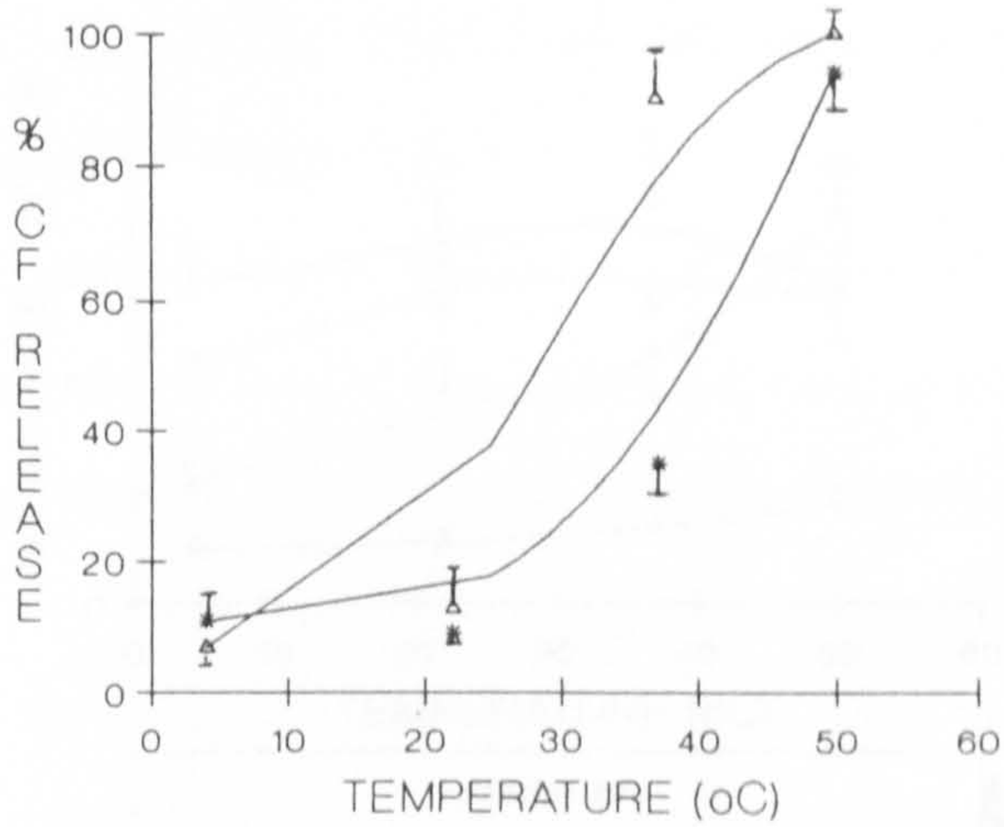


FIGURE 19a

Two surfactant vesicles were used,
niosomes, I100 and III100.

CF Release from niosomes, after **1 hour**
incubation at various **temperatures**,
containing **cholesterol**.

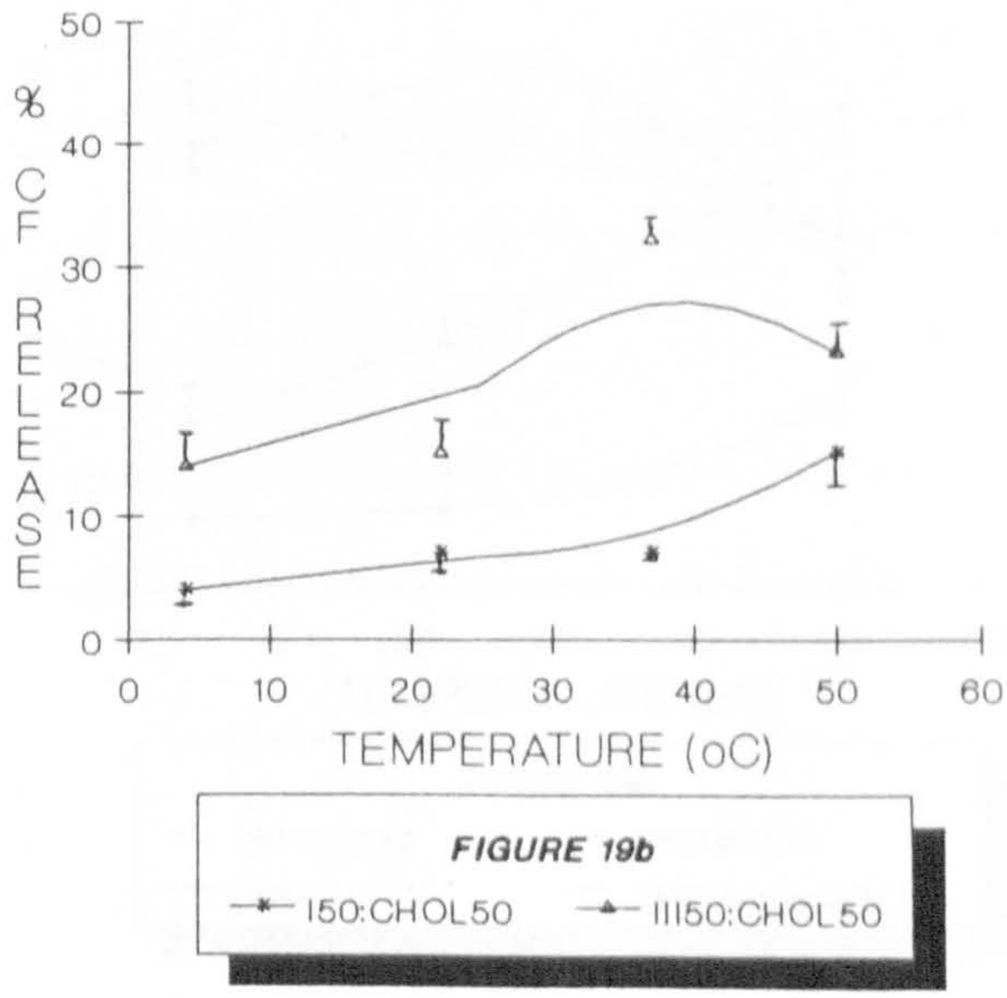


FIGURE 19b

Niosomes were prepared from
Surfactant **I** and **III** and also
included cholesterol 50 mol%

CF Release from **niosomes** containing cholesterol at **various temperatures** after **6 hours** incubation in buffer.

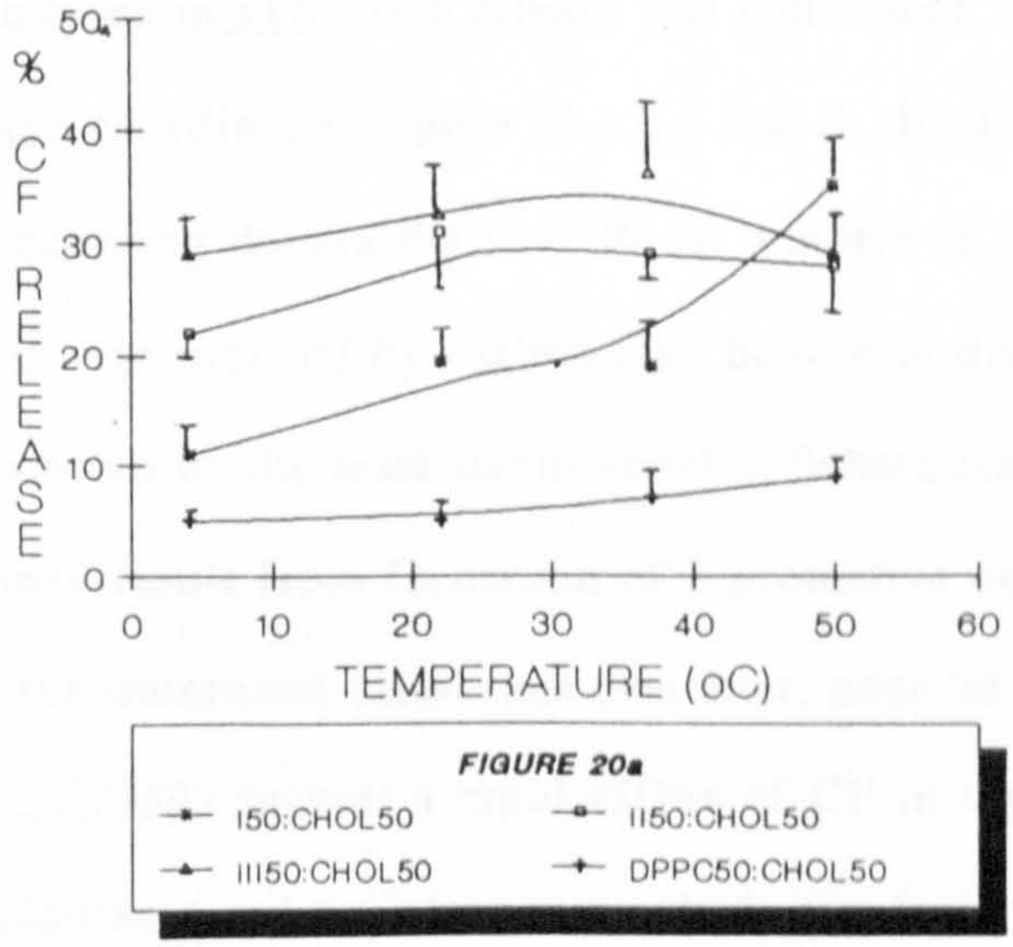


FIGURE 20a

The cholesterol content was 50 mol% in all the niosomes prepared and the buffer was PBS.

CF Release from niosomes and liposomes after **24 hours** at **various temperatures**.

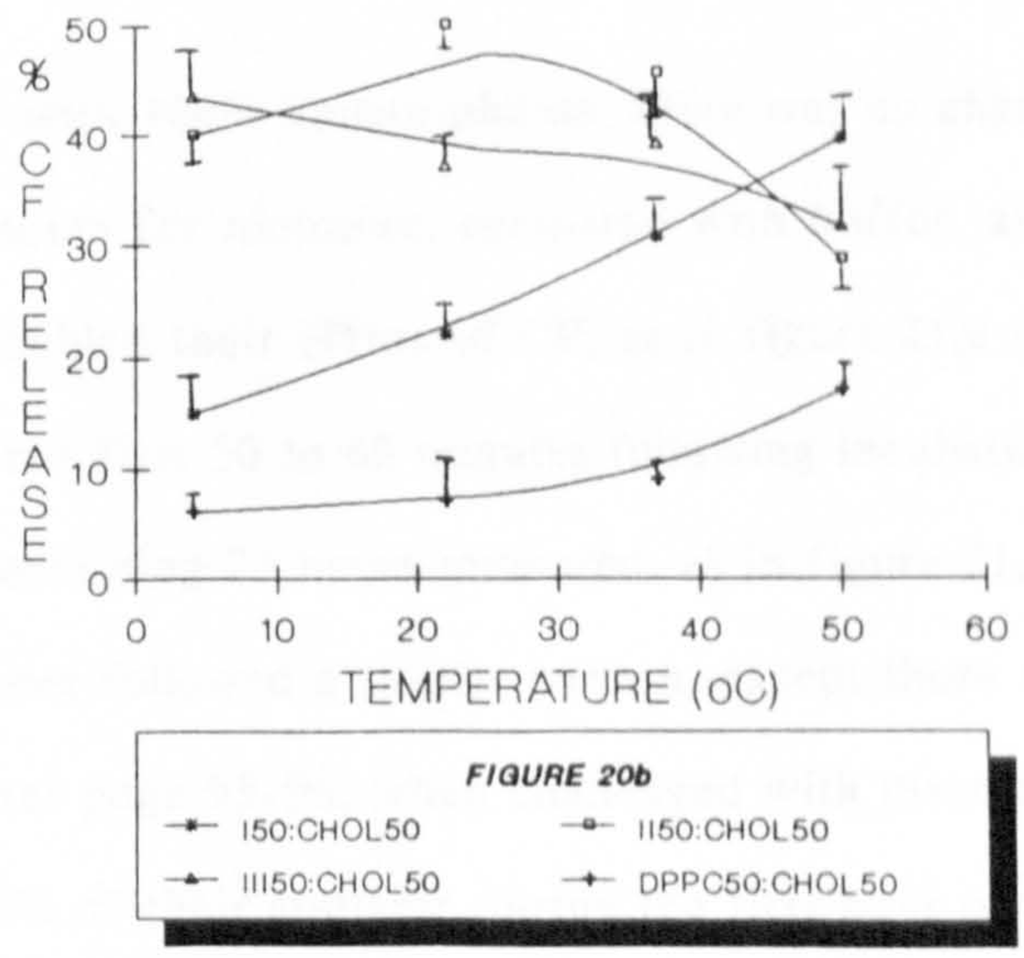


FIGURE 20b

The vesicles were incubated in buffer (PBS) and liposome was added for comparison of stability.

3.3. EFFECT OF PLASMA

To optimise the potential of niosomes as drug carriers, it is important to correlate their stability in terms of release of their contents, in vitro, with physiological conditions in vivo. Dye release was non-linear with time, when plotted on Cartesian coordinates, figure 21,a,b,c and d, displaying a substantial rapid component occuring during the first 30-60 minutes of incu- bation. This initial high leakage rate induced by plasma may be due to disturbances in the bilayers and breakdown of the least stable vesicles. Subsequent recovery of the barrier function may result from formation of a protective outer coat by plasma protein bound to the outermost membrane (see later, page 98). Similar reports on liposomes *(Guo et.al.1980)* suggest a rapid efflux of CF in the presence of plasma for the first 30 minutes, until a plateau is reached. The leakage of CF from vesicles challenged by buffer, 100% human plasma, serum and heat-inactivated serum are compared in figure 21a,b,c and d. In these environments, niosomes, **I50:CHOL50** and liposomes, **DPPC50:CHOL50** (suitable representatives), are distinctly different.

When challenged with 100% human plasma, there was no change in the leakage profile after 24 hours for niosomes, compared with buffer, although, the liposomes had more than doubled their efflux of CF, as in figure 21,a and b. This increase occurred during the first 30 to 60 minutes following incubation, attaining a plateau for the remaining 23 hours measured, as in figure 21,b. The profile seen with other niosomes followed a similar pattern, except those prepared from surfactant **II**, see later page 95-96, when challenged with plasma, loosing approximately 50% of their contents during the first hour of exposure, then remaining stable against further leakage, as in figure 21,b.

CF Release from **niosomes and liposomes** In **Buffer (PBS)** at 37oC. Surfactant **I** is a typical representative.

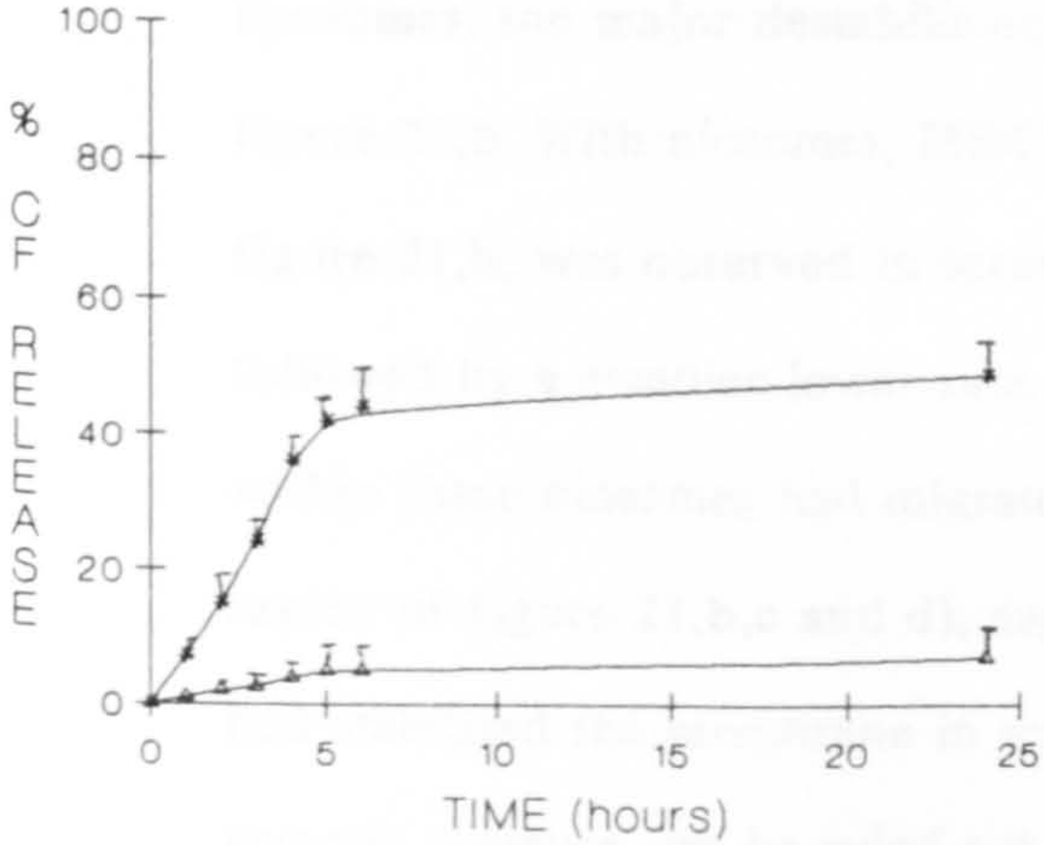


FIGURE 21a

CF Release from **niosomes and liposomes** Incubated In **100% plasma** at 37oC.

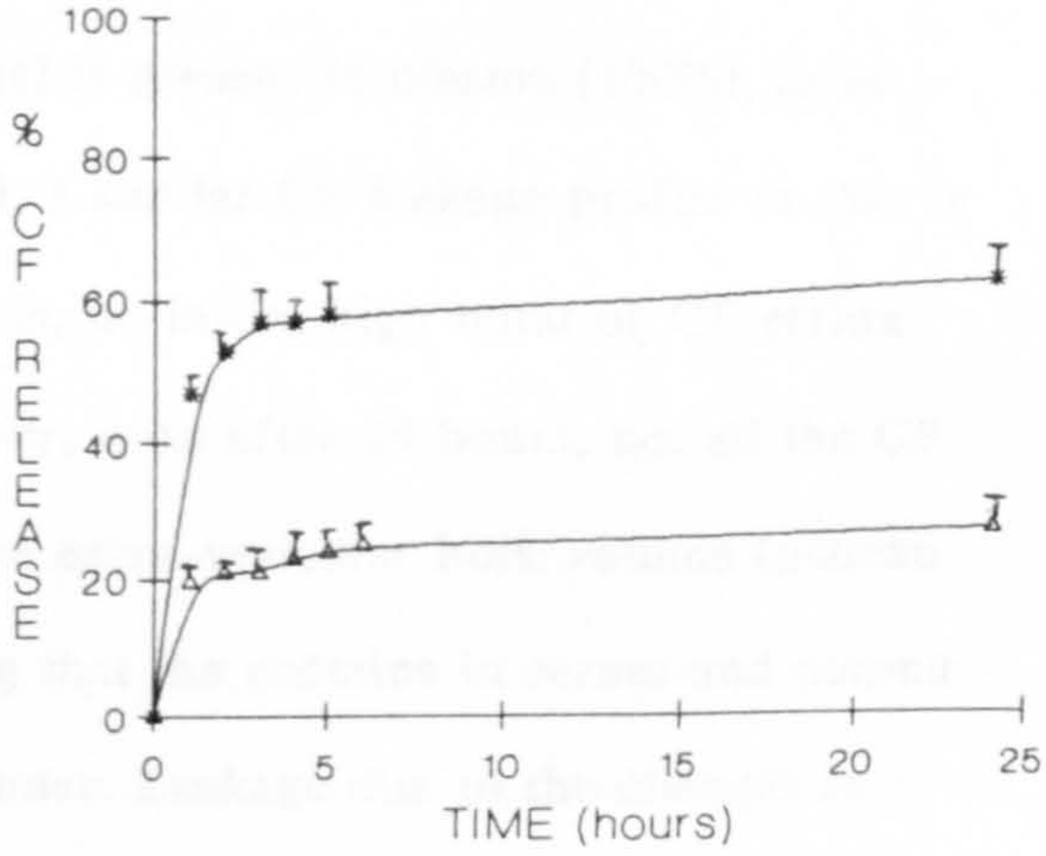


FIGURE 21b

Surfactant **I** vesicles were chosen for this comparison as a typical example and compared with an equivalent liposome

CF Release from **niosome,I50:CHOL50** and **liposome,DPPC50:CHOL50** at 37oC on incubation in **blood serum**.

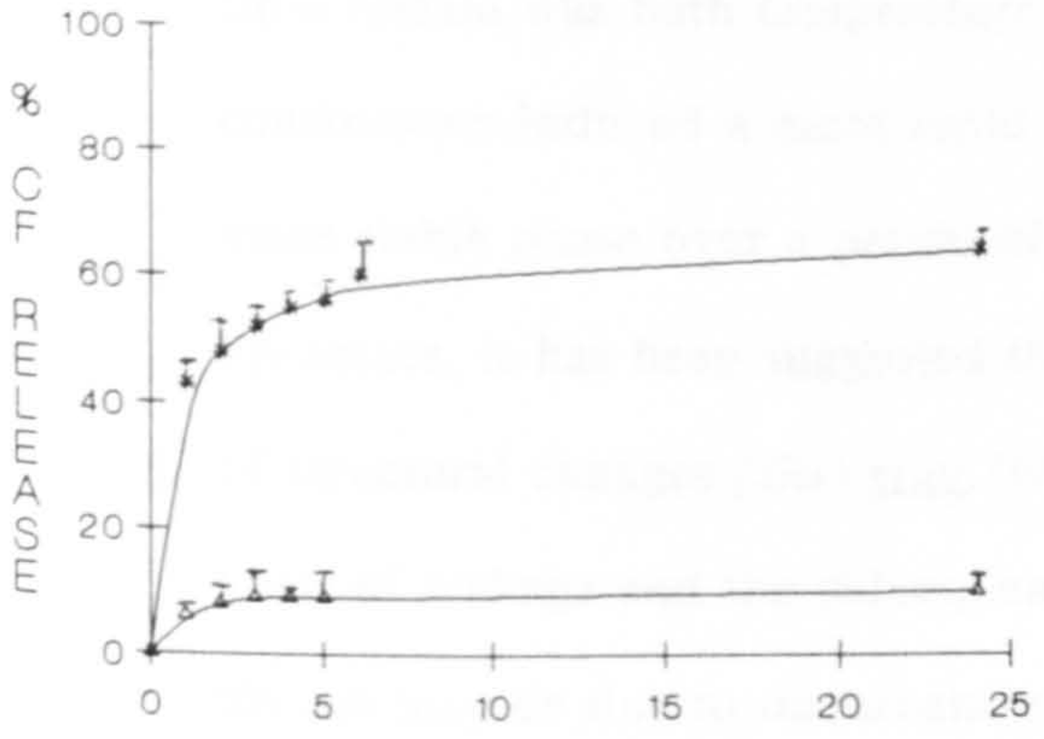


FIGURE 21c

Surfactant **I** was chosen as a typical representative and compared in similar conditions to liposomes.

CF Release from **niosomes and liposomes** after incubation in **heat-inactivated serum** at 37oC.

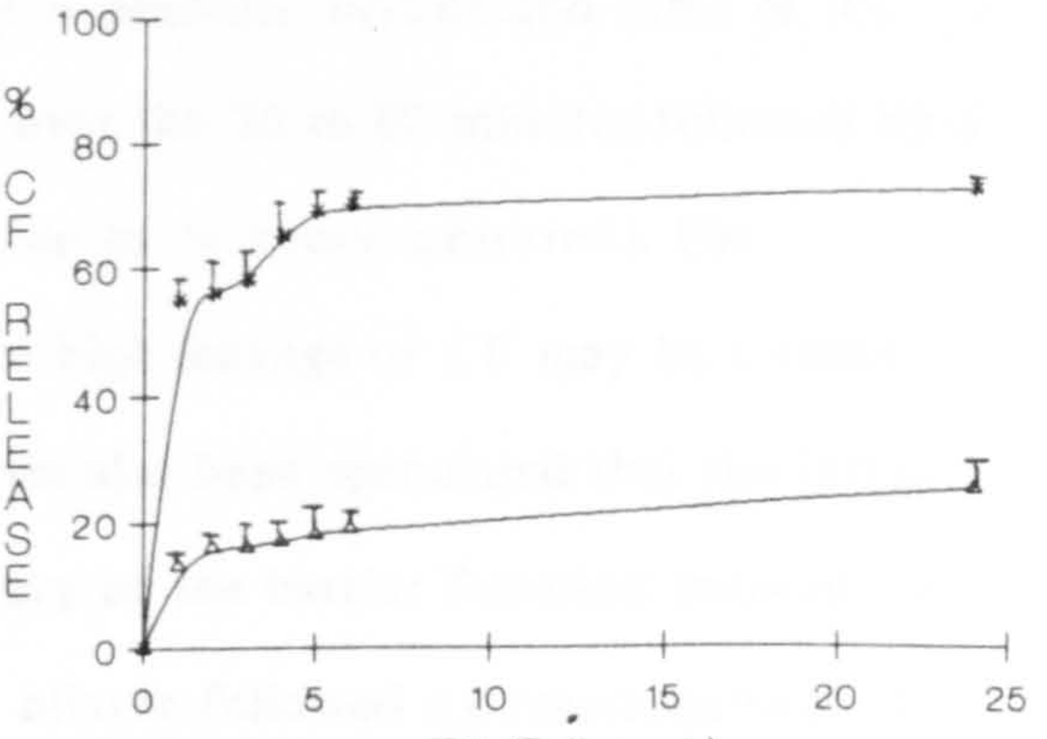


FIGURE 21d

Niosome **I50:CHOL50** and liposome **DPPC50:CHOL50** were compared as before for their individual stabilities.

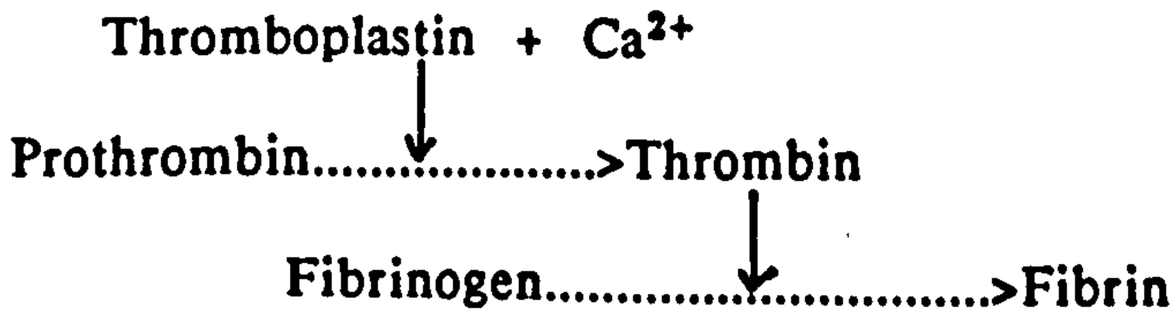
When challenged with serum (figure 21,c and d), a different response was observed. Liposomes, **DPPC50:CHOL50** displayed similar leakage kinetics to that in buffer, as in figure 21,a, when challenged with both serum and heat-inactivated serum and no increase in CF efflux was detected. This indicates that, for liposomes, the major destabilising factor(s) is present in plasma (100%), as in figure 21,b. With niosomes, **I50:CHOL50**, a similar CF leakage profile to that in figure 21,b, was observed in serum; that is, an initial high burst of CF efflux followed by a steadier lower rate. However, even after 24 hours, not all the CF within these niosomes had migrated to the extra-vesicular bulk volume (plateau region of figure 21,b,c and d), suggesting that the proteins in serum and plasma had stabilised the membrane in some manner. Leakage due to the changes in osmotic pressure can be ruled out because the osmotic pressure of plasma (average value, 6.62 atm. equivalent to 5030 mm Hg at 0^{0}C, Documenta Geigy,*1962)* is always much higher than that of CF at the working concentration.

In the absence of plasma or serum components, the enclosed dye escaped slower, hours instead of minutes, as in figure 21,a, in buffer, from these vesicles. This slow release was both temperature and pH-dependant. Plasma and some of it's constituents induced a more rapid release over the 30 to 60 minutes followed by a more stable phase over a period of hours (up to 24 hours measured). For liposomes, it has been suggested this initial high leakage of CF may be a result of structural changes *(Guo et.al,1980)*. It has also been speculated that the initial burst of leakage and the subsequent recovery of the barrier function induced by plasma may be due to disturbances of the bilayer followed by rearrangement of membrane components *(Yoshioka,1984)*. However, details of this mechanism have not been clarified.

Many reports in the literature *(Damen et.al,1982)* suggest that the removal of liposomal phospholipid with the lipids of high-density lipoprotein (HDL) causes the

release of the solute entrapped. Egg PC liposomes were shown to be transformed into smaller particles, similar to HDL, a short time (5 minutes) after intravenous administration into rats or after 5 minutes incubation with rat plasma *(Krupp et.al,1976)*. Similar results were reported *(Scherphof et.al,1978)* after incubation of liposomes with plasma HDL. The appearance of discoidal particles was observed *(Tall and Small,1977)* after incubation of DMPC liposomes with human HDL. Purified bovine apolipoprotein A-1 (Apo-1) also forms "small" complexes with DMPC liposomes containing up to 33 mol% cholesterol *(Jonas and Krajnovich,1978)*. Serum apolipoproteins were found to be most potent liposome-disrupting agents of serum in one study *(Guo et.al,1980)*. Free apolipoproteins exist in native serum, but the amounts are thought to be too small to account for the observed serum activity. However, Apo-1 is loosely associated with human HDL and may dissociate in vitro under several mild conditions *(Tall and Small,1977)*. Reports on albumin binding to liposomes *(Zborowski et.al,1977;Law et.al,1986)* may be explained by the presence of small amounts of contaminating lipoproteins or apolipoproteins. Some commercial preparations of BSA have been found to contain substantial amounts of HDL that appear as lamellar particles in electron microscopy techniques *(Hamilton,1978)*. Different commercial preparations of BSA may also contain variable amounts of contaminants that induce the release of CF from such vesicles.

Serum is produced from plasma by inducing a clot and removing it. The major constituent removed from serum is fibrinogen, a protein found in blood and tissue extracts which in the presence of thrombin is transformed into the insoluble product, fibrin.



Several clotting factors, for example, factor VIII, are also removed in this process. The results obtained using serum and heat-inactivated serum indicate that the "complement system" has no influence on the leakage of CF from niosomes, similar to results cited for liposomes *(Mayhew et.al.1980)*. However, heating serum also inactivates a protein which appears to act in conjunction with high density lipoprotein in transferring or exchanging phospholipids from liposomes *(Damen et.al.1981)*.

In addition to plasma lipoproteins, other plasma proteins are absorbed to liposomes surfaces and may be involved in their destabilisation and removal from the circulation *(Juliano and Lin,1980)*. Published data for liposomes *(Guo et.al.1980)* suggest that there is a rapid transfer of phospholipid from the vesicles to high density lipoproteins in the initial incubation period with plasma until a state of equilibrium has been reached (plateau region, similar to that in figure 21,b,c and d, page 90). Other workers *(Yoshioka et.al.1984)* however dispute this, suggesting that the initial burst of leakage may be due to reorganisation of membrane structure. It has been shown *(Agarwal et.al.1986)* that both stability in blood and the clearance rate from the circulation can be modulated by structurally modifying the ester linkages in the phospholipid component of liposomes.

Evidence has also been presented *(Bosworth and Hunt,1982)* which suggests that the effect of blood components on liposome permeability is saturable so that with an increasing number of liposomes in a fixed volume of blood, the fraction of entrapped material released decreases. The typical effects of a 100% solution of human plasma on a number of different niosomes, prepared from surfactant I, II and III,is shown in figure 22,a,b and c. This concentration of plasma (100%) caused no obvious (within experimental error) increase in CF efflux from liposomes, **DPPC50:CHOL50**, figure 22a, or from niosomes containing surfactant II (**II50:CHOL50** and **II60:CHOL30:DCP10**), as in figure 22,b.

CF Release from **niosomes** containing surfactant **I** and **liposome** when challenged with **plasma (100%)**

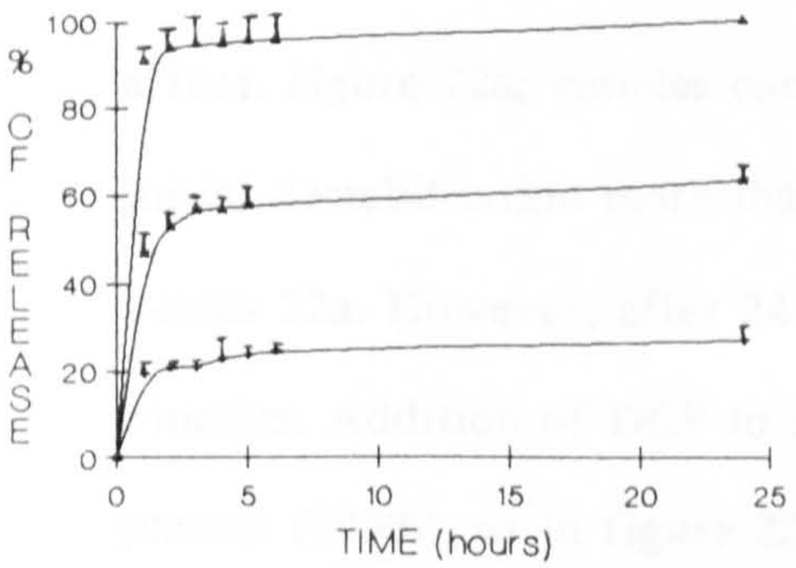


FIGURE 22a

I100 niosomes — I50:CHOL50 — DPPC50:CHOL50

Liposomes have been included for comparison, only on this graph. The added cholesterol was 50 mol%.

CF Release from **niosomes** prepared from Surfactant **II** and incubated with **plasma (100%)**

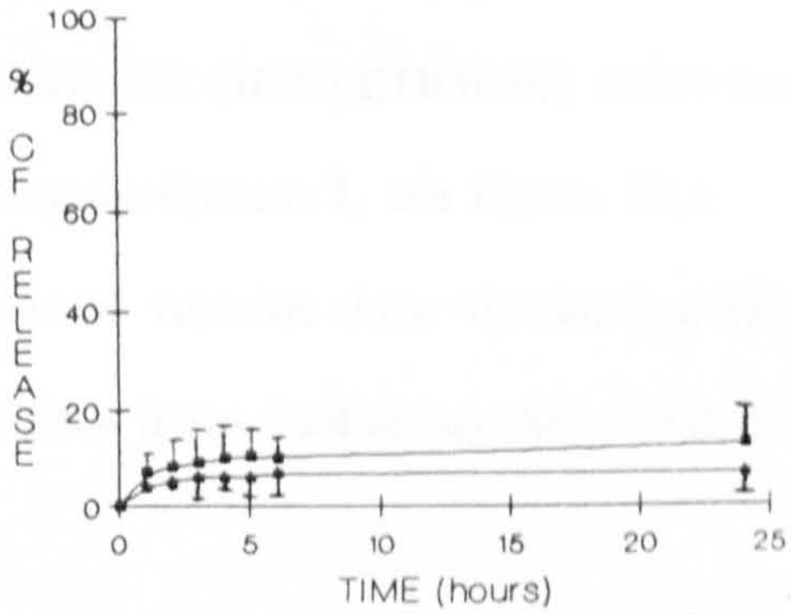


FIGURE 22b

II50:CHOL50 — II60:CHOL30:DCP10

Cholesterol (50 mol%) and DCP (10 mol%) were added to the niosomes. However, 100% vesicles did not form in this case.

CF Release from **niosomes** prepared from Surfactant **III** challenged with **plasma (100%)**.

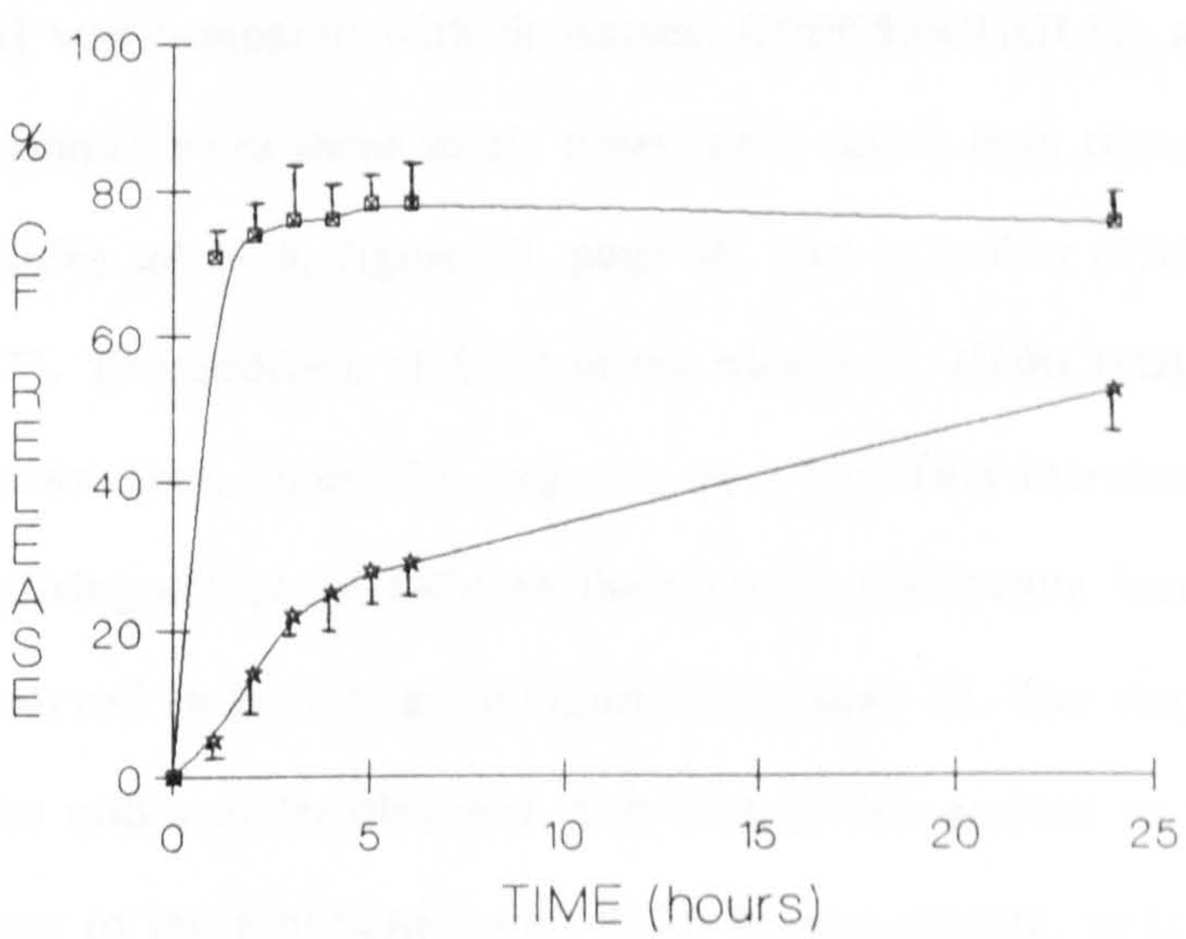


FIGURE 22c

III50:CHOL50 — III70:CHOL20:DCP10

Liposomes have not been included in this graph for clarity. The added cholesterol and DCP are 50 and 10mol%

Vesicles composed entirely of surfactant, that is, **I100** or **III100** or with cholesterol, **I50:CHOL50** or **III50:CHOL50**, were rapidly destabilised or disrupted after 1 to 2 hours and had released almost all the entrapped CF, as in figure 22,a and c, page 94. Inclusion of cholesterol into these niosomes confer a stabilising effect, figure 22a; vesicles composed of surfactant **III (III50:CHOL50)** resist the initial destabilisation more than those containing surfactant **I**, see figure 22,c versus 22a. However, after 24 hours, both types of vesicles show similar leakage kinetics. Addition of DCP to niosomes destabilises them further to the effect of plasma (100%), as in figure 22b and c.

Several reports dealing with the interaction of albumin and liposomes have been published *(Law et.al.1986;Kimelberg.1976;Sweet and Zull.1970)*. The effect of a 10% solution of bovine albumin (BSA) was measured to test it's destabilising effects on the vesicles. The niosomes most stable to challenge with 100% plasma were surfactant **II** vesicles, as in figure 22,b, page 94. Hence niosomes containing surfactant **II** was compared with liposomes, **DPPC50:CHOL50**, as in figure 23, page 96. The liposomes were three to six times more stable than these niosomes in both BSA (10%) solution, figure 23, page 96, and in buffer (PBS), as in figure 12,b, page 77. The addition of DCP to the niosomes, **II60:CHOL30:DCP10**, decreased stability, shown here, figure 23, page 96, by a two-fold increase in CF leakage. This destabilising effect, of DCP on the niosome membrane increasing CF leakage, was also observed in buffer, as in figure 12,b, page 77. The shapes of the leakage curves of the said vesicles obtained with BSA (10%), as seen in figure 23, are very different to those obtained with 100% human plasma, as in figure 22,b, page 94. This could mean that some other factor(s) are responsible for the initial high leakage rates seen in 100% plasma. The adsorption of BSA onto negatively charged, positively charged or neutral vesicles has been reported *(Law et.al.1986)* not to differ quantitatively. Therefore, the charge characteristics of vesicles are not factors affecting adsorption or release of marker. Hydrophobic interaction may

CF Release from **niosomes** prepared from Surfactant **II** and **liposomes** incubated with **BSA 10%** over time.

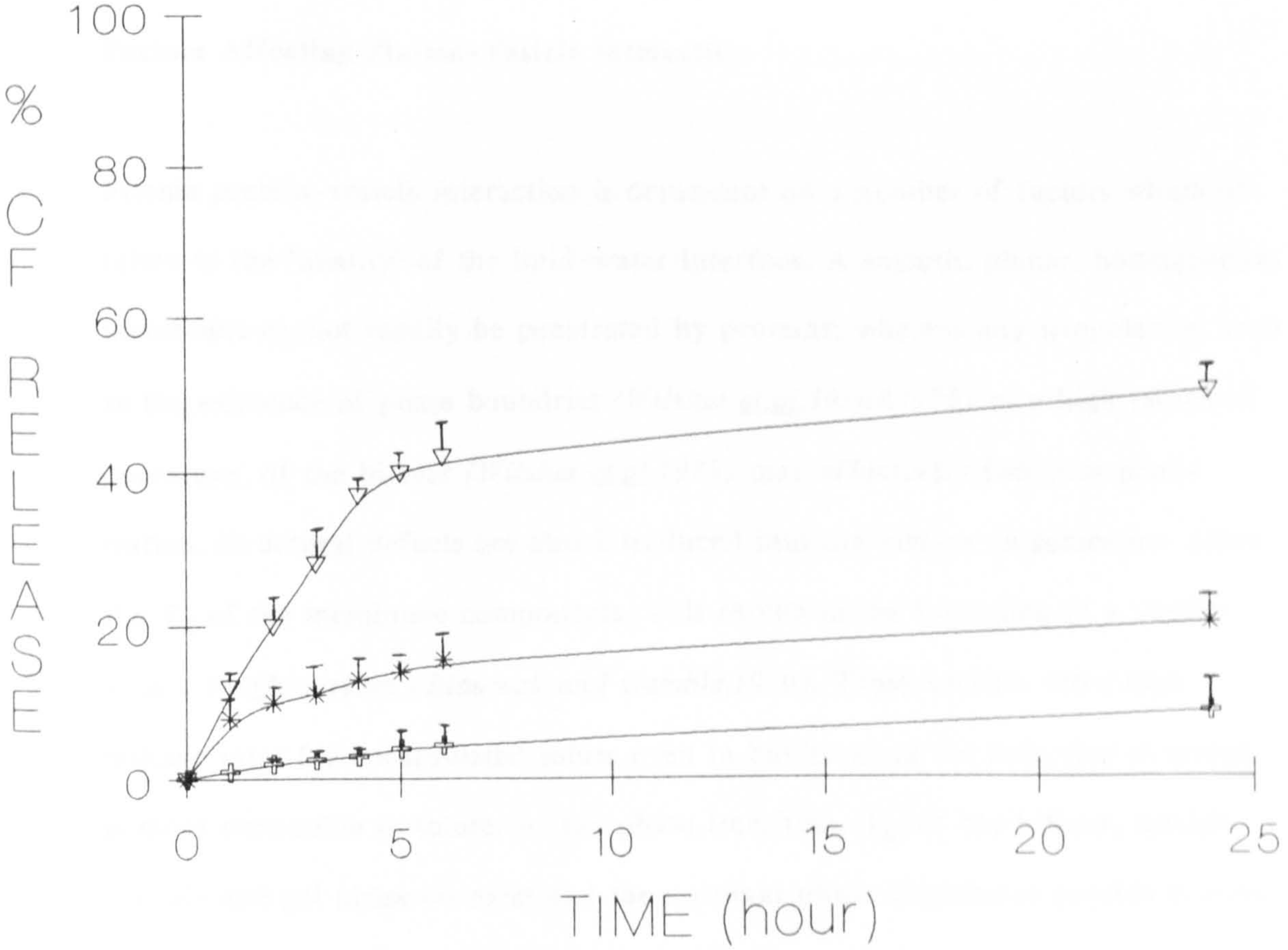


FIGURE 23

Liposome, **DPPC50:CHOL50**, is a typical example for this graph for comparison.

dominate.As concentration increases, more albumin molecules are adsorbed and the conformations on the vesicle surface rearranged into a denser molecular packing or aggregation occurs on the surface. Most of the albumin molecules can also penetrate the bilayer and bind to the hydrophobic region of the molecules by hydrophobic interaction which may be the dominant force for adsorption and its subsequent effects.

Factors Affecting Plasma-Vesicle Interaction

Plasma protein-vesicle interaction is dependent on a number of factors which all relate to the "quality" of the lipid-water interface. A smooth, planar, homogeneous membrane cannot readily be penetrated by proteins; whereas any irregularity, such as the existence of phase boundries *(Wilshut et.al.1976&1978)* or a high radius of curvature of the bilayer *(Wilshut et.al.1978)* may effectively facilitate penetration. Structural defects are also introduced into the bilayer on sonication below the T_c of the membrane components. This results in the formation of a vesicle with a rough surface *(Blaurock and Gamble,1979)*. These vesicles show high leakage rates for encapsulated solute even in buffer since the defective structure is more permeable to solute. At the phase transition, T_c, of the bilayer, liquid-crystals and gel phase co-exist and the resultant phase boundaries provide bilayer surface irregularities which facilitate protein penetration. A high degree of bilayer curvature greatly influences lipid packing in vesicles *(Scherphof and Damen et.al.1984)*, so that the susceptibility of liposomal phospholipid to lipoprotein attack depends on vesicle size. It has been reported *(Scherphof and Morselt,1984)* that larger liposomes are less susceptible to the action of plasma than smaller liposomes. In addition, multilamellar vesicles expose much less bilayer surface area per mole of lipid than small unilamellar vesicles with their high specific surface area, which like the strongly curved SUV bilayer increases susceptibility to solubilisation by lipoproteins. Cholesterol has a stabilising influence on solute

release. The ability of cholesterol to condense the packing of molecules in the bilayer *(Demel and DeKruft,1976)* abolishes the gel to liquid crystalline phase transition T_c and thereby stabilises the vesicles. Protein molecules apparently can not then easily penetrate into the hydrophobic region.

The vesicles investigated in this study are all multilamellar, thus reducing the likelihood of leakage due to bilayer stress caused by the small radius of curvature. The results substantiate the role of cholesterol which increases the stability of all the vesicles. However, to adequately explain the leakage profile of vesicles challenged with plasma, two theories must be addressed. Albumin alone may be responsible for the leakage profile. Initial interaction of albumin with the vesicle bilayer causes destabilisation allowing rapid CF efflux. It is thought that albumin molecules penetrate into the hydrophobic part of the membrane, at least, partly and attach themselves to this hydrophobic region of the bilayer. The remainder of the albumin molecule forms a "coating" around the outside of the vesicle resulting in the eventual formation of a "protective layer" and a decrease in leakage. However, although albumin and similar proteins adsorb to the outside of the vesicles resulting in a decrease in the leakage rates, the initial high efflux of entrapped substance is a direct consequence of high density lipoproteins (HDL)-amphiphile exchange. Numerous reports of this are cited in the literature for liposomes *(Guo et.al,1980;Morrisett et.al,1977)* and this may also be the the case for niosomes due to the high structural similarities between these surfactant molecules and the corresponding phospholipids. However, niosomes are apparently more leakier than liposomes. These observations require further studies to identify the actual mechanisms of leakage, for example, albumin insertion and/or HDL exchange, from vesicles in the presence of plasma. Identification of these processes would allow optimal formulation of specific drug carrying vehicles.

Ideally to establish the degree of "stability" of any vesicle preparation in blood or plasma, both the release of the lipid, or surfactant, and that of entrapped solute should be monitored. The availability of radio-labelled surfactant would make this feasible and complete these stability measurements.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was used to provide more detailed information of the adsorbed proteins on vesicles after incubation with plasma. In this study gel electrophoresis was performed to investigate the nature of proteins bound or associated with different types of vesicle, under specific conditions. Three factors were studied:

a. vesicle type and adsorbed protein;

b. effect of plasma concentration on the protein coat of vesicles; and

c. times of incubation.

Quantitative measurements were not attempted. However, in every case equal volumes of vesicle suspension were used, and, assuming the same number of vesicles were present in each volume, direct comparisons of the results could be made. A variety of vesicle types were incubated with plasma (10% and 100%) at various time intervals; 1, 2 and 24 hours, centrifuged at low speeds (1000*g*) and compared using gel electrophoresis. The gels were stained, dried and photographed as described (see experimental, page 62).

The adsorption of plasma protein to 3 different formulations of niosomes is illustrated in figure 24. This figure shows an increase in adsorption of proteins to all 3 types of vesicles with increasing time of incubation. Thus protein uptake by these vesicles appears to be time dependent. Careful examination of a number of gels indicate that a greater amount of protein is associated with vesicles

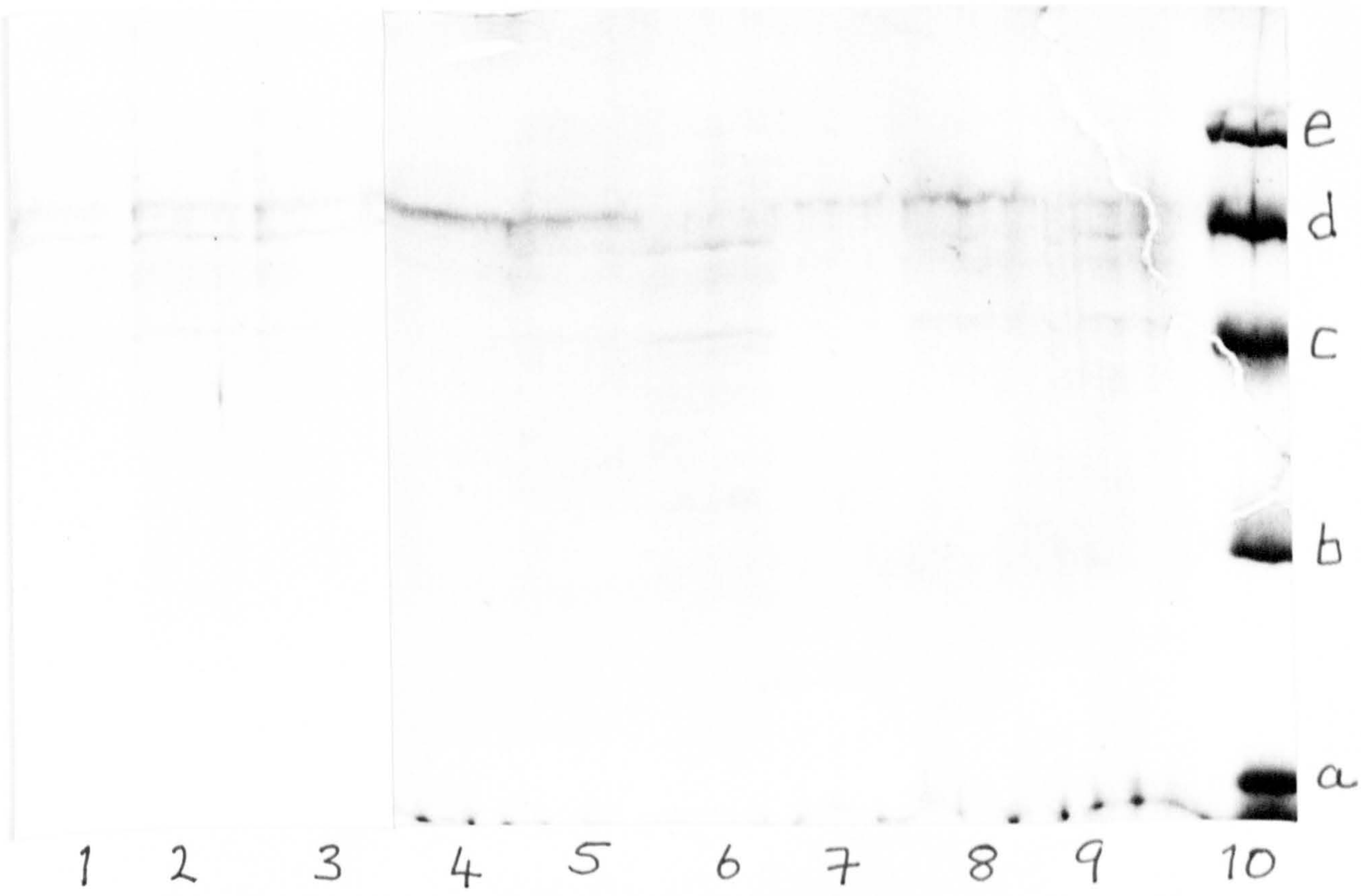


Figure 24: Niosomes, **II50:CHOL50** (1,2,3), **III50:CHOL50** (4,5,6) and **II50:CHOL50** (7,8,9) after incubation in 10% plasma for 1 hour (1,4,7), 2 hours (2,5,8) and 24 hours (3,6,9). Lane 10 are marker proteins.

a. soyabean trypsin inhibitor (20,000 daltons), **b.** carbonic anhydrase (30,000), **c.** ovalbumin (43,000), **d.** bovine serum albumin (67,000), **e.** phosphorylase B (94,000).

containing surfactant III than those containing surfactant I or surfactant II. Proteins may more tightly bind to surfactant III vesicles and so are not removed in the "washing procedure" or these vesicles have greater affinity for protein per se. The protein adsorbed in greatest abundance to vesicles has been identified as serum albumin (m.wt. approximately 66,000, visible as the darkest band, d, in figure 24, in the photographs). This protein was identified from its migration distance through the gel compared to the marker proteins. The slope and intercept of a plot of log molecular weight versus relative mobility for 5 known calibration proteins were used to convert gel position into molecular weight corresponding to that position, as shown in Appendix 3 page 163.

Figure 25 compares niosomes, **III50:CHOL50**, with liposomes, **DPPC50:CHOL50**, after incubation in plasma(10%) for 24 hours. The bands appear at similar locations to those observed previously indicating that the same types of proteins are adsorbed to both these vesicles. However, for the same quantity of vesicles and plasma proteins, niosomes, **III50:CHOL50**, show darker bands (lane 3 and 4, figure 25) than liposomes (lanes 1 and 2, figure 25), indicating the presence of more protein on the gel associated with the niosomes.

The effect of incubation times in plasma (10% and 100%) is shown in figures 26, page 103 and 27, page 104, for two separate vesicles. Both types of niosomes, **I50:CHOL50** and **III50:CHOL50**, show an increase in adsorbed proteins with time and amount of plasma, that is, 10% and 100%. Again **III50:CHOL50** vesicles, as in figure 27, showed a greater amount of protein adsorbed than **I50:CHOL50** vesicles, figure 26, for reasons similar to above. There was an increase in protein concentration and the number of different proteins adsorbed with time. For example, there was a greater number of protein bands for both types of vesicles studied after 24 hours incubation, lanes 3 and 6, figure 26 and 27. The highest number of bands was recorded when 100% plasma was used. However, again

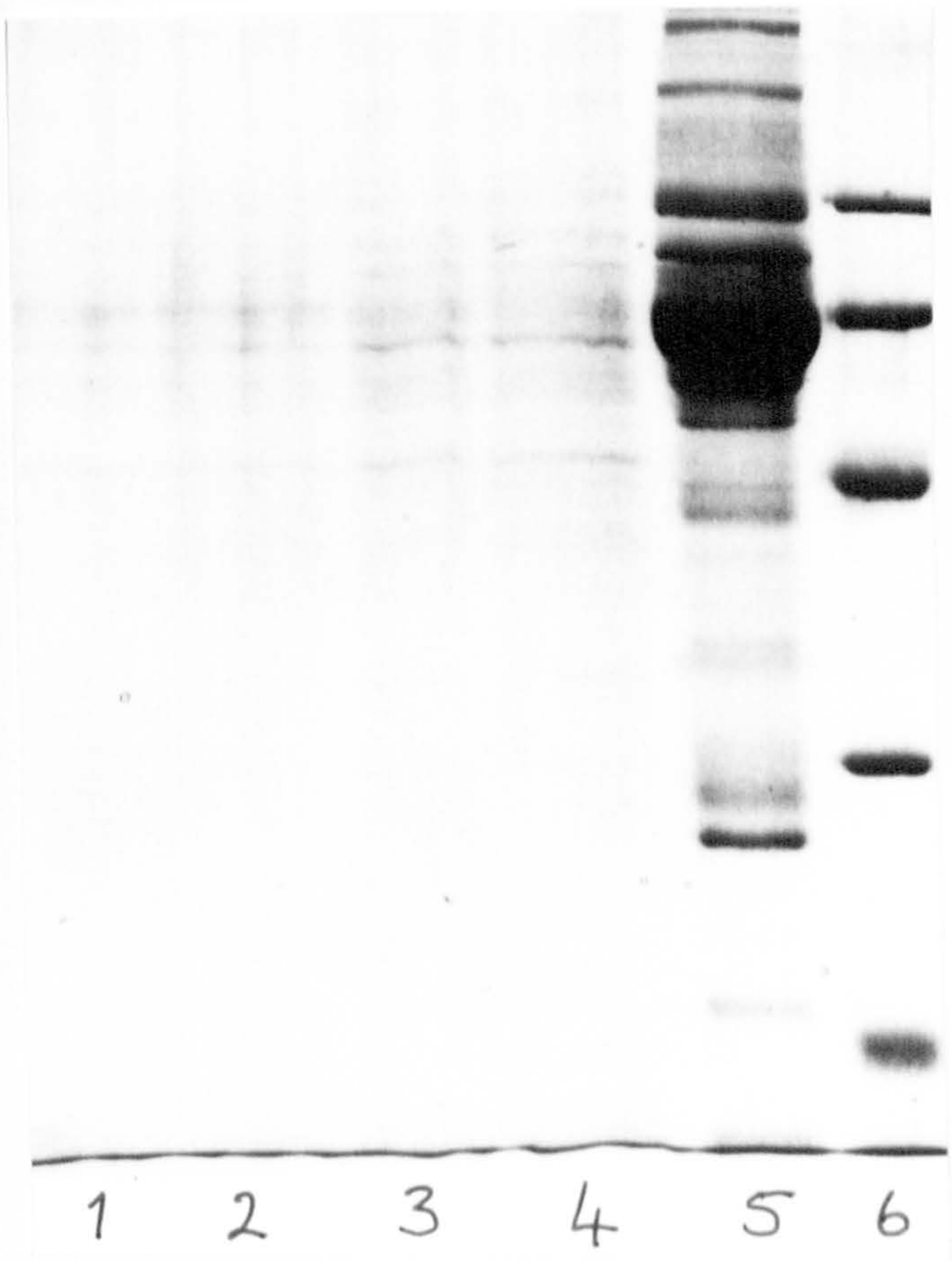


Figure 25: Incubation in 10% plasma for 24 hours.

Lanes 1 and 2 liposomes, type **DPPC50:CHOL50**

Lanes 3 and 4 niosomes, type **III50:CHOL50**

Lane 5 10% plasma; Lane 6 marker.

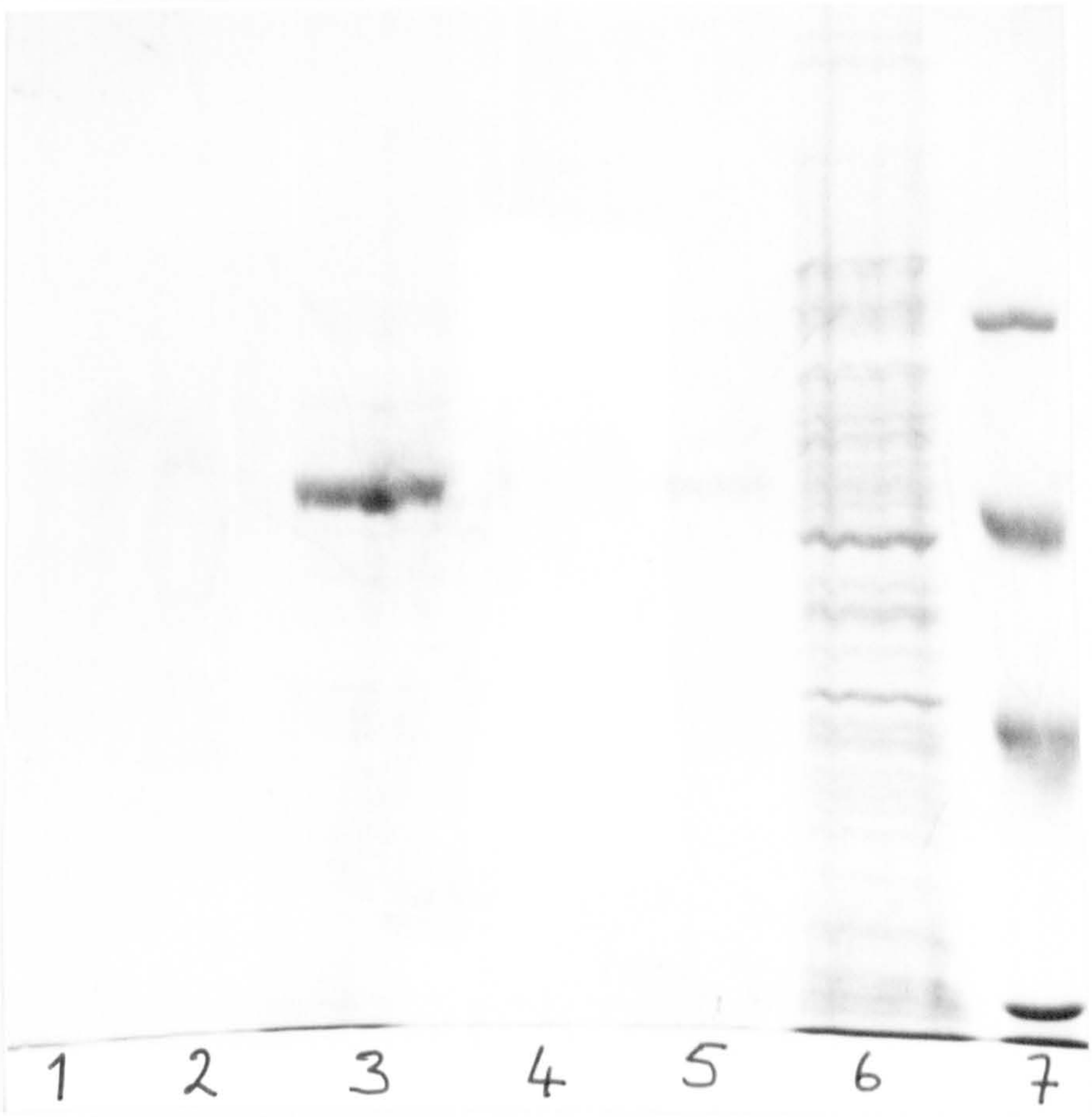


Figure 26: Effect of plasma concentration 10% (lanes 1,2,3) and 100% (lanes 4,5,6) and incubation times, 1 hour (lanes 1 and 4), 2 hours (lanes 2 and 5) and 24 hours (lanes 3 and 6) on niosomes **I50:CHOL50**. Lane 7 is the marker.

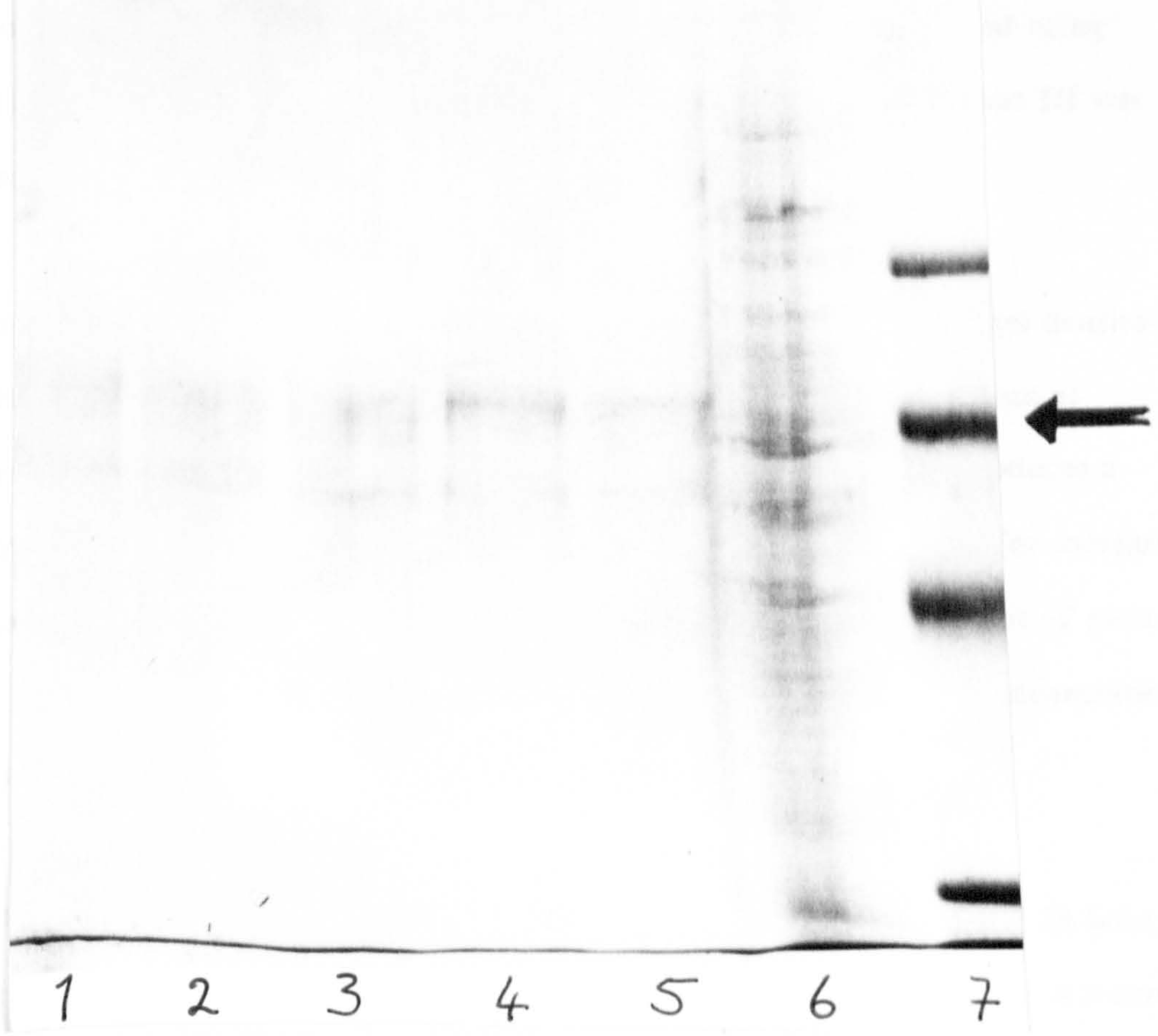


Figure 27: Niosomes, **III50:CHOL50** after incubation in 10% plasma (1,2,3) and 100% plasma (4,5,6) for 1 hour (1,4), 2 hours (2,5) and 24 hours (3,6). Lane 7 shows the marker, arrow denotes position of albumin.

surfactant III vesicles show a greater amount of protein adsorption than surfactant I vesicles, as seen by the more intense bands, lanes 3 and 6, in figure 27, page 104. As the concentration of plasma increases (10% to 100%), more molecules of protein appear to be adsorbed. It has been shown for liposomes, that there is an increase in the adsorption of BSA with increasing concentration until a saturated region is attained *(Law et.al, 1986)*. This saturated level and BSA adsorption rate was found to be identical for positive, negative and "neutral" liposomes. Although vesicles of different charge were not investigated using PAGE, the increased adsorption to niosomes, prepared from surfactant III was consistent throughout this study.

More accurate comparisons of band intensities can be made using a gel densitometer. This procedure involves the scanning of a gel using a laser beam at wavelengths sensitive to the blue dye, Coomassie blue R250. This produces a trace, measuring band intensity down the gel, with increasing peaks for increasing intensity and the area under the peaks can be correlated to the amount of protein on the gel. Therefore after measuring a standard of known protein concentration, other peaks, or bands, on the gel can be quantified.

A Western blot and ELISA were also performed on some vesicles after 24 hours incubation in 100% human plasma, in an attempt to tentatively identify a second major band appearing on the acrylamide gels. This band was thought to represent the large subunits of IgG (molecular weight approximately 50,000) and was present on all gels. A second faint band corresponding to a molecular weight around 25,000 was frequently detected and may correspond to the light chain subunit of IgG. Identification of these bands was attempted using a commercially produced antibody for IgG and the results are shown in figure 28, overleaf. Unfortunately this product was non-specific and bound to several other proteins on the blot, especially albumin. This was a serious problem with this IgG antibody

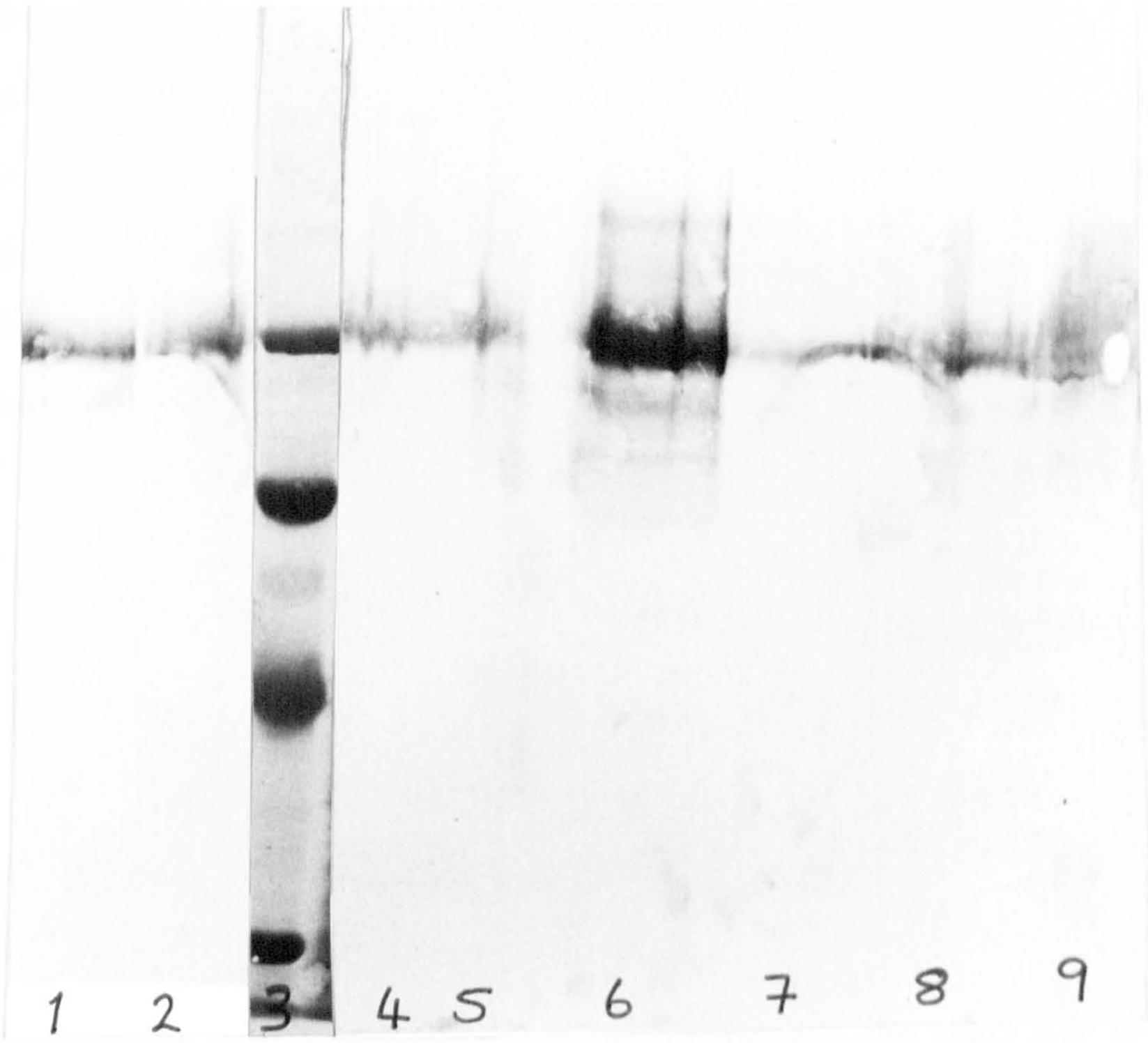


Figure 28: Results from the Western blot after ELISA for IgG with vesicle types;

1. **II60:CHOL30:DCP10**
2. **II50:CHOL50**
3. Marker
4. **DPPC70:CHOL20:DCP10**
5. **DPPC50:CHOL50**
6. **I60:CHOL30:DCP10**
7. **I100**
8. **III70:CHOL20:DCP10**
9. **III50:CHOL50.**

and the Scottish Antibody Production Unit (SAPU), the manufacturers, also confirmed the non-specific binding (*personal communication* with technical service). Therefore it was decided not to repeat this part of the work.

Attempts were also made to remove bound proteins using various agents, such as high ionic strength solutions eg. 1M NaCl, chelating agents eg. EDTA, chaotropic agents eg. 4M urea or proteases eg. trypsin (1mM for 30 minutes at 37^{0}C). No significant change was observed with any of these agents. However, this experiment was only performed once. Juliano and Lin *(1980)*, reported binding of high molecular weight, (HMW), >200,000 dalton protein components, dependent on vesicle composition (eg.inclusion of cholesterol) and on the surface charge on the vesicle. They also observed a time dependance in protein adsorption over the first 30 minutes in both serum or plasma and this adsorption was temperature sensitive; protein binding occurs much faster at 37^{0}C than at 4^{0}C. When challenged with low doses of protease, such as trypsin, cleavage and subsequent removal of the proteins from the liposomes resulted. These HMW proteins appear to be loosely attached to the outer surface of the vesicles but other lower molecular weight serum proteins which are more tightly associated to liposomes were not displaced by chelating or chaotropic agents or by high ionic strength solution. The apparent lack of activity with trypsin and the vesicles prepared in this study may be due to the relatively small quantity of high molecular weight protein attached to these vesicle surface, as is apparent from the fewer bands on the gels in the HMW region, figures 24-27, see previous pages; the upper regions of the gels.

The main purpose of these experiments was to identify the protein(s) which adsorb to these vesicles and to study the effect of composition on type and amount of protein bound. The results show that albumin is the most abundant protein adsorbed under all test circumstances and suggest that IgG may also be found associated with these vesicles. Fibrinogen is also known to bind to vesicles

but in amounts too small to be detected under these conditions. More sensitive techniques are required. The abundance or relative concentration of fibrinogen in plasma has been shown to have little effect on adsorption tendency *(Uniyal and Brash,1982)*. It has been found *(Vroman et.al,1980)* that fibrinogen adsorption from plasma is highly time dependent and that initially adsorbed fibrinogen is replaced by other proteins, notably high molecular weight kininogen (HMWK), within a few minutes of contact. Therefore, it is essential to specify time of contact when discussing protein adsorption. It is also possible to quantify these experiments in terms of a protein and/or CF evaluation to check the amounts present before gel application. Alternatively, an assay of the surfactants used would quantify the sample under study. However, such a test for these surfactants has not yet been fully investigated.

In vivo, the rate of elimination of particles from the vascular compartment is controlled by factors such as size and surface charge. For instance, large liposomes are removed more rapidly than small ones. This was anticipated from the biphasic rate of clearance of liposomes of mixed sizes *(Gregoriadis and Ryman,1972)* and later confirmed *(Juliano and Stamp,1975)*. With surface charge, it appeared that negative liposomes are removed more rapidly than those which are made to bear positive or neutral charges *(Tagesson et.al,1977)*. This may be related to the net negative charge on the surface of the vesicle following contact with plasma proteins *(Black and Gregoriadis,1976)* but the mechanism by which such a charge controls the affinity of these vesicles to cells in vivo is unclear. It is possible that the surface charge originally present in the vesicles modulates the extent to, or even the manner in which plasma components bind onto vesicles and thus cause association of the latter with cell surfaces. It seems likely, therefore, that it may be possible to manipulate the interaction of liposomes with reticuloendothelial cells and thus influence the clearance and distribution by imparting an appropriate protein coat to the particles prior to injection.

Electrophoresis

To investigate the surface potential of vesicles, their electrophoretic mobilities were measured. These vesicles were prepared entrapping NaCl (2.0 X 10^{-3}M, pH 5.4). The electrophoretic mobility (μ) was measured as a function of pH as described (see experimental, page 60).

The mobility was determined by measuring the time taken for the vesicles to travel a distance of 2cm on the optical measuring grid, under the influence of a constant electric field. The field was produced by a constant current source of 2mA and its magnitude was calculated from the conductivity of the solution. During preliminary work it was found that vesicle mobility was dependent on location, therefore all electrophoretic measurements were made in the centre of the cell. To ensure reproducible results short, low current passage times and the reversal of current direction through the cell was maintained. Heat effects were minimised by these low currents. Data obtained for vesicle movement in either direction did not differ significantly. The mobilities for at least 3 different batches of each vesicle type were recorded, each reading, an average of 40 measurements at the stationary level; 20 at the front wall and 20 at the back wall, and a standard deviation of ± 0.5 $\mu m.s^{-1}$ per $V.cm^{-1}$ was obtained.

Electrophoretic mobility, μ, can be related to the zeta potential, ξ, using the Helmholtz-Smoluchowski equation, see introduction, page 38.

$$\xi = \frac{\eta \mu}{\epsilon_o \epsilon_r} \qquad \text{.....[4]}$$

In aqueous solutions of low ionic strengths, the physical constants for pure water apply. Assuming zeta potential, ξ, equals the surface potential, ψ_o and substitution

into equation [7], page 39, allows calculation of surface charge density, σ. For a working example, consider niosomes, **I50:CHOL50**, from table 3, page 111;

C_b = bulk molar concentration of the electrolyte, (NaCl)= 2.0×10^{-3} mol l^{-1}.

Z = valency of the electrolyte = 1.

q = absolute charge on the electron = 1.6×10^{-19} Coulomb.

T = absolute temperature = 298K (25^0C).

k = Boltzman constant = 1.38×10^{-23} Jk^{-1}.

Substituting, $\xi = \psi_0 = 13.6 \times 10^{-3}$ V (from table 3, page 111) into equation [7], from page 39, we have,

$$\sigma = 11.77\ C_b^{\frac{1}{2}}\ .\text{sin.h.}\ (Zq\psi_0 / 2kT).$$

$$\sigma = 11.77\ (2.0\times10^{-3})^{\frac{1}{2}}\ .\text{sin.h}\ \frac{(1 \times 1.6\times10^{-19} \times 13.6 \times 10^{-3})}{2 \times 1.38\times10^{-23} \times 298}$$

σ = -* 0.14 $\mu C.cm^{-2}$.

(* = the minus sign originates from the negative electrophoretic mobility).

Values for μ, ξ (ψ_0) and σ at pH 5.4 are presented in table 3, page 111. The "neutral" vesicles, **I100**, **I50:CHOL50**, **II50:CHOL50**, **III50:CHOL50** and **DPPC50:CHOL50**, all showed a small negative charge. A plausible explanation is the polarisation in the head groups of the surfactants wherein the oxygen atom carries a small negative charge causes the hydrogen atom to become slightly positive. At the outer surface of the vesicles, this produces a small electropositive force which attracts negatively charged hydroxyl groups from the external media, resulting in the overall negative charge on the vesicle. It is possible to "titrate" the small negative charge on the "neutral" vesicles by inclusion of a positive charge to the bilayer. This was achieved by the addition of small amounts of

TABLE 3

Vesicle Type	μ ($\mu m.s^{-1}$ per $V.cm^{-1}$)	$\zeta(=\psi_0)$ $(V)X10^{-3}$	σ ($\mu C.cm^{-2}$)
I100	-1.1	-13.6	-0.14
I50:CHOL50	-1.1	-13.6	-0.14
I60:CHOL30:DCP10	-4.6	-60.0	-0.76
I68:CHOL30:SA2	+0.02	+0.3	+0.003
II50:CHOL50	-1.2	-16.2	-0.17
III100	-1.2	-16.2	-0.17
III50:CHOL50	-1.6	-20.6	-0.22
III70:CHOL20:DCP10	-4.6	-60.0	-0.76
DPPC50:CHOL50	-1.3	-16.1	-0.17
DPPC70:CHOL20:DCP10	-5.0	-64.1	-0.84

stearylamine (2 mol%) to the surfactant/cholesterol mixture. These vesicles, **I68:CHOL30:SA2**, had a slight positive surface charge at pH 5.4 of σ = +0.003 $\mu C.cm^{-2}$. It should, however, be noted that at pH 10.0 (high hydroxyl ion concentration) the charge on these positive vesicles became slightly negative (σ = -0.001 $\mu C.cm^{-2}$). Inclusion of DCP (10mol%) to the vesicles, **I60:CHOL30:DCP10**, **III70:CHOL20:DCP10** and **DPPC70:CHOL20:DCP10**, introduces a further negative charge into the bilayer and this is demonstrated by an increase in electrophoretic mobility and a parallel increase in surface charge, table 3, page 111.

A representative curve of surface charge as a function of pH is shown in figure 29, page 113. The change in pH to the external medium (NaCl electrolyte) is effected by addition of a few drops of acid or base respectively. The graph shows the relationship between pH and surface charge for niosomes, **I50:CHOL50**, a typical representative and liposomes, **DPPC50:CHOL50**. No differences in surface charges were observed between these 2 types of vesicles when measured with NaCl as the entrapped solution, the solid lines in figure 29, page 113. It appears therefore, that the surface charge on the surfactant molecules in niosomes and phospholipids in liposomes are affected to the same extent, irrespective of the change in pH outside the vesicles. Similar results were obtained for all other types of surfactant vesicles measured in this study. These other graphs were not included for brevity. Also the addition of DCP to niosomes and liposomes was shown to increase the surface charges in the same orders of magnitude.

This trend, for the said niosomes and liposomes, was also found when the vesicles were first incubated in plasma (50% human) for 2 hours at 37^0C, washed and resuspended in NaCl electrolyte solution. However, in this case the overall order of surface charge was lower, as seen by the dotted lines in figure 29, overleaf.

Changes in **Surface charge** with **pH** for **niosomes and liposomes** when in **NaCl** and in **human plasma (50%)**.

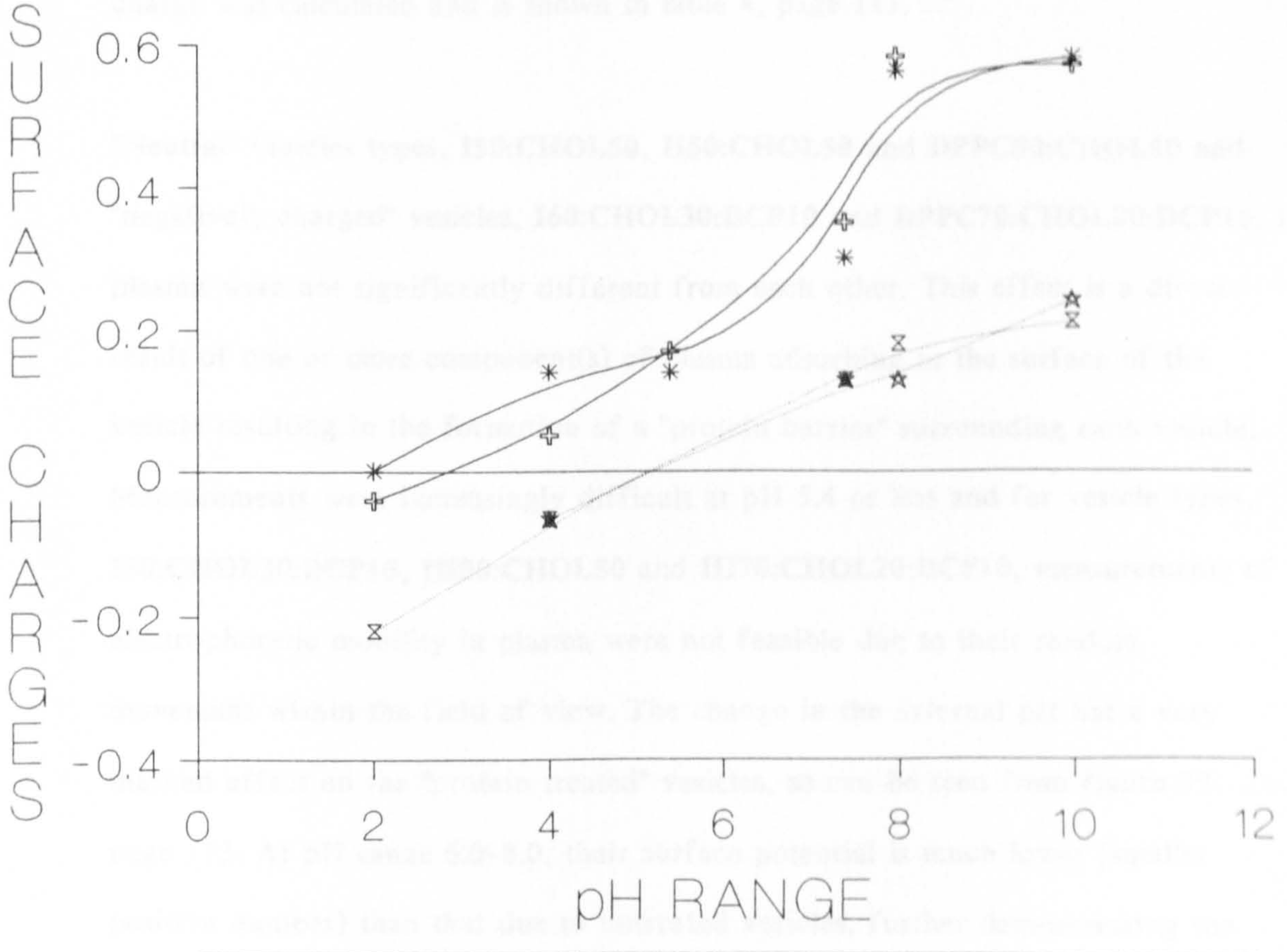


FIGURE 29

Cholesterol 50 mol% was added to both niosomes and liposomes and vesicles were incubated in plasma for 2 hrs at 37oC.

To measure the effects of plasma, vesicles were prepared entrapping NaCl electrolyte ($2.0X10^{-3}M$) in glucose solution (0.2M). This inclusion raised the internal osmotic pressure of the vesicles which would otherwise disrupt when challenged with plasma. The vesicles thus maintained a constant surface area and the surface charge remained relatively insensitive to changes in the ionic strength of the surrounding electrolyte. The effect of plasma on μ was measured, the surface charge was calculated and is shown in table 4, page 115.

"Neutral" vesicles types, I50:CHOL50, II50:CHOL50 and DPPC50:CHOL50 and "negatively charged" vesicles, I60:CHOL30:DCP10 and DPPC70:CHOL20:DCP10, in plasma were not significantly different from each other. This effect is a direct result of one or more component(s) of plasma adsorbing to the surface of the vesicle resulting in the formation of a "protein barrier" surrounding each vesicle. Measurements were increasingly difficult at pH 5.4 or less and for vesicle types, I68:CHOL30:DCP10, III50:CHOL50 and III70:CHOL20:DCP10, measurements of electrophoretic mobility in plasma were not feasible due to their random movement within the field of view. The change in the external pH has a very marked effect on the "protein treated" vesicles, as can be seen from figure 29, page 113. At pH range 6.0-8.0, their surface potential is much lower (smaller positive number) than that due to untreated vesicles, further demonstrating the "coating effect" of plasma proteins on the outer surface of these vesicles. This coat protects the polar heads from the high external OH^- environment thus reducing their effect on the overall surface charge. In fact, at pH 4.0 and below (high H^+ environment), the surface charge is positive due to the measured positive electophoretic values. The effect of surface charge due to the changes in pH with vesicles treated with plasma, must be therefore a direct effect of the charge on the protein surrounding these vesicles.

TABLE 4

Vesicle type	Surface Charge, σ ($\mu C.cm^{-2}$)	
	NaCl	Plasma
I50:CHOL50	0.14 (0.17)	0.13
I60:CHOL30:DCP10	0.76 (0.87)	0.17
II50:CHOL50	0.16	0.11
DPPC50:CHOL50	0.17	0.11
DPPC70:CHOL20:DCP10	0.84	0.13

NB. The numbers in parentheses for vesicle types, I50:CHOL50 and I60:CHOL30:DCP10, in NaCl solution were calculated from direct measurements using a Malvern Zetasizer IIc^{R*}.

These numbers are in the same order as those obtained with the manual method, using the Rank^{R*} microelectrophoresis apparatus.

It is known that albumin is the major constituent of plasma proteins (70-80%). Albumin, typical of other proteins can be represented as below:

$$R\text{----}HN\text{-----}(CH_2)_n\text{-----}COOH.$$

It has an isoelectric point of 5.4. At low pH, that is, high hydrogen ion concentration, the nitrogen atom (N) on the molecule becomes protonated and there is no charge on the carboxyl group (COOH). However, at high pH (low hydrogen ion concentration) the nitrogen atom looses it's proton and the COOH group becomes ionised (that is, acquires a negative charge). The curve (surface charge against pH, figure 29, page 113) cuts the X-axis at around 5.4 (within experimental limits) which is the pH at which albumin exists as a "neutral" molecule, that is, it's isoelectric point. This suggest that the major constituent of blood adsorbed on the vesicles is probably serum albumin. All the other vesicles investigated show similar trends and have profiles as shown in figure 29.

3.4. STUDIES WITH *TETRAHYMENA ELLIOTTI* CELLS

The rate of uptake and subsequent breakdown of niosomes containing surfactants I, II and III and liposomes by the micro-organism T.elliotti were measured. The rate of uptake is expressed as an average number of food vacuoles formed per T.elliotti cell in a given time period. Although there are wide variations in the numbers of ingested vesicles in the food vacuoles between individual cells, the results show a trend and conform to a general pattern. T.elliotti cells used in this study remained fully motile and active in the vesicle suspensions with little or no signs of toxicity after 24 hours. This lack of toxicity was repeatedly noted with every preparation.

The results showed little variation between the rates of uptake for the niosomes of all types and liposomes, as seen in figure 30. This was expected since T. elliotti ingests most materials at a steady rate. The vesicles were broken down within the food vacuoles resulting in the appearance of a diffuse intracellular fluorescence, as in figure 30. This occured more readily for liposomes and niosomes prepared from surfactant III, that is, **III100, III50:CHOL50** and **III70:CHOL20:DCP10**, than for vesicles with surfactants I, for example, **I100, I50:CHOL50** and **I60:CHOL30:DCP10**, or surfactant type II, such as, **II50:CHOL50** and **II60:CHOL30:DCP10**. An explanation for this observation is that surfactant III is more susceptible to intracellular degradation than the ether linked surfactants I and III. The efflux of vesicular CF into the cytoplasmic space of the T.elliotti cell may also be a function of the low pH within the food vacuole which has been approximated as pH 4.0 to 5.0 *(Nuccitelli and Deamer,1982)*. Alternatively, intracellular degradation above pH 4.0 may be caused by enzymes, normally used to digest matter within the food vacuoles. These enzymes may be responsible for the apparent vesicle degradation observed in vivo, or the CF leakage is a product of the two processes simultaneously.

The uptake of vesicles and eventual release of entrapped CF from vesicles was examined using epifluorescence microscopy. The uptake and breakdown of vesicles was recorded by delayed time sequence photography and was seen clearly, using the self-quenching properties of CF. Carboxyfluorescein entrapped within vesicles was self-quenched and localised inside the food vacuoles after ingestion. These vacuoles were visible as distinct fluorescent spheres, as seen in figure 31, page 119 for liposomes and figure 32, pages 120-122 for niosomes. Released vesicular CF was diluted throughout the T.elliotti cell and hence produced a diffuse intracellular fluorescence which increased with time. However the breakdown of vesicles was also dependent on composition and the most rapid rate was seen with

Apparent uptake of **niosomes** and **liposome** by ***T.elliotti.*** cells over a given time period.

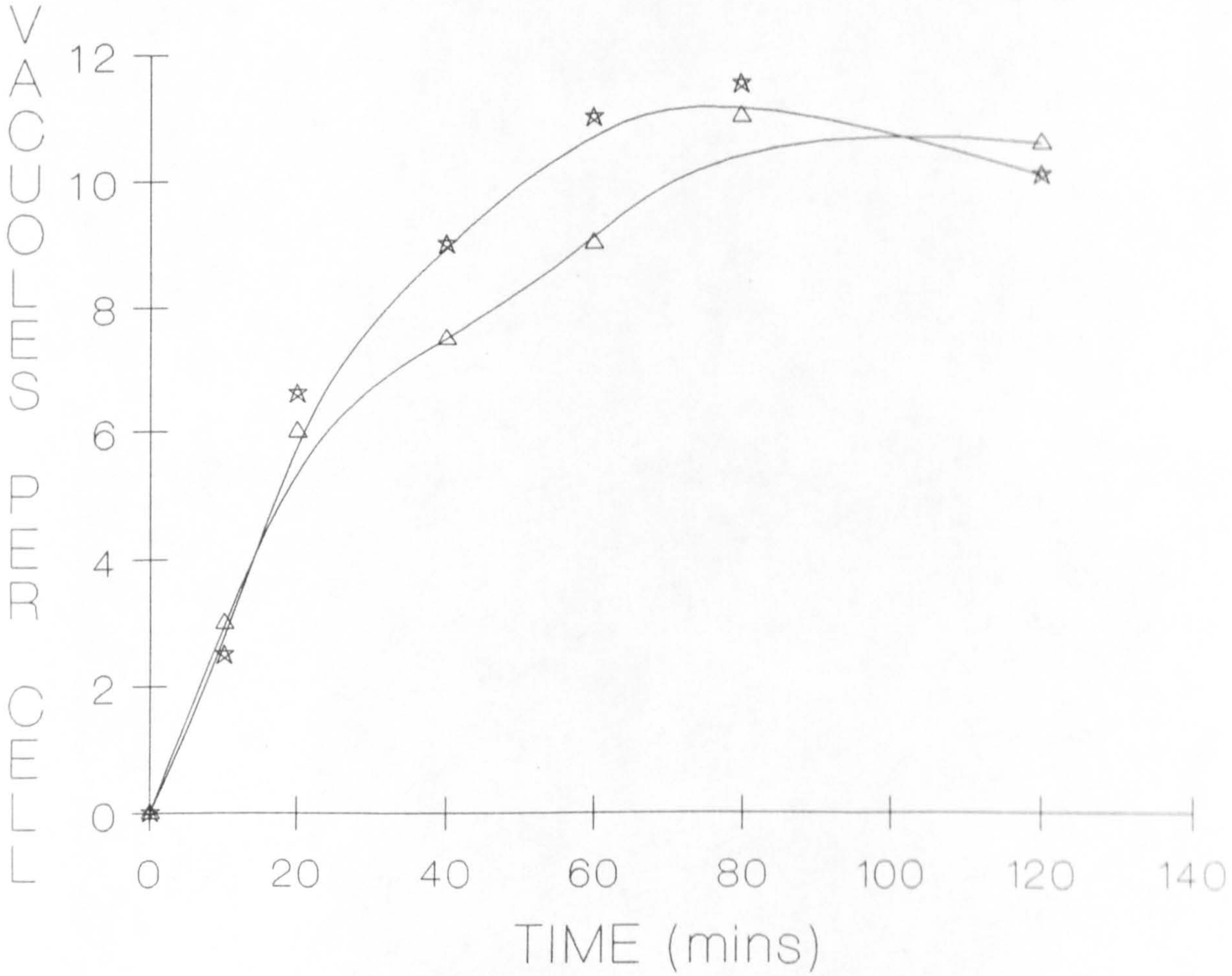
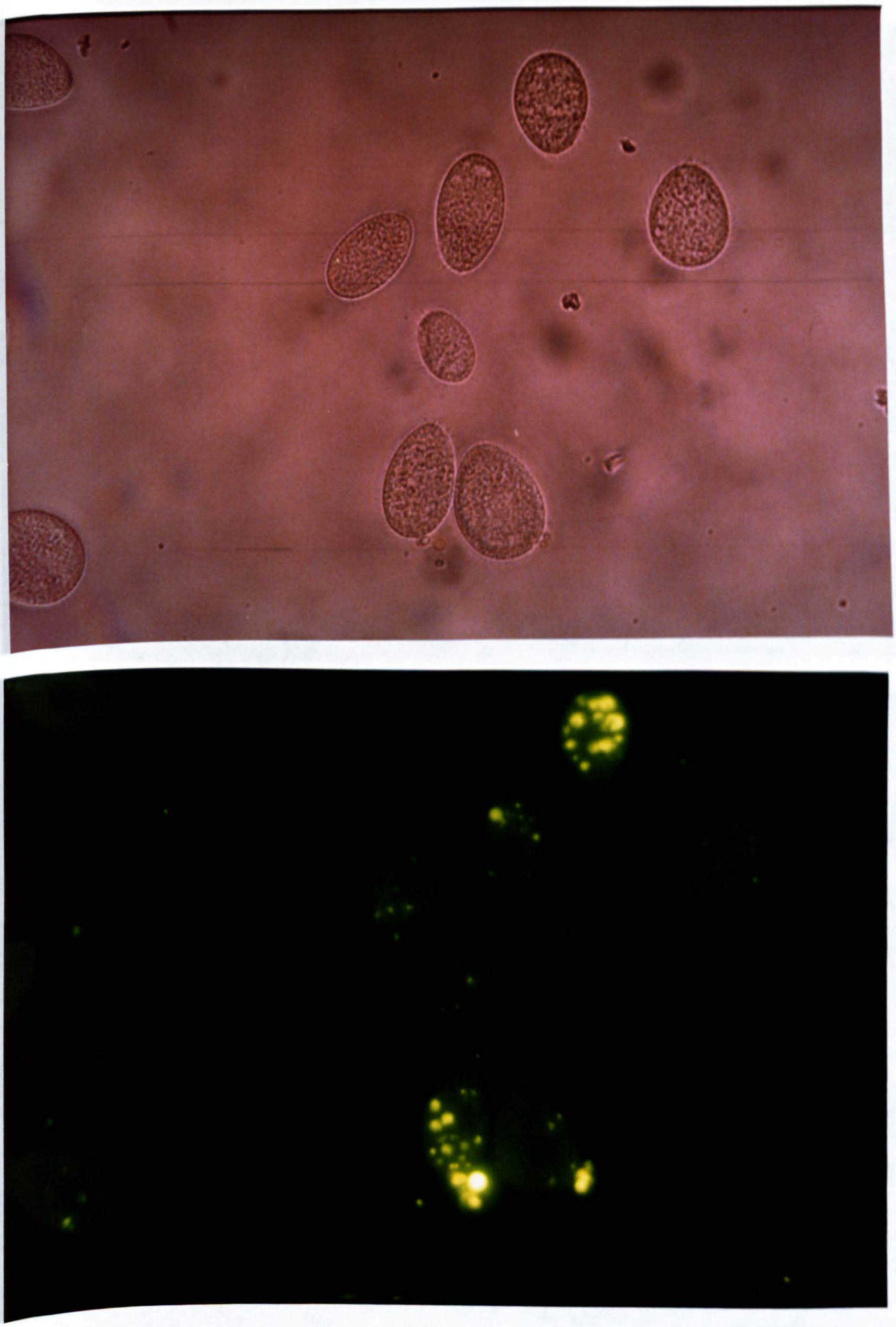


FIGURE 30

Each point is an average of **100 counts** of cells. The Y-axis is the number of fluorescent vacuoles per **100 cells**.



TOP

BOTTOM

Figure 31: Apparent uptake by T.elliotti cells after 3 hours incubation in a suspension containing liposomes, **DPPC50:CHOL50**, under normal light (top) and after ultraviolet illumination (bottom).

Magnification = 200X.

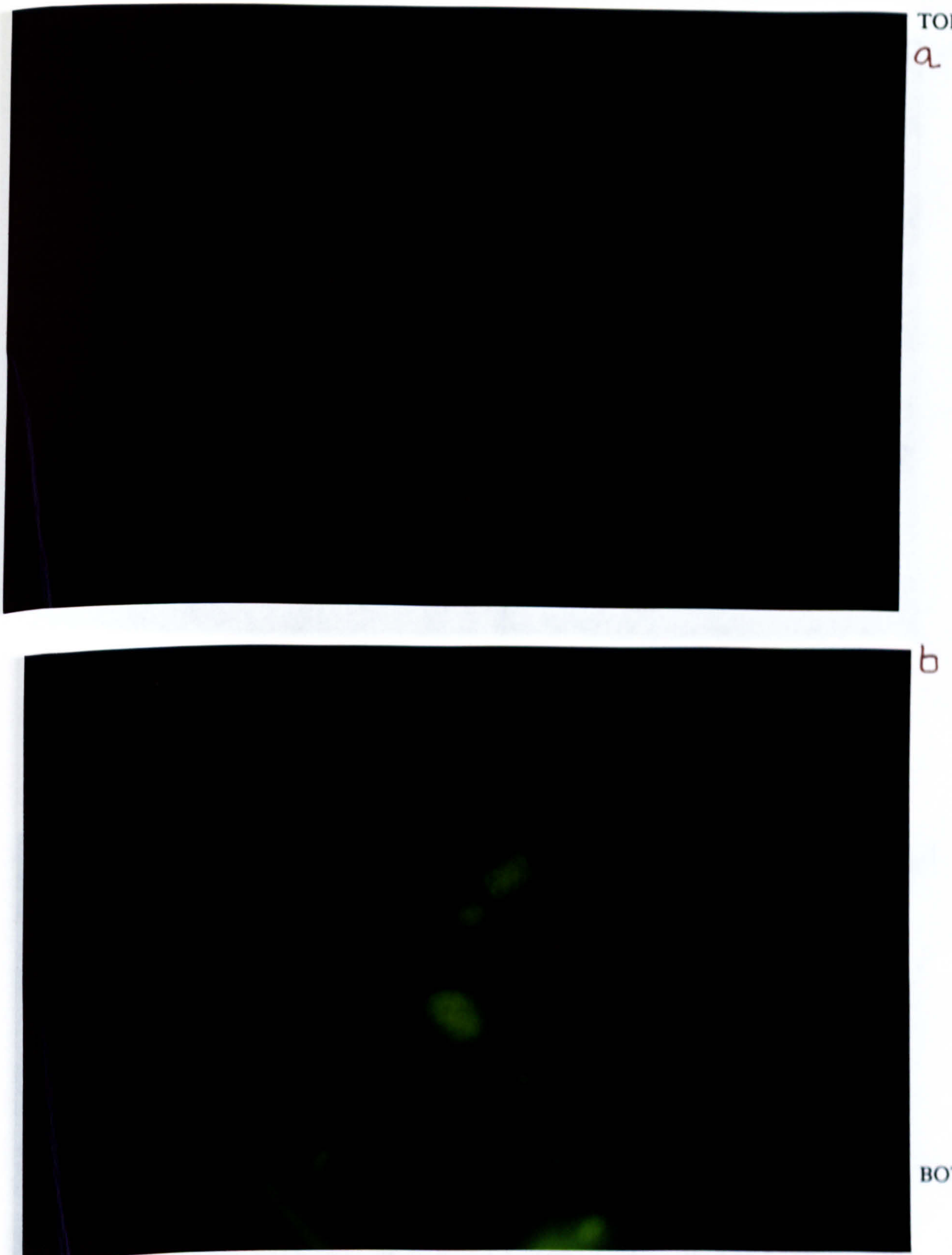
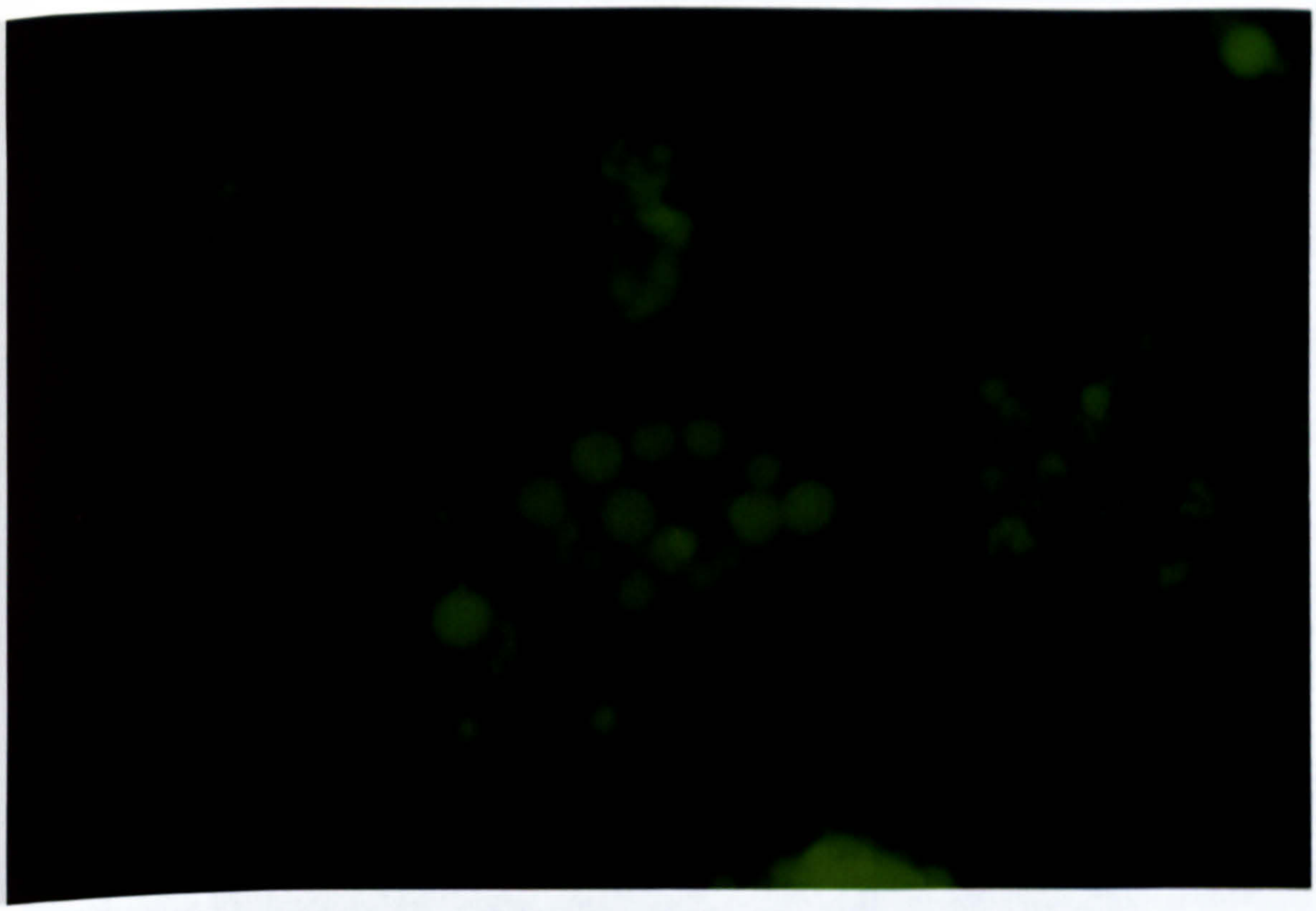
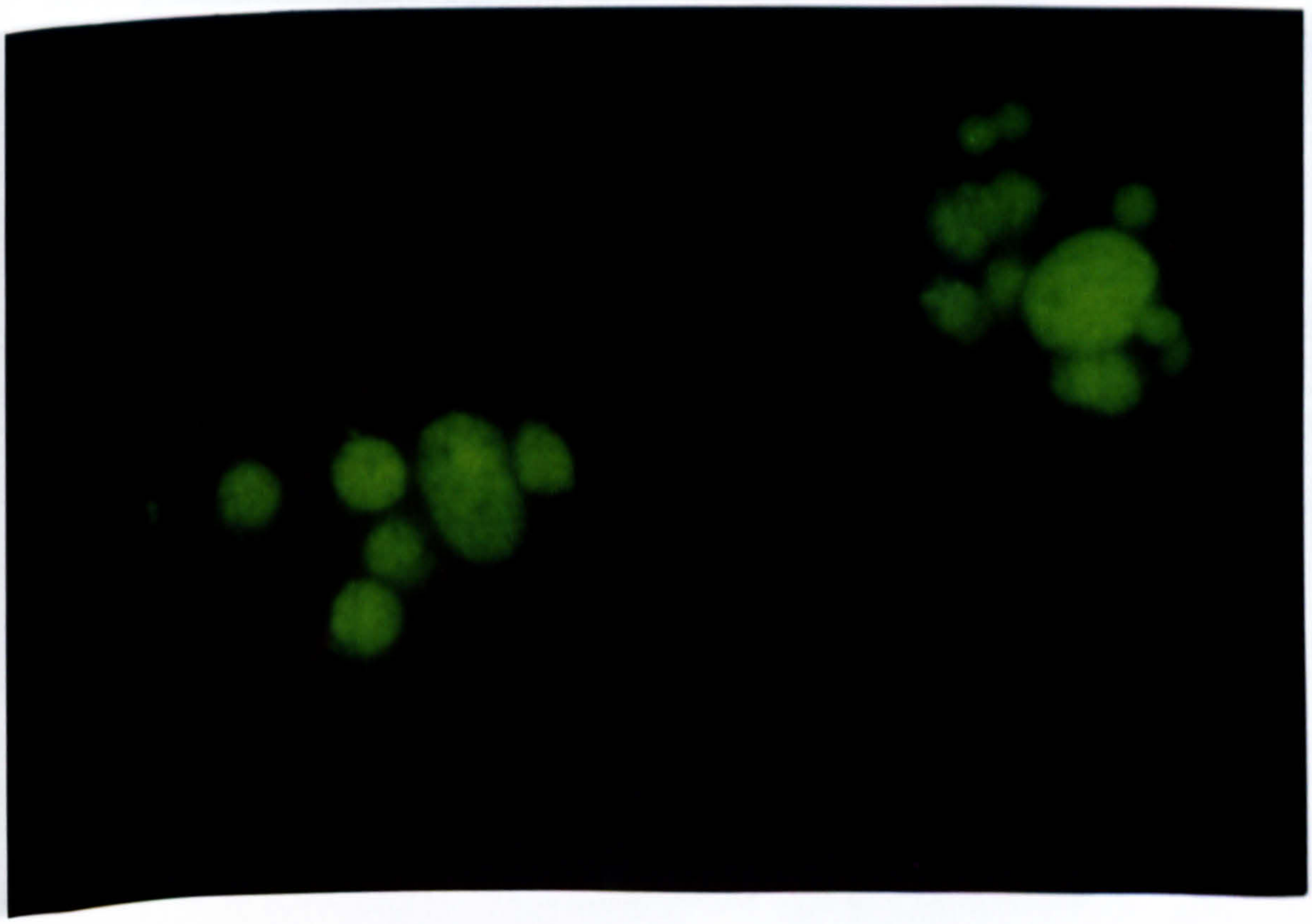


Figure 32: Fluorescence photographs of T.elliotti at various times (1 to 6 hours, **a-f**) incubation in a suspension containing niosomes, **I50:CHOL50** (Photographs **a-f** under normal illumination are to be found inside the back cover of the thesis.) Magnification = 500X.

TOP



c

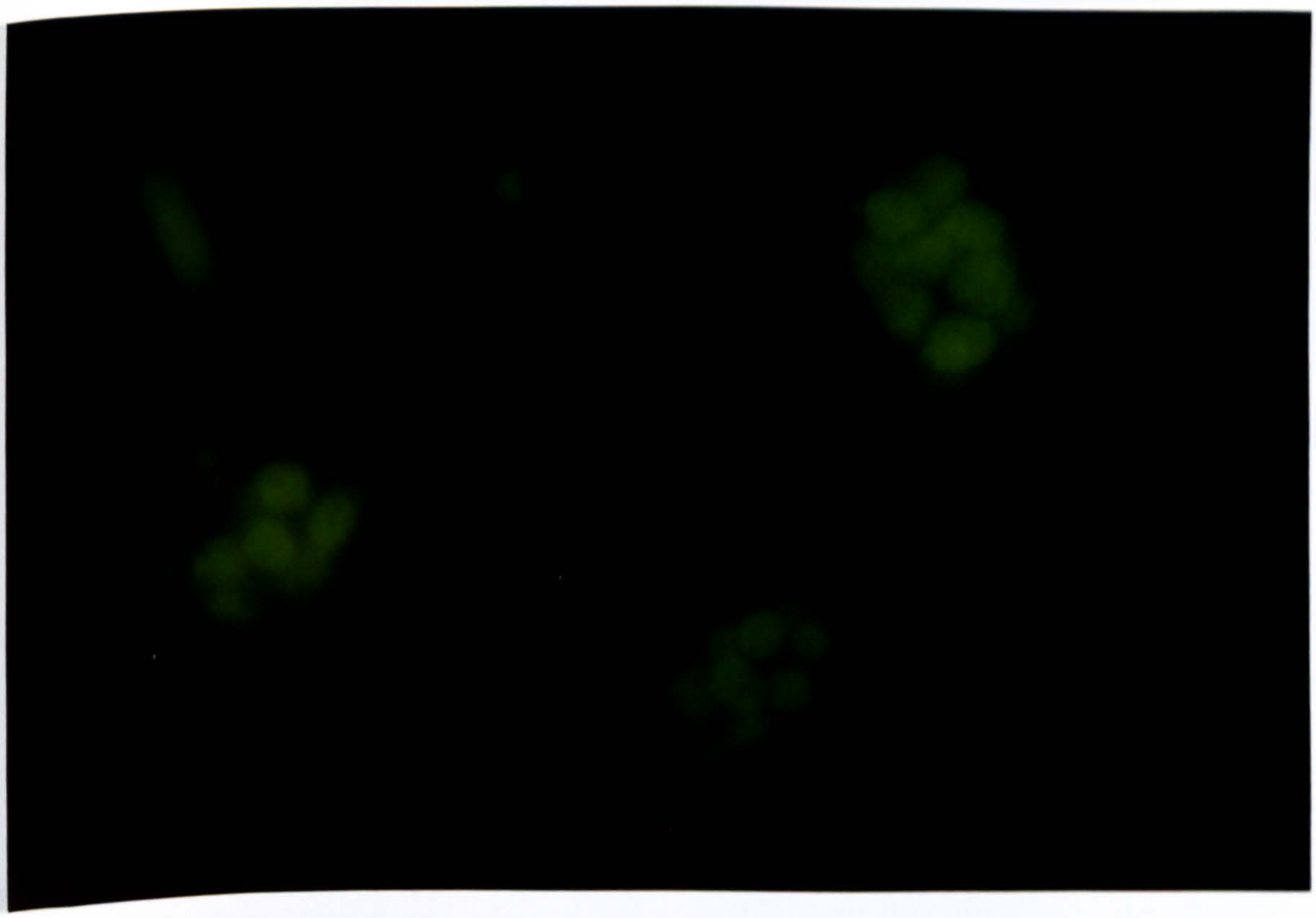


d

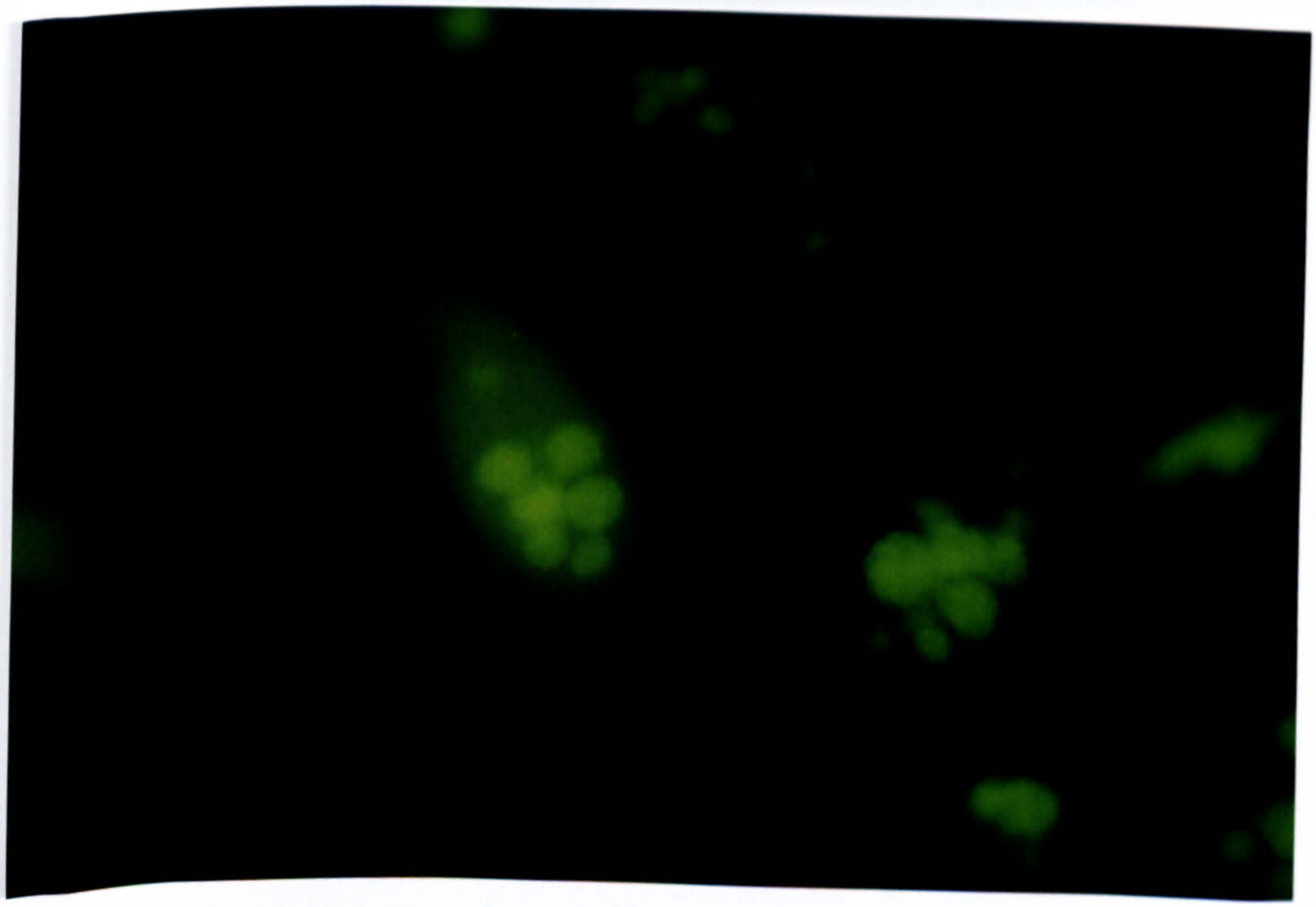
BOTTOM

TOP

e



f



BOTTOM

TABLE 5

Vesicle Type	Time (in hours)							
	0	½	1	2	3	4	5	6
I50:CHOL50	-	-	-	-	-	-	-	+
I60:CHOL30:DCP10	-	-	-	-	-	-	+	+
II50:CHOL50	-	-	-	+	+	+	+	+
II60:CHOL30:DCP10	-	-	-	+	+	+	+	+
III50:CHOL50	-	-	-	+	+	+	+	+
III70:CHOL20:DCP10	-	-	+	+	+	+	+	+
DPPC50:CHOL50	-	-	+	+	+	+	+	+
DPPC70:CHOL20:DCP10	-	+	+	+	+	+	+	+

+ = presence of diffuse intracellular fluorescence.

- = no diffuse fluorescence observed.

liposomes since this resulted in the appearance of diffuse intracellular fluorescence within an hour of incubation (table 5, page 123). With niosomes containing the ester-linked molecule, surfactant III, types III50:CHOL50 and III70:CHOL20:DCP10, release of CF was apparent only after 1 to 2 hours and with surfactant II vesicles, II50:CHOL50 and II60:CHOL30:DCP10, and surfactant I, types I50:CHOL50 and I60:CHOL30:DCP10, after 2 and 6 hours respectively (table 5). In general, inclusion of cholesterol in niosomes increases the stability of the vesicles within the food vacuole, and the resultant diffuse fluorescence within the cell body appears much later. This process was reversed when DCP (10 mol%) was added to the niosomes and the vesicles appeared to release their contents at a much faster rate. These observations are consistent with studies on liposomes containing cholesterol and added DCP (table 5). The inclusion of DCP may attract lysosomal enzymes to the outer membrane of liposomes hence increasing destabilisation of the entire shell. This leakage is further enhanced by the effect of the lower pH of the vacuole (around 4.0). Previous studies (see page 71) on vesicle behaviour in buffer at pH 4.0, have shown a steady increase in leakage of CF with time, as shown in figure 33a,b and c, page 125. Vesicles containing surfactant III and cholesterol, III50:CHOL50, exhibit the fastest leakage at pH 4.0, figure 33c, followed by vesicles containing surfactant I and II with added cholesterol respectively, figure 33a and b. In all cases inclusion of DCP (10 mol%) into the vesicle membrane increases the rate of CF release. The trend shown in figure 33a,b and c, was measured after incubation of the vesicles in buffer at pH 4.0 for various time intervals at room temperature, 22^{0}C. These results suggest that pH is an important factor in the intracellular destabilisation of niosomes, whether within or outside the food vacuole of the T.elliotti.

The other possible destabilising factor is the presence of lysosomal enzymes, many proteolytic in nature within the food vacuole. Two typical enzymes were chosen to study their effects on vesicle stability; phospholipase A_2, because the natural

CF Release from **niosomes** prepared from Surfactant **I** after incubation at **pH 4.0 in BUFFER**.

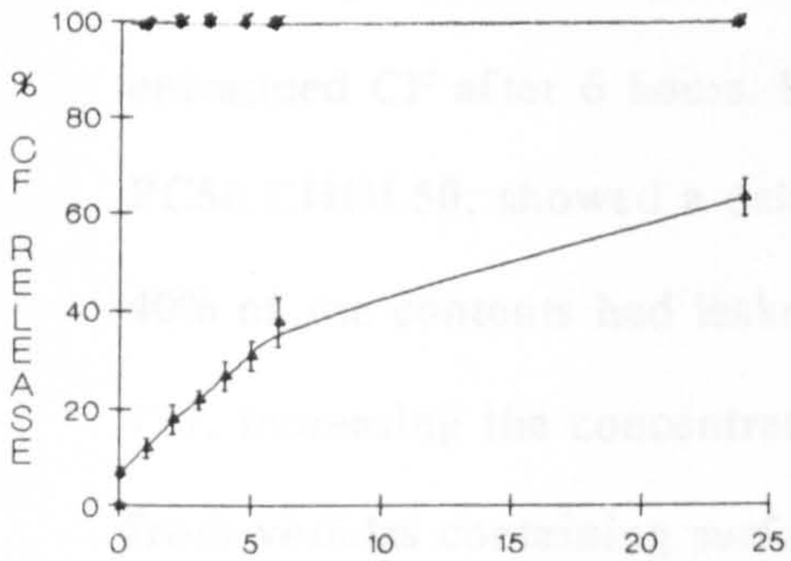


FIGURE 33a

I50:CHOL50 — I60:CHOL30:DCP10

Cholesterol and DCP added are 50 mol%, 30 mol% and 10 mol% respectively.

CF Release from **niosomes** prepared from Surfactant **II** at **pH 4.0 in BUFFER**.

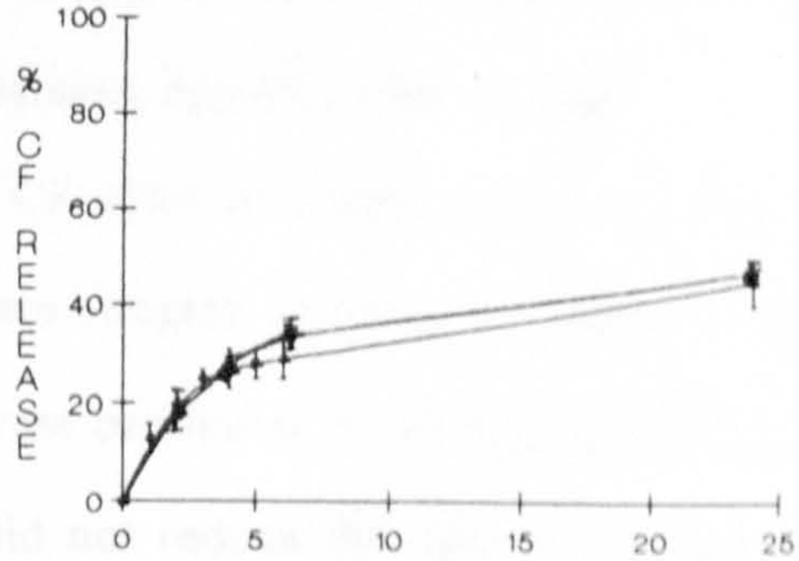


FIGURE 33b

II50:CHOL50 — II60:CHOL30:DCP10

The added DCP is 10 mol% and that of cholesterol 30 and 50 mol% . The error bars are standard error of means.

CF Release from **niosomes** prepared from Surfactant **III** at **pH 4.0 in BUFFER**.

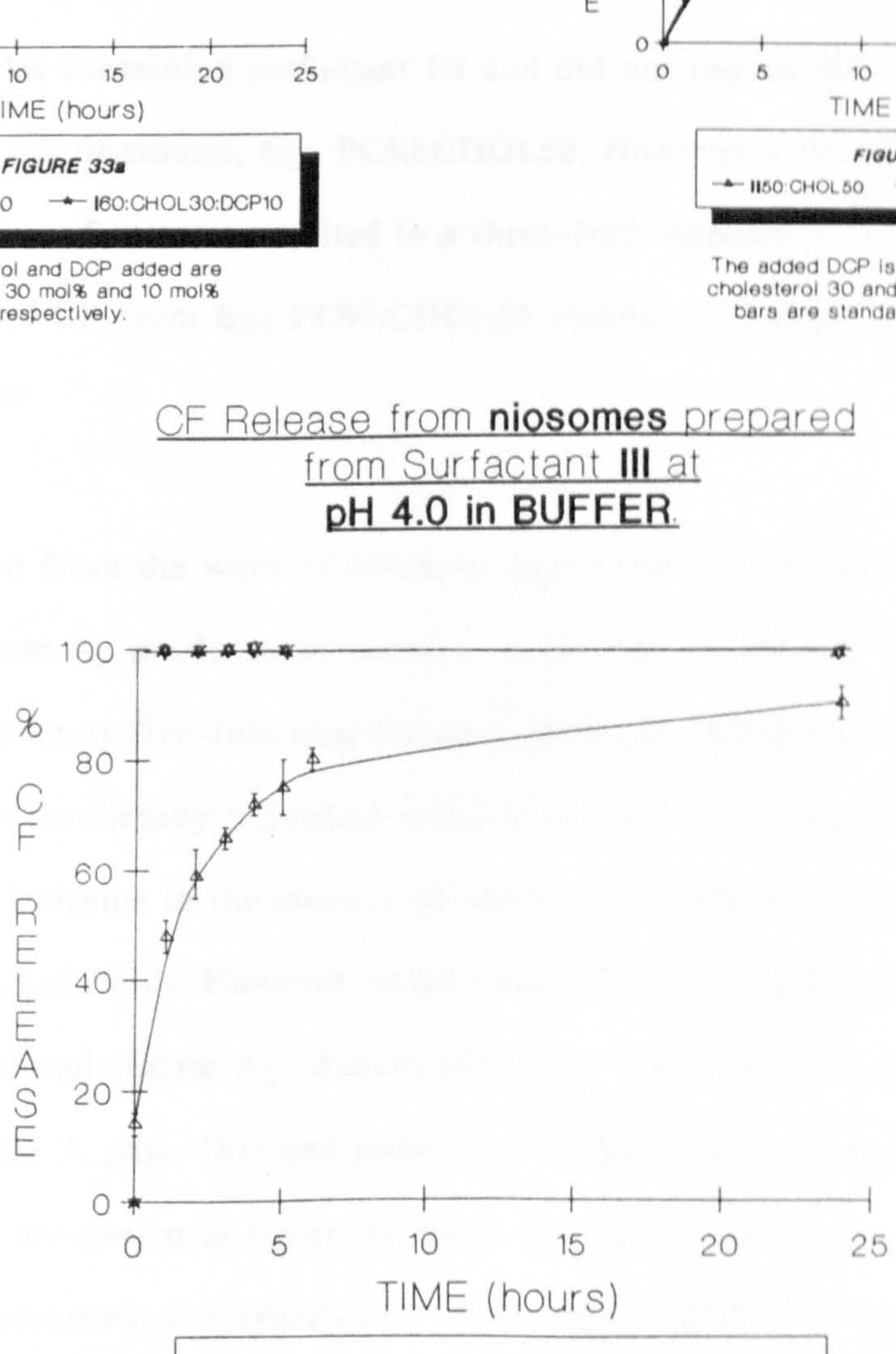


FIGURE 33c

III50:CHOL50 — III70:CHOL20:DCP10

Added cholesterol and DCP are in the same proportions as stated in the text.

substrate for this enzyme is phospholipid and carboxylic ester hydrolase, since this enzyme is known to break ester bonds. Carboxylic ester hydrolase produced leakage of CF only in vesicles, **III50:CHOL50** and **III70:CHOL20:DCP10**, that is, containing the ester linked surfactant **III**, resulting in approximately 40% loss of entrapped CF after 6 hours. Liposomes, containing egg PC, that is, **Egg PC50:CHOL50**, showed a delayed release of CF after 24 hours incubation where 40% of the contents had leaked, with this same enzyme, as seen in figure 34, page 127. Increasing the concentration of the enzyme produced no change in leakage from vesicles containing surfactant **III** and did not reduce the measured 6 hours lag period for liposomes, **Egg PC50:CHOL50**. However a five-fold increase in the concentration of esterase resulted in a three-fold increase in the 24 hour release of entrapped CF from **Egg PC50:CHOL50** vesicles; this latter result is not shown in figure 34.

As expected from the work of Slotboom et.al *(1982)*, pure porcine pancreatic phospholipase A_2 produced no increase in CF leakage for any vesicle types measured even at five-fold concentration increases and excess calcium ions. This agrees with the already published inability of pancreatic phospholipase to attack long chain lecithins in the absence of added detergents or organic solvents *(Slotboom et.al,1982)*. However, snake venom from Russell's viper provides a good source of phospholipase A_2 *(Russell,1980*; this venom also contains other enzymes, see Appendix 2, page 161) and produced CF efflux from several types of vesicles. The results are shown in figure 35, page 128; all liposome types examined and niosomes composed of surfactant **III**, for example, **DPPC100**, **DPPC50:CHOL50**, **DPPC70:CHOL20:DCP10**, **Egg PC50:CHOL50**, **DMPC50:CHOL50**, **III50:CHOL50** and **III70:CHOL20:DCP10**, displayed relatively high rates of leakage. Inclusion of cholesterol decreased leakage and addition of DCP stimulated CF efflux similar to the vesicles tested in vivo in Tetrahymena, as shown in Table 5, page 123. No increase in CF leakage from **II50:CHOL50** vesicles was recorded during the

Effect of **carboxylic ester hydrolase** on CF Release from **niosome and liposome** preparations.

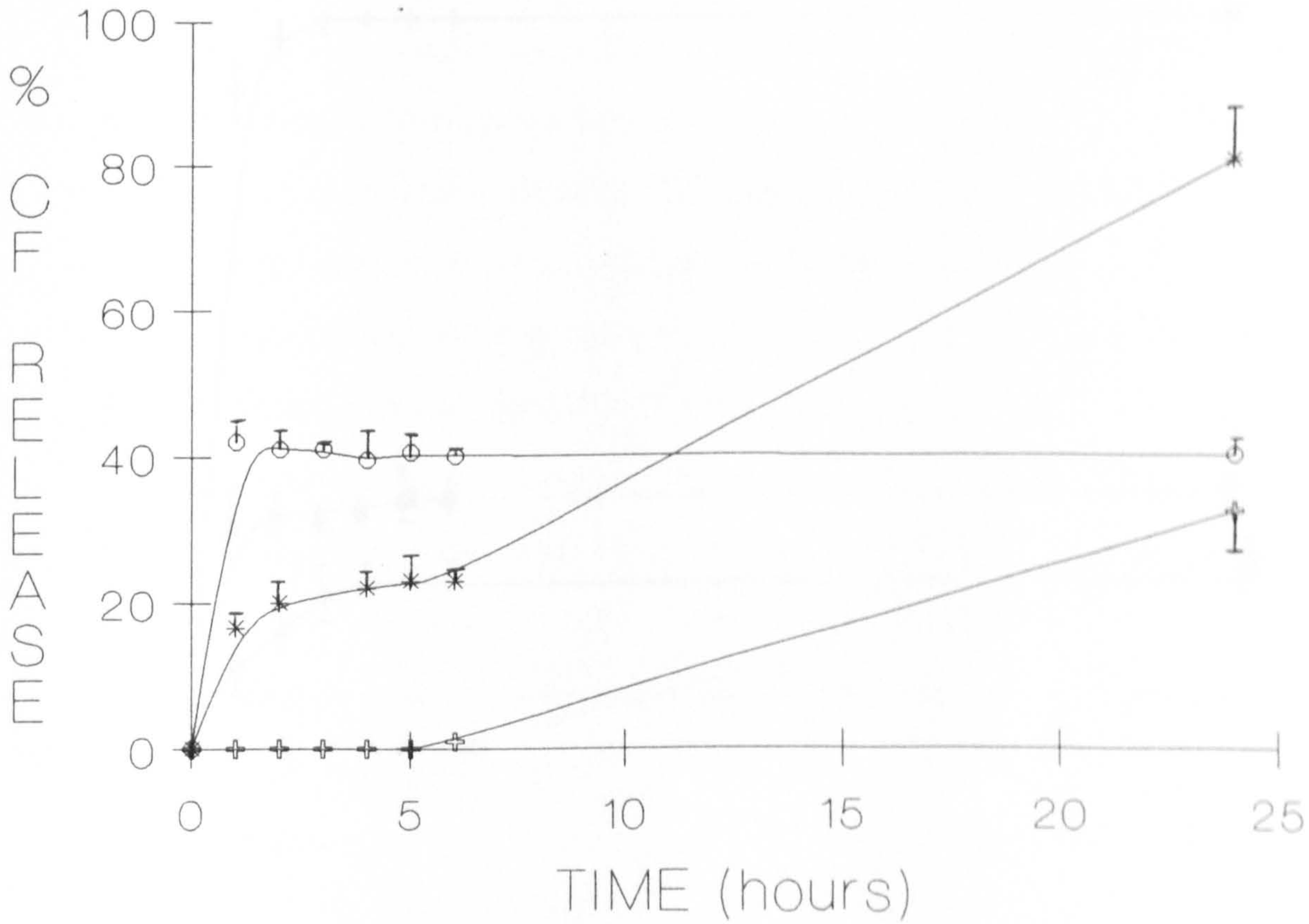


FIGURE 34

Surfactant **III** vesicles are the most sensitive to this enzyme. The liposome preparation is detailed in the text.

Effect of **snake venom phospholipase A2** on CF Release from **niosome and liposome** preparations.

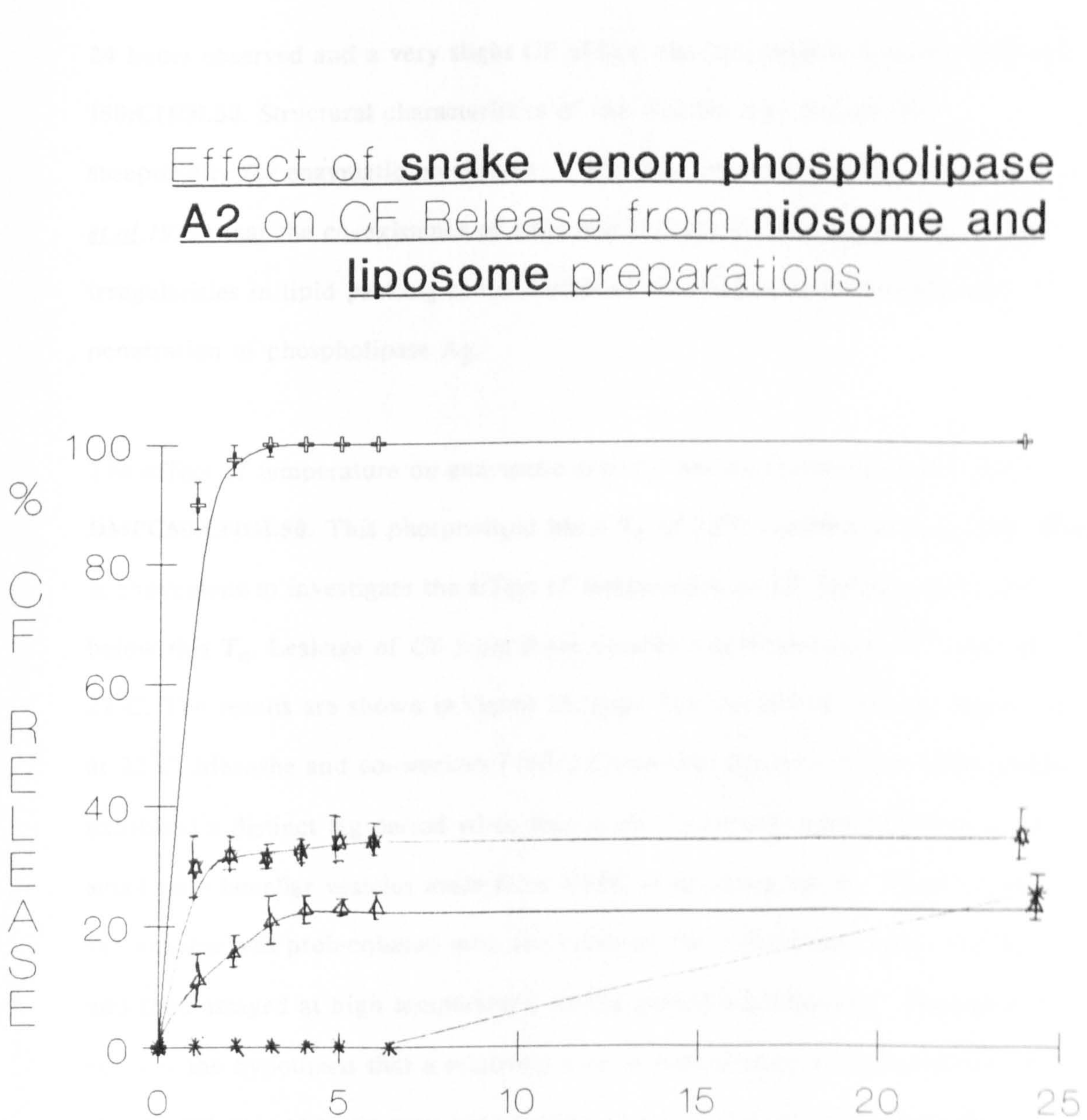


FIGURE 35

The niosomes used were prepared from Surfactant **III**, the ester type and had varying propotions of cholesterol added.

24 hours observed and a very slight CF efflux was measured in vesicles, **I100** and **I50:CHOL50**. Structural characteristics of the vesicles may govern their suseptibility for enzymatic breakdown. It has been reported *(Op den Kamp et.al,1975)* that the co-existence of solid and liquid phases in the bilayer create irregularities in lipid packing at the border of these domains facilitating the penetration of phospholipase A_2.

The effect of temperature on enzymatic activity was measured using vesicles, **DMPC50:CHOL50**. This phospholipid has a T_c of 23°C *(Ladbrooke et.al,1969)* which is convenient to investigate the effect of temperature on CF leakage above and below this T_c. Leakage of CF from these vesicles was measured at 37°C and at 22°C. The results are shown in figure 36, page 130 and the CF leakage was lower at 22°C. Menashe and co-workers *(1981)* found that the time course of hydrolysis exhibited a distinct lag period when they mixed pancreatic phospholipase A_2 and small uni- lamellar vesicles made from DPPC at or above the T_c. However when the enzyme was preincubated with the substrate for a short time below the T_c and then assayed at high temperature, no lag period was observed. These results support the hypothesis that a relatively slow substrate-enzyme organisational step is required as part of an activation process and this is most rapid when the substrate is in the gel state. However, the intrinsic enzymatic activity (after the initial step of activation) is maximal when the substrate is in the liquid-crystalline state. Hence, the lag time may reflect a penetration step of the enzyme into the lipid-water interface *(Langer,1980;Langer and Peppas,1981)*. It has been reported *(Wilschut et.al,1979)* that phospholipase A_2 can degrade the entire pool of phospholipid in the outer half of a vesicle without releasing any entrapped CF. Liposomes, **DPPC50:CHOL50**, were produced by sonication *as reported, (Menashe et.al,1981)* and incubated with pancreatic phospholipase A_2 and calcium ions. Sonicated vesicles have a high radius of curvature which may produce irregularities in the surface so aiding enzyme penetration. CF efflux was

CF Release from liposome **above** and
below the **Tc** of the lipid **(Tc=23oC)**
on addition of **snake venom phospholipase**

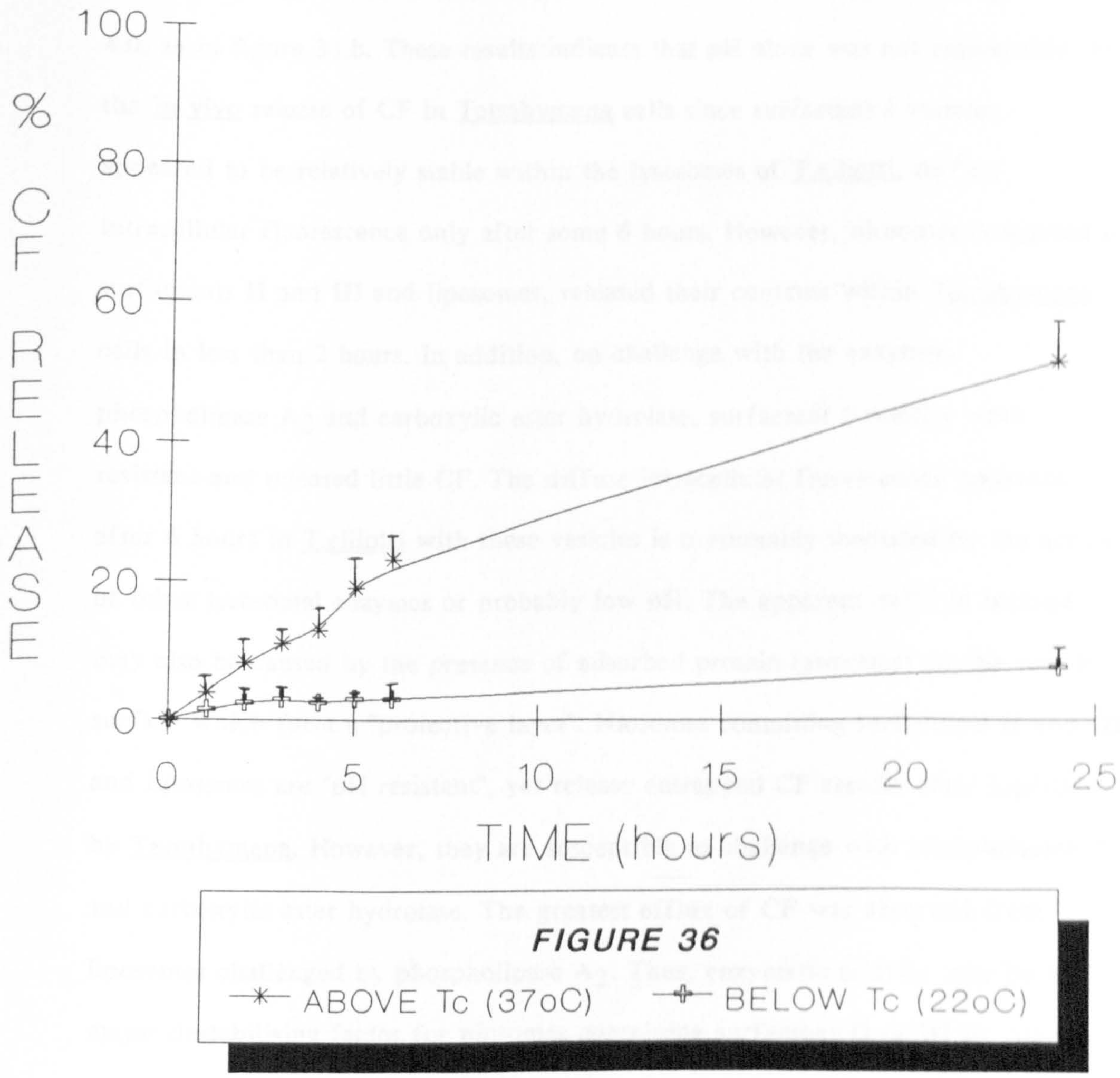


FIGURE 36

The preparation, **DMPC50:CHOL50**, was used
due to the low **Tc** of the lipid and to
study the effect of **phospholipase A2**.

monitored over 24 hours and no substantial increase was observed at either 22°C or at 37°C.

The effect of hydrolytic enzymes and pH were studied separately in the laboratory. The leakage was greatest at pH 4.0, which is seen typically with surfactant I and III vesicles, as shown in figure 33,a and c, page 125. Niosomes produced using surfactant II and liposomes were more resistant to leakage at pH 4.0, as in figure 33,b. These results indicate that pH alone was not responsible for the in vivo release of CF in Tetrahymena cells since surfactant I vesicles appeared to be relatively stable within the lysosomes of T.elliotti, diffuse intracellular fluorescence only after some 6 hours. However, niosomes composed of surfactants II and III and liposomes, released their contents within Tetrahymena cells in less than 2 hours. In addition, on challenge with the enzymes, phospholipase A_2 and carboxylic ester hydrolase, surfactant I vesicles were resistant and released little CF. The diffuse intracellular fluorescence apparent after 6 hours in T.elliotti with these vesicles is presumably mediated by the action of other lysosomal enzymes or probably low pH. The apparent delay in leakage may also be caused by the presence of adsorbed protein (enzymes) on the vesicle surface which form a "protective layer". Niosomes containing surfactants II and III and liposomes are "pH resistant", yet release entrapped CF rapidly after ingestion by Tetrahymena. However, they are susceptible to challenge with phospholipase A_2 and carboxylic ester hydrolase. The greatest efflux of CF was observed from liposomes challenged by phospholipase A_2. Thus, enzymatic activity may be a major destabilising factor for niosomes containing surfactant II or III in vitro in Tetrahymena cells, similar for liposomes. It is not clear why surfactant II niosomes should be destabilised in vitro by phospholipase A_2. Presumably it is not an enzyme phenomenon since there are no apparent points of attack, that is, no susceptible bonds within the molecule for the "reagent enzyme" to attack.

However, snake venom also contains many other enzymes, as in Appendix 2, page 161 and the observed activity may be due to the other enzymes.

Enzymatic CF release appears to be more effective than that caused by low pH. This demonstrates that drug release and hence distribution at the subcellular level can be tailored by suitable formulations of niosome preparations. This ability to finely tune the release of active ingredient at the cellular level appear to be a definite advantage of niosomes with respect to liposomes.

SECTION 4

CONCLUSIONS AND FUTURE WORK

4.1. GENERAL CONCLUSIONS

The proposed aims at the outset of this project have been successfully completed. A study of niosomes, prepared from three different non-ionic surfactants, have been compared to liposomes. The niosomes have been fully characterised in terms of their composition, stability and behaviour in a variety of systems, by analogy to the well-documented liposome delivery system. The ability of these bodies to interact with biological fluids have been studied thoroughly and attempts were made to quantify this interaction by studying their electrophoretic mobilities and changes in surface charges when added to blood plasma.

These vesicles were all found to bind to proteins in the blood and using poly-acrylamide gel electrophoresis, the nature of the proteins adsorbed to their outer surface were studied and in every case, albumin was found to be present. This was not surprising since albumin constitutes the major protein in blood and is expected to make the biggest contribution, as found in studies with liposomes. However, such studies have never before been carried out with niosomes and these findings reported here in should aid future formulation work with these novel carrier systems.

The rate of survival of these vesicles in a living, eukaryotic cell, such as T.elliotti, was a new approach to evaluate their stability within an in vitro system. This organism, which served as a simple model, has a number of lytic enzymes within it's digestive tract (food vacuoles) and the observation that niosomes were more stable than liposomes in this environment was encouraging for future development work for specific targeting related to the present model. By formulating the chemotherapeutic agent within the desired niosome system, their profile of breakdown within macrophages, containing living bacteria can be

evaluated. This ultimately will benefit the patient undergoing therapy by reducing toxicity: the final goal of such targeting systems.

Specific Conclusions

The size and nature of the surfactant used in the preparation of niosomes explains their stability in a given system. Irrespective of the composition, there were little variations in their ability to encapsulate drugs. In this project, the water soluble dye, CF was used while colleagues within our laboratories have used drugs such as doxorubicin, methotrexate and the antimony salt, Pentostam^{R*}. However, the ability to release the marker were different depending on the surfactant under investigation. The most stable vesicles, as a general rule, over a wide pH and temperature range, were those containing surfactant II. It appears that the greater number of methylene units (-CH_2-) in surfactant II, increases the lipophilicity of the molecule which packs this lamellar structure more tightly than the other two types of surfactant vesicles studied. Addition of cholesterol into the bilayer further stabilised these vesicles, irrespective of the types of surfactant used, while addition of DCP destabilised them, in a variety of conditions and environments.

The overall effect of blood proteins, on every type of vesicle prepared for this study, remained the same and this has been shown to be due to the "protective coat" of the albumin surrounding the outer membrane. As a consequence, their behaviour in biological fluids was governed by the nature of this external "coat", which in turn affects the stability of entrapped drug. This effect was further demonstrated when vesicles prepared from these three different types of surfactants, which varied in their individual stabilities, electrophoretic mobilities and charge densities were shown to have little difference when dispersed in plasma. Since only a "qualitative" study of the influence of this "protective coat"

was undertaken in this study a detailed and "quantitative" estimate will identify further differences which would help to optimise these niosome preparations as drug delivery systems. Gel electrophoresis is an excellent technique for such quantitative studies and is certainly a consideration for future work.

It was not surprising to find that niosomes containing surfactant III, an ester-linked amphiphile, were the least stable when challenged with carboxylic ester hydrolase, both in vitro (experiments using purified enzyme) and in vivo (within the food vacuole in T.elliotti cells). The relative stability of these niosomes, when compared to liposomes, to phospholipid A_2 was an interesting observation. Due to their phospholipid content, liposomes are more susceptible to degradation and eventual release of contents. However niosomes containing the single chain surfactant I were more stable under these conditions than those niosomes with surfactant II. This must be a direct consequence of the nature of the surfactant which affects the packing within the bilayer membrane.

The exact choice of non-ionic surfactant together with cholesterol produce niosomes that provide some degree of protection against release of drug content into the desired area. Further niosomes can be separated into unilamellar and multilamellar vesicle types containing a drug of choice within the aqueous compartment. Subjecting these vesicles to a systematic study, on a protocol outlined in this thesis, can accurately determine their behaviour in a chosen model. Together with a thorough knowledge of the biochemistry of the target cell(s), the release of contents from such vesicles can be predicted and the desired effects obtained. Such studies are part of the ongoing projects within our laboratories.

4.2. FUTURE WORK

The present investigations have been satisfactory in that the systematic study of a novel drug delivery system has produced a number of new ideas which can be further developed.

The most promising of these is the observation that although the niosome system is anologous in many respects to the well-characterised liposomal system, their subtle differences in properties can be exploited. In an attempt to acheive similar and other specific systems, a number of controlled studies are suggested:

1. Separation and analysis of multilamellar and unilamellar vesicles after preparation, by particle size extrusion to obtain niosomes of a particular size range and study their individual characteristics in a chosen system of interest.

2. The use of radioactive drug within the aqueous compartment or a radiolabelled surfactant incorporated into the membrane, for example, tritium or ^{14}Carbon are ideal, relatively inexpensive radiotracers, will allow the progress of the leakage in vivo to be monitored more closely in a system which can be tailored to the biological environment under study. This alleviates the need of markers which ultimately can only serve as an approximate model and may not be specific. A better understanding of the interactions involved in drug release, whether it is due to distortions in the outer membrane of the vesicle or simple drug diffusion into the surrounding media, can be ascertained accurately.

3. Quantitative gel electrophoresis to further amplify the protein-membrane interactions, as mentioned previously, page 108.

4. A reappraisal of studies in which the liposomal drug delivery system has been found to be non-specific or ineffective. In particular, applications in immunology: a very "specific" area ideally suited for "tailor made" targeting.

4.3. PERPECTIVES

Practical applications of liposomes have been explored in three main areas of research, namely in model membrane studies, in controlled and targeted drug delivery in vivo and in transfer of genetic and other materials into cells, in culture. Of these, the drug delivery concept, including the immunological adjuvant potential has attracted predominant interest. Promising results have been obtained in specific applications in experimental therapy related to the RES as in, for example, the accumulation of toxic metals in the liver *(Rahman,1980)* and in visceral leishmaniasis *(Alving,1986)*. The successful use of macrophage activating factors within liposomes directed towards alveolar macrophages for the activation of the eradication of lung metastases in cancer chemotherapy by utilising the liposome carrier with cytotoxic drugs, in laboratory animals have also been reported *(Rahman et.al,1986)*.

The use of liposomes as a model for biological membranes is confined to the research laboratory, but their successful application for drug delivery and/or genetic engineering will depend on several factors, such as a demonstration of the superiority of the liposome carrier for the intended purpose, and also upon the technical and economic feasibility of the system in practice. One of the fundamental questions is whether it is possible to prepare, isolate and characterise the particular liposomal system on an industrial scale with clearly defined and reproducible properties. Furthermore, the physical and chemical stability of such vesicles is of vital importance.

The future objectives of drug carrier systems can be considered as less ambitious than they were a few years ago. These are no longer used to deliver highly specific cytostatic agents to tumour cells. The emphasis is more on improving therapeutic treatment of certain types of disease by combining some of the

characteristics of liposome delivery systems such as preferential uptake by the RES cells, slow release effect for the entrapped drug and eventually preferential tissue localisation. The stimulation of macrophage mediated destruction of tumour cells and invading micro-organisms as well as the reduction of some side-effects of drugs are also areas of major therapeutic interest. There are numerous diseases that involve pathological modifications of cellular blood constituents, for example, sickle cell anaemia, or require active modification of cellular competance, as in the oxygen carrying capacity or tumouricidal properties. In these cases, the specific and/or non-specific interactions with vesicles carrying cellular effectors either in their membranes and/or in their aqueous space could be used to modify the recipient blood-cell physiology. For non-phagocytic and/or non-fusogenic target cells like the erythrocytes, hydrophobic compounds could be incorporated into the bilayer and transformed via exchange mechanisms into the plasma membrane. Also, non-specific cellular absorption of the carrier with subsequent release of it's contents could provide a circulating depot and/or create a locally high concentration of drug in question in the vicinity of it's target, the blood cells. To name but a few possibilities: Gersonde and Nicolau *(1979)* have shown that the oxygen-carrying capacity of erythrocytes can be markedly enhanced in vitro upon interaction with liposomes loaded with the allosteric effector, inositol hexaphosphate. Circulating peripheral monocytes *(Fidler et.al.1981)* have been shown to internalize liposomes containing muramyl dipeptide, with subsequent activation of their tumouricidal properties. Gorecki et.al *(1980)* have synthesised a series of benzyl esters of aromatic or hydrophobic amino acids that are potentially useful therapeutic agents for the treatment of sickle cell disease but cannot as yet be transferred effectively into the sickle erythrocytes *(Gorecki et.al.1980)*. The action of such drugs might be improved if they were incorporated into vesicular carriers that act either as depot systems with "sustained" survival in the blood stream and/or could be "homed" specifically to surface determinants on target cells contained in the circulation.

The vesicles described in this dissertation do not constitute a universal drug carrier and their real possibilities are likely to be fairly limited. However, they show very specific advantages for certain applications.

REFERENCES
&
APPENDICES

REFERENCES

Agarwal, K., *Bali, A and Gupta, C.M.* **(1986)** *Biochim. Biophys. Acta. 856, 36-40.*

Allen, T.M. and Cleland, L.G. (1980) *Biochim. Biophys. Acta. 597, 418-425.*

Allen, T.M. (1984) *In: Liposome Technology, Vol.III, (Gregoriadis, G., ed.) pp.177-182, CRC Press, Boca Raton, Florida.*

Alving, C.R., *Steck, E.A., Chapman, G.M.Jr., and Hanson, W.L.* **(1978)** *Proc. Natl. Acad. Sci. USA 75, 2959-2963.*

Alving, C.R. (1986) *In: Int. Encycl. Pharm. Ther. Section 120, Methods in Drug Delivery (Ihler, G.M., ed.) pp.281-300.*

Andersson, L.O., *Rehnstrom, A and Eaker, D.L.* **(1971)** *Eur. J. Biochem. 20, 371-380.*

Attwood, D and Florence, A.T. (1983) *Surfactant Systems:Their Chemistry, Pharmacy and Biology. Chapman and Hall, London.*

Avegard, R. and Haydon, D.A. (1973) *In: An Introduction to the Principles of Surface Chemistry. pp.40-47, Cambridge University Press, London.*

Azmin, M.N., *Florence A.T., Handjani-Vila, R.M., Stewart, J.S.B., Vanlerberghe, G and Whittaker, J.S.* **(1985)** *J. Pharm. Pharmacol. 37, 237-242.*

Azmin, M.N., *Florence A.T., Handjani-Vila, R.M., Stewart, J.S.B., Vanlerberghe, G and Whittaker, J.S.* **(1986)** *J. Microencapsulation. 3, 95-100.*

Baier, R.E and Dutton, R.C. (1969) *J. Biomed. Mater. Res. 3, 191-206.*

Baillie, A.J., *Florence, A.T., Hume, L.R., Muirhead, G.T. and Rogerson, A.* **(1984)** *J. Pharm. Pharmacol.Supp. 36, 48P.*

Baillie, A.J., *Florence, A.T., Hume, L.R., Muirhead, G.T and Rogerson, A.* **(1985)** *J. Pharm. Pharmacol. 37, 863-868.*

Baillie, A.J., *Coombs, G.H., Dolan, T.F and Laurie, J.* **(1986)** *J. Pharm. Pharmacol. 38, 502-505.*

Bakker-Woudenberg, I.A., *Lokerse, A.F., Roerdink, F.H., Regts, D and Michel, M.F.* **(1985)** *J. Infect. Dis. 151, 917-924.*

Bangham, A.D., *Standish, M.M. and Watkins, J.C.* **(1965)** *J. Mol. Biol. 13, 238-252.*

Barenholz,Y., *Amselem, S and Lichtenberg, D.* **(1979)** *FEBS Lett. 99, 210-214.*

Batzri, S. and Korn, E.D. (1973) *Biochim. Biophys. Acta. 298, 1015-1020.*

Black, C.D.V. and Gregoriadis, G. (1976) *Biochem. Soc. Trans. 4, 253-256.*

Blaurock, A.E. and Gamble, R.C. (1979) *J.Membr. Biol., 50, 187-192.*

Blum, R.H. and Carter, S.K. (1979) *Ann. Intern. Med. 80, 249-259.*

Blumenthal, R., *Weinstein, J.N., Sharrow, S.O. and Henkart, P.* **(1977)** *Proc. Natl. Acad. Sci. USA 74, 5603-5607.*

Bosworth, M.E. and Hunt, C.A. (1982) *J. Pharm. Sci., 71, 100-104.*

Brash, J.L., *Szota, P and Thibodeau, J.A.* **(1984)** *Trans. Soc. Biomat. 7, 253-256.*

Brown, P.J and Juliano, R. L. (1985) *Science, 228, 1448-1451.*

Caride, V.J. and Sostman, H.D. (1984) *In: Liposome Technology, Vol.II (Gregoriadis, G., ed.) pp.107-124. CRC Press, Boca Raton.*

Chen, R.F. (1977) *Anal. Lett. 10, 787-795.*

Chen, W.C. and Bittman, R. (1977) *Biochemistry, 16, 4145-4149.*

Chien, Y.W. (1980) *In: Drug delivery systems: Characteristics and biomedical applications. (Juliano, R.L., ed.) pp. 11-83, New York, Oxford, Oxford University Press.*

Clarke, H.G.M and Freeman, T. (1968) *Clin. Sci. 35, 403-413.*

Dahl, G., *Azarnia, R. and Werner, R.* **(1981)** *Nature 289, 683-685.*

Damen, J., *Regts, J and Scherphof, G.* **(1981)** *Biochim. Biophys. Acta., 665, 538-545*

Damen, J., *Regts, J and Scherphof, G.* **(1982)** *Biochim. Biophys. Acta., 712, 444-452.*

Darke, A., *Finer, E. G., Flook, A. G and Phillips, M. C.* **(1971)** *FEBS Lett. 18, 326-330.*

De Kruyff, B., *van Dijck, P. W. M., Demel, R. A.,Schuijff, A., Brants, F and van Deenen, L. L. M.* **(1974)** *Biochim. Biophys. Acta., 356, 1-7.*

De Haas, G.H., *Postema, N.M., Nieuwenhuizen, W. and van Deenen, L.M.M.* **(1968)** *Biochim. Biophys. Acta., 159, 103-117.*

Deamer, D. and Bangham, A.D. (1976) *Biochim. Biophys. Acta., 443, 629-634.*

Deamer, D. (1984) *In : Liposome Technology, Vol.I (Gregoriadis, G., ed.) pp.29-35. CRC Press, Boca Raton, Florida.*

Deliconstantinos, G., *Gregoriadis, G., Abel, G., Jones, M. and Robertson, D.* **(1977)** *Biochem. Soc. Trans. 5, 1326-1328.*

DeLoach, J.R. (1986) *Med. Res. Rev. 6(4), 487-504.*

Demel, R.A. and De Kruyff, B. (1976) *Biochim. Biophys. Acta., 457, 109-132.*

Desiderio, J.V. and Campbell, S.G. (1983a) *J. Reticuloendothel. Soc. 34, 279-287.*

Desiderio, J.V. and Campbell, S.G. (1983b) *J. Infect. Dis. 148, 563-570.*

Diehl, H. and Ellingboe, J.L. (1956) *Anal. Chem. 28, 882-895.*

Dingle, J.T. (1972) *Lysosomes. North Holland Publishing Co., Amsterdam.*

Documenta Geigy.(1962) *p.548.*

Edwards, D.I. (1980) *Antimicrobial Drug Action, University Park Press, Baltimore, USA.*

Ehrlich, P. (1906) *In : Collected Studies on Immunity Vol 2. pp.442-447., Wiley, New York.*

Elliott, A.M. (1965) *Science 149, 640-641.*

Elliott, A.M. and Clemmons, G.L. (1966) *J. Protozool. 13, 311-314.*

Fendler, J. H and Tundo, P. (1984) *Acc.Chem Res. 17, 3-8.*

Fidler, I.J., *Sone, S., Fogler, W.E. and Barnes, Z.L.* **(1981)** *Proc. Natl. Acad. Sci. USA 78, 1680-1684.*

Fildes, F.J.T. (1981) *In: Liposomes: from Physical Structure to Therapeutic Applications (Knight, C.G., ed.) pp.465-485. Elsevier/ North Holland Biomedical Press, Amsterdam.*

Forssen, E.A. and Tokes, Z.A. (1981) *Proc. Natl. Acad. Sci. USA 78, 1873-1877.*

Fountain, M.W., *Weiss, S.J., Fountain, A.G., Shen, A and Lenk, R.P.* **(1985)** *J. Infect. Dis. 152, 529-535.*

Freise, J., *Muller, W., Broelsch, C.H and Magersteds, P* **(1981)** *Hepato-gastroenterol. 28, 90-92.*

Frokjaer, S., *Hjorth, E.L. and Worts, O.* **(1982)** *In: Optimisation of drug delivery*

(Bundgaard, H., Hansen, A.B. and Kofod, H., eds.) pp.384-408, Munksgaard, Copenhagen.

Fukuzawa, K., *Ikeno, H., Tokumura, A and Tsukatani, H.***(1979)** *Chem. Phys. Lipids 23, 13-22.*

Gabizon, A., *Dagan, A., Goren, D., Barenholz, Y and Fuks, Z.* **(1982)** *Cancer Res. 42, 4734-4739.*

Gabizon, A., *Meshover, A and Barenholz, Y.* **(1986)** *J Natl. Cancer Inst. 77, 459-469.*

Gaffney, B.J. *and Mich, R.J.* **(1976)** *J. Am. Chem. Soc. 98, 3044-3045.*

Gersonde, K. and Nicolau, C. (1979) *Blut. 39, 1-7.*

Gorecki, M., *Votano, J.R. and Rich, A.* **(1980)** *Biochemistry 19, 1564-1568.*

Grahame, D.C. (1947) *Chem. Rev. 41, 441-501.*

Gregoriadis, G., *Leathwood, P.D. and Ryman, B.E.* **(1971)** *FEBS Lett. 14, 95-99.*

Gregoriadis, G. and Ryman, B.E. (1972) *Biochem. J. 129, 123-133.*

Gregoriadis, G. (1974) *Biochem. Soc. Trans. 2, 117-119.*

Gregoriadis, G. (1976) *Meth. Enzymol. 44, 698-709.*

Gregoriadis, G. (1977) *Nature 265, 407-409*

Gregoriadis, G. and Allison, A.C. (1980) *Liposomes in biological systems. John Wiley and Sons, Chichester, England.*

Gregoriadis, G., *Kirby, C., Meehan, A. and Senior, J.* **(1981)** *In: Liposomes, drugs and immunocompetent cell functions (Nicolau, C. and Paraf, A., eds.) pp.29-40, Acad. Press. London.*

Gregoriadis, G. (1982) *Drugs 24, 261-266.*

Gregoriadis, G. (1983) *Pharmacy International: 33-37.*

Grimes, P.A., *Stone, R.A., Laties, A.M. and Li, W.* **(1982)** *Arch. Ophthalmol. 100, 635-639.*

Guo, L.S.S., *Hamilton, R.L., Goerke, J., Weinstein, J.N. and Havel, R.J.* **(1980)** *J. Lipid Res. 21, 993-1003.*

Hamilton, R.L. (1978) *In:Disturbances in Lipid and Lipoprotein Metabolism. (Dietschy, J.M., Gotto, A.M.Jr. and Ontko, J.A., eds.) pp.155-171. American Physiological Society, Bethesda, MD.*

Hamilton-Miller, J.M. (1973) *Bacteriol.Rev. 37, 166-169.*

Handjani-Vila, R. M., *Ribier, A., Rondot, B. and Vanlerberghe, G.* **(1979)** *Int. J. Cos. Sci., 1, 303-314.*

Hargreaves, W.R. (1978) *Biochem. 17, 3759-3767.*

Hawkins, D., *Pinekard, R.N. and Farr, R.S.* **(1968)** *Science 160, 780-782.*

Haydon, D.A. and Myers, V.B. (1973) *Biochim. Biophys. Acta. 307, 429-443.*

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

Heiple, J.M and Taylor, D.L (1982) *In: Intracellular pH: its measurement, regulation and utilisation in cellular functions. (Nuccitelli, R and Deamer, D.W., eds) pp. 21-54. New York*

Hope, M.J., *Bally, M. B., Webb, G and Cullis, P.R.* **(1985)** *Biochim. Biophys. Acta. 812, 55-65.*

Horbett, T.A. (1981) *J Biomed. Mater. Res. 15, 673-695.*

Horbett, T.A and Weathersby, P.K. (1981) *J.Biomed. Mater. Res. 15, 403-423.*

Hsia, J.C., *Schneider, H. and Smith, I.C.P.* **(1971)** *Can. J. Biochem. 49, 614-622.*

Hsu, M.J and Juliano, R.L. (1982) *Biochim. Biophys. Acta. 720, 411-419.*

Huang, C.H. and Charlton, H.P. (1971) *J. Biol. Chem. 246, 2555-2562.*

Hunt, A.C. (1982) *Biochim. Biophys. Acta. 719, 450-463.*

Hunter, R.J. (1981) *Zeta Potential in Colloidal Science. Acad. Press, London.*

Ihlenfield, J.V and Cooper, S.L (1979) *J. Biomed. Mater. Res. 13, 577-591.*

Ihler, G.M. (1983) *Pharmac. Ther. 20, 151-169.*

Ihler, G.M. (1986) *Methods in Drug Delivery. Int. Encycl. Pharm. Ther., Section 120 and references therein.*

Inoue, K. (1974) *Biochim. Biophys. Acta. 339, 390-402.*

Jonas, A. and Krajnovich, D.J. (1978) *J. Biol. Chem. 253, 5758-5763.*

Juliano, R.L. and Stamp, D. (1975) *Biochim. Biophys. Res. Commun. 63, 651-658.*

Juliano, R.L. and Stamp, D. (1978) *Biochem. Pharmacol. 27, 21-26.*

Juliano, R.L. and Lin, G. (1980) *In "Liposomes and Immunobiology" (eds., Tom, B. and Six, H.) pp. 49-66, Elsevier, New York.*

Juliano, R.L. (1981) *In: Liposomes: from Physical Structure to Therapeutic Application (Knight, C.G.,ed.) pp.21-32. Elseiver North Holland Biomedical Press, Amsterdam.*

Juliano, R.L., *Lopez-Berestein, G., Hopfer, R.L., Mehta, R., Mehta, K and Mills, K.* **(1985)** *Ann. N.Y. Acad. Sci. USA. 446, 390-402.*

Kaneda, A., *Nishimura, K and Sezaki, H.* **(1974)** *Chem. Pharm. Bull. 23, 523-528.*

Kano, K and Fendler, J.H. (1977) *Life Sci. 20, 1729-1734.*

Kaye, S.B. (1981) *Cancer Treat. Rev. 8, 27-50.*

Kimelberg, H.K. (1976) *Mol. Cell. Biochem. 10, 171-190.*

Kimelberg, H.K. and Mayhew, E. (1978) *CRC Crit. Rev. Toxicol. 6, 25-29.*

Kimelberg, H.K., *Tracy, T.F., Watson, R.E., Kung, D., Reiss, F.L. and Bourke, R.S.* **(1978)** *Cancer Res. 38, 706-715.*

Kinsky, C.B., *Haxby, J.A and Kinsky, S.C.* **(1966)** *Proc. Natl. Acad. Sci. USA 61, 300-304.*

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

Kirby, C., *Clark, J. and Gregoriadis, G.* **(1980b)** *FEBS Lett. 111(2), 324-328.*

Kirby, C and Gregoriadis, G. (1984) *Biotechnology, No. 11, 979-984.*

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

Kunitake, T and Okahata, Y (1977) *J. Am. Chem. Soc. 99, 3860-3861.*

Kunitake, T and Okahata, Y (1978) *Bull. Chem. Soc. Jpn. 51, 1877-1879.*

Kunitake, T and Okahata, Y (1980) *J. Am. Chem. Soc. 102, 549-553.*

Kunitake, T. (1986) *Ann. N.Y. Acad. Sci. USA 471, 70-82.*

Ladbrooke, B. D and Chapman, D. (1969) *Chem. Phys. Lipids 3, 304-309.*

Laemmli, U.K. (1970) *Nature 227, 680-685.*

Langer, R. (1980) *Chem. Eng. Commun. 6, 1-48.*

Langer, R. and Peppas, N.A. (1981) *Biomaterials 2, 195-210.*

Langer, R. and Peppas, N.A. (1983) *J. Macromol. Sci. 4, 247-252.*

Law, S.L., *Lo, W.Y., Pai, S.H., Teh, G.W. and Kou, F.Y.* **(1986)** *Int. J. Pharm. 32, 237-241.*

Lelkes, P.I. and Tandeter, H.B. (1982) *Biochim. Biophys. Acta. 716, 410-419.*

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

Lesslauer, W., *Cain, J.E and Blasie, J. L.* **(1972)** *Proc. Natl. Acad. Sci. 69, 1499-1506.*

Leung, J.G.M. (1980) *Biochim. Biophys. Acta. 597, 427-432.*

Levine, Y.K and Wilkins, M. H. F. (1971) *Nature New Biol. 230, 69-72.*

Levy, M. and Elliott, A.M. (1968) *J. Protozool. 15, 208-212.*

Lopez-Berestein, G., *Mehta, R., Hopfer, R.L., Mills,K., Kasi, L., Mehta, K., Fainstein, V., Luna, M., Hersh, E.M and Juliano, R.L.* **(1983)** *J Infect. Dis. 147, 939-945.*

Lopez-Berestein, G., *Fainstein, V., Hopfer, R., Sullivan, M., Rosenblum, M., Mehta, R., Luna, M., Hersh, E., Reuben, J., Mehta, K., Juliano, R.L and Bodey, G.* **(1985)** *J. Infect. Dis. 151, 704-710.*

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

Madden, T.D., *Bully, N.B., Hope, M.J., Cullis, P.R., Schieren, H.P. aqnd Janoff, A.S* **(1985)** *Biochim. Biophys. Acta. 817, 67-74.*

Martin, F.J. and MacDonald, R.C. (1976) *J. Cell Biol. 70, 515-526.*

Marty, J.M., *Oppenheim, R.D. and Speiser, P.* **(1978)** *Pharm. Acta. Helv. 53, 17-23.*

Mathias, C., *Thambi Dorai, D. and Bachhawat, B.K.* **(1977)** *Ind. J. Biochim. Biophys. 14, 142-148.*

Mayhew, E., *Papahadjopoulos, D., Rustum, Y.M. and Dave, C.* **(1976)** *Cancer Res. 36, 4406-4411.*

Mayhew, E., *Papahadjopoulos, D., O'Malley, J.A., Carter, W.A. and Vail, W.J.* **(1977)** *Mol. Pharmacol. 13, 488-495.*

Mayhew, E., *Gotfredsen, C., Schneider, Y.J and Trouet, A.* **(1980)** *Biochem Pharmacol. 29, 877-886.*

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

McLaughin, S. (1977) *In:Current Topics in Membrane and Transport. Vol. 9. (Bronner, F. and Kleinzeller, A., eds.) pp.71-144, Acad. Press, New York.*

Medoff, G., *Brajtburg, J., Kobayashi, G.S and Bolard, J* **(1983)** *Ann. Rev. Pharmacol. Toxicol. 23, 303-304.*

Menashe, M., *Lichtenberg, D., Gutierrez-Merino, C. and Biltonen, R.L.* **(1981)** *J. Biol. Chem.* 256, *4541-4543.*

Milsmann, M.H.W., *Schwendener, R.A. and Weder, H.G.* **(1978)** *Biochim. Biophys. Acta.* 512, *147-154.*

Morrisett, J.D., *Jackson, R.L. and Gotto, A.M.* **(1977)** *Biochim. Biophys. Acta.* 472, *93-103.*

Mosher, D.S. (1984) *Ann. Rev. Med.* 35, *561-575.*

Muller, M., *Baudhuin, P. and De Duve, C.* **(1966)** *J. Cell Physiol.* 68, *165-170.*

New, R.B.C., *Chance, M.L., Thomas, S.C. and Petas, W.* **(1978)** *Nature* 272, *55-56.*

Newman, G.C and Huang, C (1975) *Biochem.* 14, *3363-3369.*

Nilsson, J.R. (1970a) *C.R. Trav. Lab. Carlsberg* 38, *87-90.*

Nilsson, J.R. (1970b) *C.R. Trav. Lab. Carlsberg* 38, *107-112.*

Nuccitelli, R and Deamer, D.W. (1982) *Intracellular pH: its measurement, regulation and utilisation in cellular functions, New York, A. R. Liss.*

Okahata, Y., *Tanamachi, S., Nagai, M and Kunitake, T* **(1981)** *J. Coll. Inter. Sci.* 82, *401-417.*

Olson, F., *Hunt, C.A., Szoka, F.C., Vail, W.J and Papahadjopoulos, D.* **(1979)** *Biochim. Biophys. Acta.* 557, *9-23.*

Olson, F., *Mayhew, E., Maslow, D.,Rustum, Y. and Szoka, F.* **(1982)** *Eur. J. Clin. Oncol. 18, 167-176.*

Op den Kamp, J.A.F., *Kauerz, M.T. and Van Deenen, L.L.M.* **(1975)** *Biochim. Biophys. Acta. 406, 169-177.*

Oppenhenheim, R.C. (1981) *Int. J. Pharm. 8, 217-234.*

Ostro, M. (1987) *Sci. Amer. 256, 102-111*

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

Papahadjopoulos, D., *Jacobson, K., Nir, S. and Isac, T.* **(1973)** *Biochim. Biophys. Acta. 311, 330-334.*

Papahadjopoulos, D., *Mayhew, E., Poste, G., Smith, S. and Vail, W.J.* **(1974)** *Nature 252, 163-166.*

Parker, C.A. (1968) *Photoluminescence of Solutions. Elsevier, New York.*

Pethig, R., *Gascoyne, P.R.C., McLaughlin, J.A. and Szent-Gyorgyi, A.* **(1984)** *Proc. Natl. Acad. Sci. 81, 2088-2091.*

Piard, J.C., *Elsoda, M., Alkhalaf, W., Rousseau, M., Desmazeaud, M., Vassal, L. and Gripon, J.C.* **(1986)** *Biotech. Lett. 8(4), 241-246.*

Poste, G. (1983) *Biol. Cell. 47, 19-38.*

Poznansky, M.J. and Juliano, R.L. (1984) *Am. Soc Pharmacol. Exp. Ther. 36, 277-336.*

Pratt, W.B. (1977) *Chemotherapy of Infection. Oxford University Press, New York.*

Quaissi, N. A and Cabron, A. (1985) *Ann. Inst. Pasteur Immun. 136C, 169-185.*

Rahman, Y.E. (1980) *In: Liposomes in biological systems (Gregoriadis, G. and Allison, A.C., eds.) pp. 265-298, John Wiley and Sons, Chichester, England.*

Rahman, Y.E., *Cerny, E.A., Patel, K.R., Lau, E.H. and Wright, B.J.* **(1982)** *Life Sci. 31, 2061-2063.*

Rahman, A., *Carmichael, D., Harris, M and Roh, J.K.,* **(1986)** *Cancer Res. 46, 2295-2299.*

Rahman, A., *Kessler, A., More, N., Sikic, B., Rowden, G., Wooley, G. and Schein, P.S.* **(1980)** *Cancer Res. 40, 1532-1536.*

Ralston, E., *Hjelmeland, L.M., Klauser, R.D., Weinstein, J.N and Blumenthal, R.* **(1981)** *Biochim Biophys. Acta. 649, 133-137.*

Rao, L.S. (1984) *In: Liposome Technology, Vol. I (Gregoriadis, G., ed.) pp.247-257, CRC Press, Boca Raton, Florida.*

Readio, J.D and Bittman, R. (1982) *Biochim. Biophys. Acta. 685, 219-224.*

Rogerson, A. (1986) *Ph.D. Thesis, University of Strathclyde.*

Rogerson, A., *Cummings, J., Wilmott, N and Florence, A.T.* **(1988)** *in press.*

Rossi, J. D and Wallace, B. A. (1983) *J. Biol. Chem. 258, 3327-3331.*

Russell, F.E. (1980) *Snake Venom Poisoning, J.B.Lippincott Company, Philidelphia, Toronto.*

Ryan, P.J., *Davis, M.A., De Gaeta, L.R., Woda, L.R. and Melcior, D.L.* **(1984)** *Radiology 152, 759-762.*

Ryman, B.E. and Tyrrell, D.A. (1980) *Essays Biochem. 16, 49-98.*

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

Scherphof, G., *Damen, J. and Hoekstra, D.* **(1981).** *In: Liposomes: from physical structure to therapeutic actions. (Knight, C.G., ed) pp.299-332. Elsevier/ North Holland, Amsterdam.*

Scherphof, G., *Damen, J and Wilschut, J* **(1984)** *In: Liposomes Technology, Vol. III (Gregoriadis, G., ed.) pp. 205-224. CRC Press, Boca Raton, Florida.*

Scherphof, G. and Morselt, H. (1984) *Biochem. J. 221, 423-429.*

Schieren, H. (1978) *Biochim. Biophys. Acta. 542, 137-140.*

Schneider, M. (1977) *Battelle Memorial Institute US patent 4' 224' 179.*

Seltzer, S.E., *Davis, M.A., Adams, D.F., Shulkin, P.M., Landis, W.J. and Havron, A.* **(1984)** *Invest. Radiol. 19, 142-151.*

Shaw, D.J. (1968) *Electrophoresis, Acad. Press, London.*

Shinozawa, S., *Araki, Y and Oda,T* **(1981)** *Acta. Med. Okayama 35, 395-405.*

Sjoholm, I. and Edman, P. (1984) *In: Microspheres and Drug Therapy. Pharmaceutical, Immunological and Medical Aspects. (Davis, S.S., Illum, L., Mc.Vie, J.G. and Tomlinson, E., eds.) pp.245-262.*

Slotboom, A.J., *Verheij, H.M and de Haas, G.H* **(1982)** *In: Phospholipids (Hawthorne, J.N and Ansell, G.B.,eds.) pp. 359-434. Elsevier, Amsterdam.*

Spector, A.A and Fletcher, J.E. (1977) *In: Albumin: structure, biosynthesis, function. (Peters, T and Sjoholm, I., eds.) pp.51-60, Pergamon Press.*

Strauss, G., *Schurtenberger, P and Hausser, H* **(1986)** *Biochim. Biophys. Acta. 858, 169-180.*

Sweet, C. and Zull, J.E. (1970) *Biochim. Biophys. Acta. 219, 253-262.*

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

Szoka, F. and Papahadjopoulos, D. (1980) *Ann. Rev. Biophys. Bioeng. 9, 467-508.*

Tagesson, C., *Stendahl, O. and Magnusson, K.E.* **(1977)** *Stud. Biophys. 64, 151-160.*

Tall, A.R. and Small, D.M. (1977) *Nature 265, 163-164.*

Tall, A. R and Lange, Y. (1978) *Biochim. Biophys. Acta. 513, 185-197.*

Tanford, C. (1980) *The Hydrophobic Effect. Formation of Micelles and Biological Membranes. 2nd. Edition, Wiley-Interscience, New York.*

Tomlinson, E., *Burger, J.J., Schoonderwoerd, E.M.A. and McVie, J.G.* **(1984)** *In: Microspheres and Drug Therapy. Pharmaceutical, Immunological and Medical Aspects, (Davis, S.S., Illum, L., McVie, J.G. and Tomlinson, E., eds.) pp. 75-91*

Towbin, H., *Staehlin, T and Gordon, J.* **(1979)** *Proc. Natl. Acad. Sci. USA 76, 4350-4354.*

Tyrrell, D.A., *Ryman, B.E., Keeton, B.R. and Dubowitz, V.* **(1976)** *Brit. Med. J. 2, 88-91.*

Tyrrell, D.A., *Heath, T.D., Colley, C.M. and Ryman, B.E.* **(1976)** *Biochim. Biophys. Acta. 457, 259-302.*

Ugoretz, R.J. (1976) *J. Am. Med. Assoc. (JAMA) 236, 295-296.*

Uniyal, S and Brash, J.L. (1982) *Thromb. Haemostas. 47, 285-290.*

Van Berkel, T.J.C., *Kar Kruijt, J., Harkes, L, Vagelkerke, J.S., Spanjer, H and Kempen, H.J.M.* **(1986)** *In: Site-specific drug delivery, (Tomlinson, E and Davis, S.S., ed.), pp. 49-68, John Wiley and Sons Ltd; Chichester.*

Van Deenen, L.L.M. and de Haas, G.H. (1964) *Adv. Lipid Res. 2, 167-234.*

Van Renswoude, J. and Hoekstra, D. (1980) *Biochemistry 20, 540-546.*

Vanlerberghe, G., *Ribier, A and Handjani-Vila, R.M* **(1978)** *Communication au Colloque du CNRS sur la physicochemie des composes amphiphiles, Bordeaux.*

Vroman, L., *Adams, A.L., Fisher, G.C and Munoz, P.C* **(1980)** *Blood 55, 156-159.*

Wallach, D.F.H., *Surgenor, D.M., Soderberg, J. and Delano, E.* **(1959)** *Anal. Chem., 31, 456-461.*

Wallach, D.F.H and Steck, T.L. (1963) *Anal. Chem. 35, 1035-1044.*

Weast, R.C. (1984) *Handbook of Chemistry and Physics pp. F-37, E-50. 65th Edn. CRC Press, Cleveland.*

Weinstein, J.N., *Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A.* **(1977)** *Science 195, 489-492.*

Weinstein, J.N., *Ralston, E., Leserman, L.D., Klausner, R.D., Dragsten, P., Henkart, P. and Blumenthal, R.* **(1984)** *In: Liposome Technology, Vol III (Gregoriadis, G., ed.) pp.183-204. CRC Press, Boca Raton, Florida.*

Weinstein, J.N., *Blumenthal, R. and Klausner, R.D.* **(1986)** *Meth. Enzymol. 128, 657-668.*

Weissmann, G., *Sessa, G. and Weissman, S.* **(1966)** *Biochem. Pharmacol. 15, 1537-1551.*

Weissmann, G., *Bloomgarden, D., Kaplan, R., Cohen, C., Hoffstein, S., Collins, T., Gotlieb, A. and Nagle, D.* **(1975)** *Proc. Natl. Acad. Sci. USA 72, 88-92.*

Weissmann, G., *Cohen, C. and Hoffstein, S.* **(1977)** *Biochim. Biophys. Acta. 498, 375-385.*

Whitmore, A., *Brooks, L.G and Wheeler, K.P* **(1979)** *J. Pharm. Pharmacol. 31, 277-283.*

Williams, R. M and Chapman, D. (1970) *In: Progress in the Chemistry of Fats and Other Lipids, 21, 1-10 (Holman, R. T., ed.,) Pergamon Press, Oxford.*

Wilschut, J.C., *Regts, J., Westenburg, H. and Scherphof, G.* **(1976)** *Biochim. Biophys. Acta. 433, 20-31.*

Wilschut, J.C., *Regts, J., Westenberg, H. and Scherphof, G.* **(1978)** *Biochim. Biophys. Acta. 580, 185-196.*

Wilschut, J. and Papahadjopoulos, D. (1979) *Nature 281, 690-692.*

Wilschut, J.C., *Regts, J. and Scherphof, G.* **(1979)** *FEBS Lett. 98, 181-186.*

Wilson, T., Papahadjopoulos, D. and Taber, R. (1977) *Proc. Natl. Acad. Sci. USA 74, 3471-3475.*

Yatvin, M.B., *Weinstein, J.N., Dennis, W.H. and Blumenthal, R.* **(1978)** *Science 202, 1290-1293.*

Yoshioka, S. (1984) *Chem. Pharm. Bull. 33, 308-312.*

Yoshioka, S., *Ishibashi, M., Shibazaki, T and Uchiyama, M.* **(1984)** *J. Par. Sci. Tech. 38(6), 222-227.*

Zborowski, J., *Roerdink, F. and Scherphof, G.* **(1977)** *Biochim. Biophys. Acta. 497, 183-191.*

APPENDIX 1

THERMAL TRANSITIONS

Compound	Temperature for gel-to-gel crystal (lamellar) T_c, in (^{0}C)
DPPC (C16)	41
DMPC (C12)	23
egg PC	-15/-7
surfactant 1 (m.wt.473)	41-43
surfactant 2 (m.wt.972)	41-47
surfactant 3 (m.wt.404)	40-42

APPENDIX 2

Enzymes of Snake Venom. [Reproduced from *Russell,F.E (1980)*]

Acetylcholinesterase: catalyses the hydrolysis of acetylcholine to choline and acetic acid.

Arginine ester hydrolase: non-cholinesterase. The substate specificities are directed to the hydrolysis of the ester or peptide linkage, to which an arginine residue contributes the carboxyl group.

Collagenase: specific kind of proteinase that digests collagen.

Hyaluronidase: catalyses the cleavage of internal glycoside bonds of certain acid mucopolysaccharides.

Lactate dehydrogenase: reversibly catalyses the conversion of lactic acid to pyruvic acid.

L-aminoacid oxidase: catalyses the oxidation of L-α-amino acids and α-hydroxy acids and is the most active of the known amino acid oxidases. This gives the venom it's yellow colour.

NAD-nucleotidase: catalyses the hydrolysis of the nicotinamide N-riboside linkage of NAD, yielding nicotinamide and adenosine diphosphate riboside.

5'-nucleotidase: specifically hydrolyses phosphate monoesterase which links with a 5' position of DNA and RNA.

Phospholipase A_2: catalyses the hydrolysis of one of the fatty ester linkages in diacyl phosphatides, forming lysophosphatides and releasing both saturated and unsaturated fatty acids.

Phospholipase B: hydrolyses lysophosphatides.

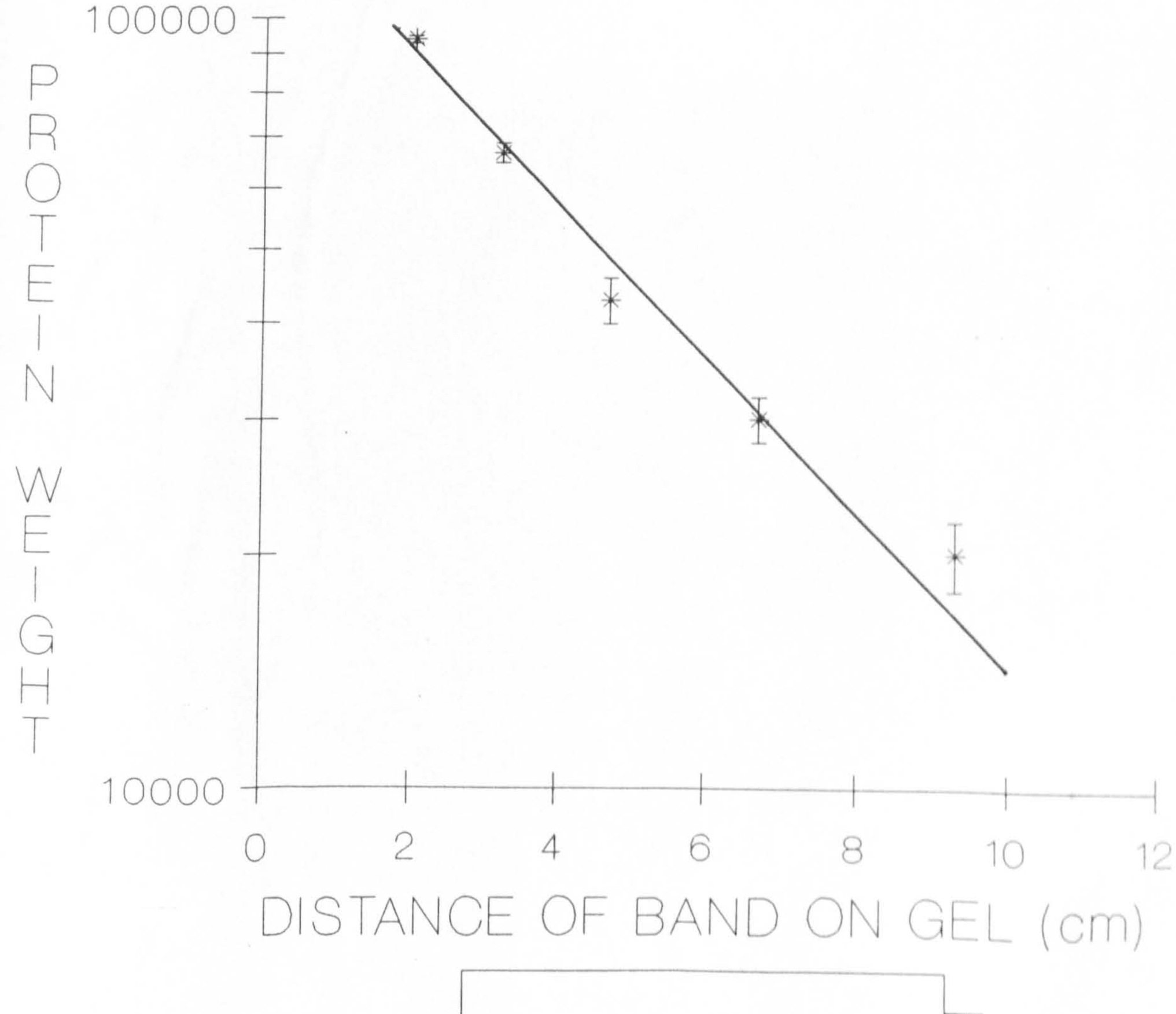
Phospholipase B and C: hydrolyses lysophosphatides.

Phosphomonoesterase: hydrolyses mono esters.

Phosphodiesterase: an orthophosphoric diester phosphohydrolase that releases 5-mononucleotide from the polynucleotide chain and thus acts as an exonucleotidase.

Proteolytic enzymes: trypsin-like enzymes that digest tissue proteins and peptides.

A **semi-logarithmic** plot of
distances migrated on the gel
against **molecular weight of proteins**.



APPENDIX 3

* Protein location

The protein used were all standards
as explained in the text.

figure 32 d

p 120 - 122

(c)

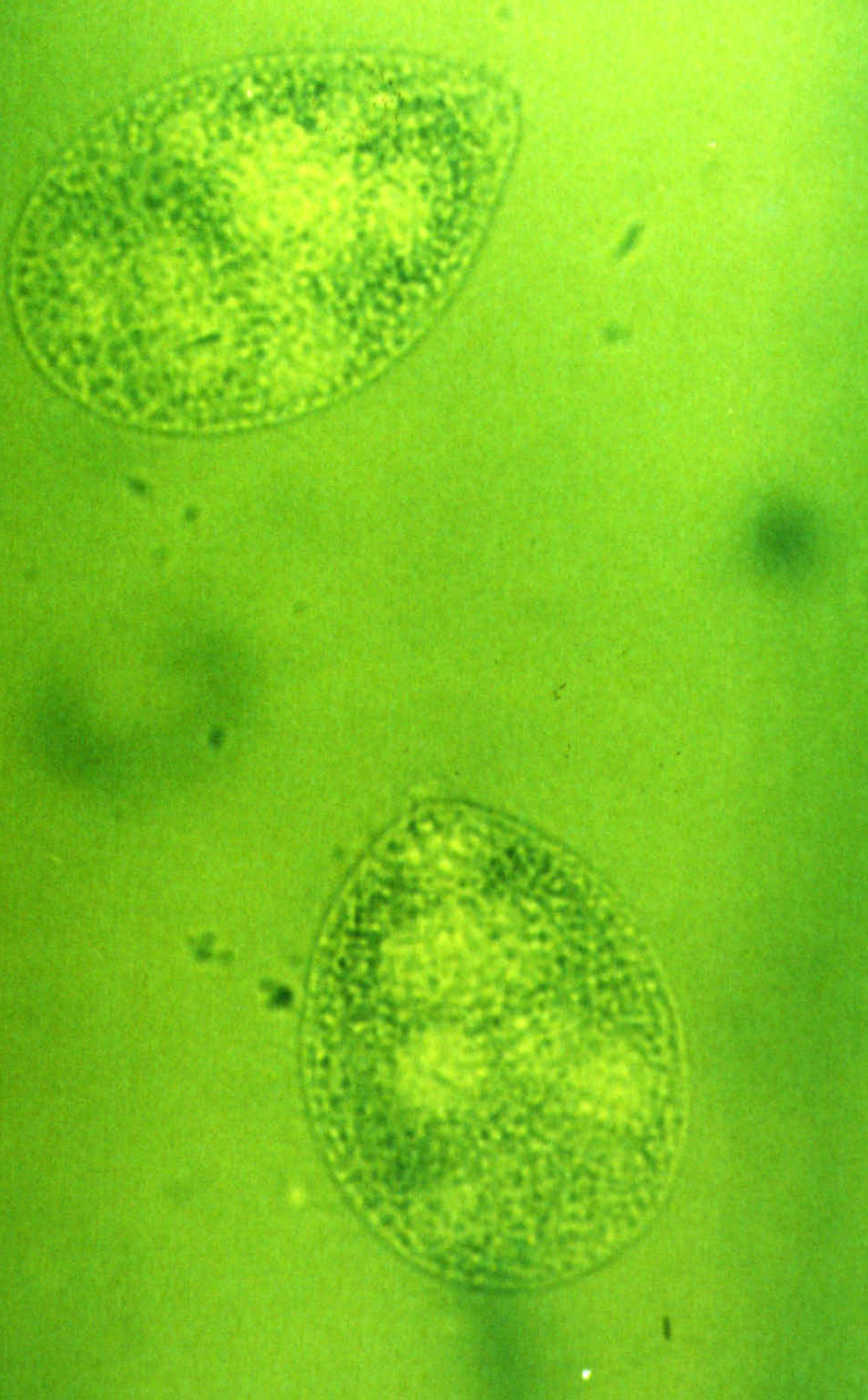


figure 32 b

p. 120 - 122

figure 32c

p 120-122

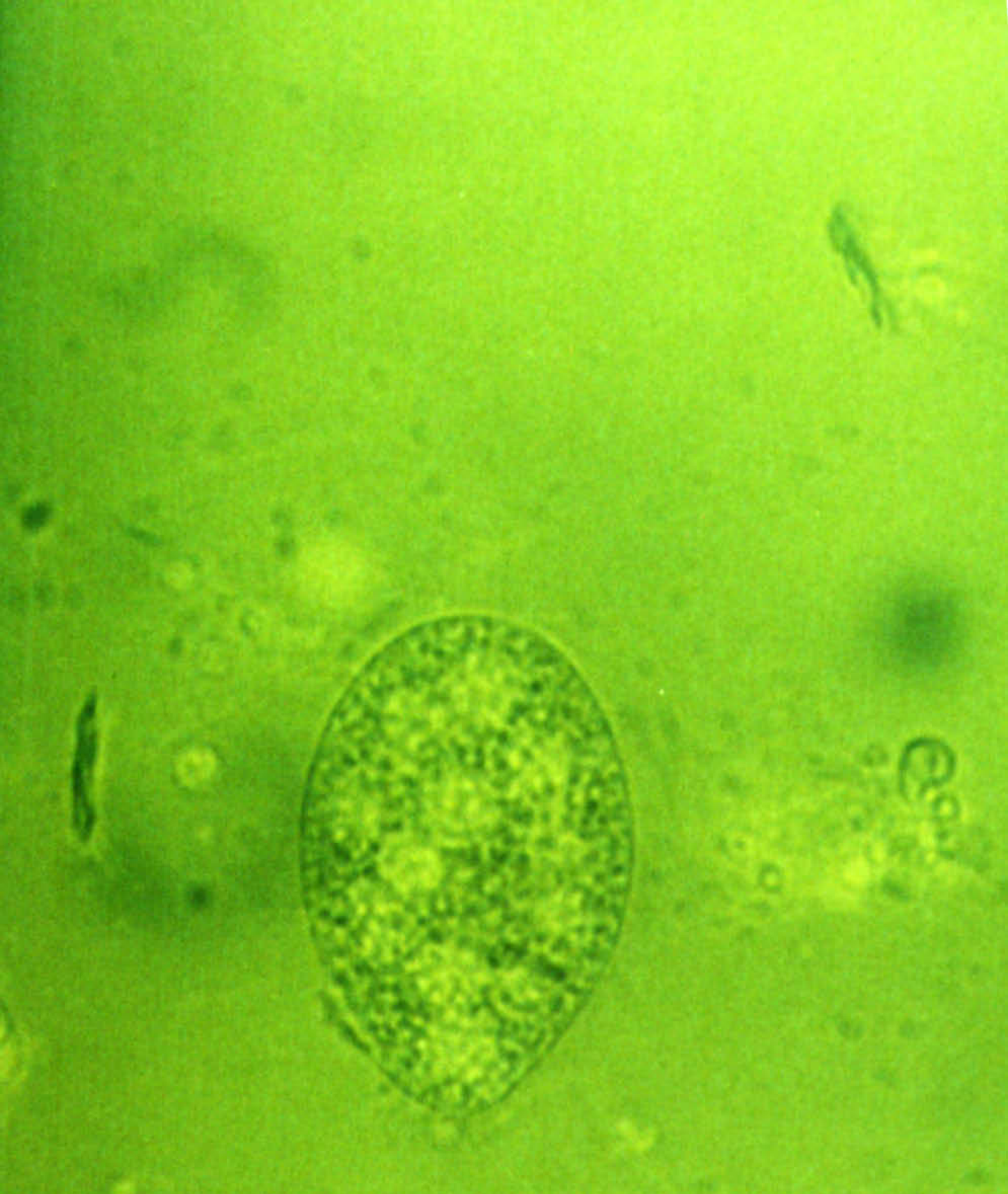


figure 32a

p120-122