AN EXAMINATION OF THE EFFECT OF SURFACE MODIFICATIONS ON THE PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES OF NON-IONIC SURFACTANT VESICLES.

by

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This thesis is presented in partial fulfilment of the requirement for the award of the degree of Doctor of Philosophy of the University of Strathclyde.

February 1990

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Scotland

Acknowledgments

I wish to thank my supervisor Professor A.T. Florence, formerly of the Department of Pharmacy, University of Strathclyde, Glasgow and now Dean of the School of Pharmacy, University of London, London for all his help, advice, encouragement and enthusiasm throughout this work.

All members of staff and postgraduate students in the Pharmaceutics Division of the Department of Pharmacy have my thanks for making my stay so enjoyable, especially since I did hail from "that other city". I am especially indebted to Dr. A.J. Baillie and Dr. G.W. Halbert who were of particular help in the latter stages of my work.

I am grateful to Professor S.B. Kaye of the CRC Department of Medical Oncology, University of Glasgow and his staff who all made me welcome during my stay at their department. In particular, I wish to mention Dr. Jim Cassidy, Gilmour Morrison, Albert Setanoians and Tom Hamilton for their expert help and technical assistance during my time within their laboratories.

I also wish to express my gratitude to Dr. Margaret Burgoyne, Senior Registrar, Pathology Department, Southern General Hospital, Glasgow for her assistance in preparing the fluorescent photomicrographs and for her helpful expertise and discussion in their interpretation.

I am indebted to L'Oreal, who funded this work, and in particular Mr.J. Whittaker and Dr. G. Vanlerberghe for their encouragement, advice and help, particularly during the two weeks I spent in their laboratories in France.

On a more social note, I feel I must mention all the "athletes" who frequented the University sports centre at lunchtimes especially

those who went running. I take many memories with me from our lunchtime burn-ups, and characters such as "wee Roy", Drew, Sam and the Ironman will never be forgotten.

My wife, Gwen, will have my lifelong thanks and gratitude for enabling me to undertake this work, for supporting me in all senses of the word and for putting up with me during all the ups and downs. Both my family and Gwen's family have helped and supported us during my Ph.D. and is something I am particularly grateful for.

Finally, my Mum and Dad, Harriet and Jack, deserve a special mention for all the opportunities they have given me. I shall never forget my Mum who was the inspiration behind undertaking this work, and in whose memory this thesis was written.

The following note was one of the last things written by my mother and expresses now she felt about her condition and how she saw other cancer patients dealing with their own situations.

To Colin.

Cancer is a dreaded word to many people. It seems that it is no longer the disease described by many people as a terminal illness. With modern drugs success has come a long way in the last 20 years. Radiotherapy and chemotherapy both have some unpleasant side effects such as hair loss and severe nausea. Other drugs such as Interferon have influenza type side effects as well as a few other minor ones. Leukaemia can be treated with tablets with practically no side effects. It appears that quite a few people are grateful for the treatments they receive while others are apparently not grateful. Cancer appears to be accepted by many people as an illness instead of the dreaded word it once was.

Love Mum

Description of Vesicles Prepared from Non-Ionic Surfactants

In earlier publications from this department the name "niosome" was given to vesicles prepared from non-ionic surfactants. However, since L'Oreal wish to restrict the use of the term Niosome® as a trade mark for their cosmetic products, the term niosome has not been used in this thesis. Throughout this thesis, vesicles prepared from non-ionic surfactant molecules have been called, simply, non-ionic surfactant vesicles, abbreviated to NSV's. Any reference to niosomes made in earlier publications from this department has been substituted in this thesis by the term NSV. The terms non-ionic surfactant vesicle (NSV) and niosomes should be regarded as being synonyms.

Abstract

The physicochemical properties of non-ionic surfactant vesicles (NSV's), formed from the non-ionic surfactants I, II and IV (monoalkyl and dialkyl polyglyceryl ethers), were examined. The effects of incorporating polyoxyethylene cholesteryl ethers and charged molecules into the vesicular bilayer on these properties were examined and their potential as drug delivery vehicles for doxorubicin (DOX) investigated.

The size and aqueous entrapment volume of vesicles were related to the preparation method, although charged molecules or increasing the hydrophilicity did affect both parameters.

The vesicular surface charge, due to adsorbed hydroxyl ions or incorporated charged molecules, was reduced as the polyoxyethylene chain length of incorporated cholesteryl ethers was increased. The vesicular charge of vesicles containing cholesteryl polyoxyethylene(24)ether (SOL24) was unaffected by the encapsulation of DOX but encapsulation of the drug in positively charged vesicles markedly affected the charge against pH profile.

Increasing the vesicular cholesterol content reduced the enthalpy of the phase transition, ultimately abolishing the peak between 33-50mol% cholesterol, while the peak shape was altered by the addition of charged molecules.

Vesicular aggregation was induced by NaCl, although incorporation of charged molecules or SOL24 to the vesicular bilayer prevented this flocculation.

DOX release from drug loaded vesicles was markedly reduced by the inclusion of cholesterol or stearylamine (SA) in the bilayer, while the incorporation of SOL24 produced a slight reduction. Empty vesicles containing SOL24 and SA molecules interacted with DOX

molecules; SOL24 produced an immediate and reversible interaction and SA produced a delayed but apparently irreversible interaction.

Administration of DOX loaded, SOL24 containing vesicles to mice reduced the peak cardiac concentration and produced a higher terminal plasma concentration compared with free drug. The co-administration of free drug with empty vesicles increased drug concentrations in the heart and kidney.

The intravenous injection of DOX loaded, SA containing vesicles into mice bearing a subcutaneously implanted ROS tumour gave inconclusive results due to analytical problems, although the injection of empty vesicles with free drug behaved similarly to free drug alone. Drug loaded vesicles were much less effective at retarding tumour growth than either of the free drug containing samples despite fluorescence photomicrographs showing the majority of the vesicles residing within the tumour vasculature.

Contents

1 Introduction

1.1 Liposomes - Preparation and Properties

1.1.1 Methods of Preparing Liposomes

Vesicles composed of phospholipids were first described by Bangham and Horne (1964) and were later termed "liposomes". Since then a vast volume of literature has been published on the subject.

When phospholipids are suspended in aqueous solution above the phase transition temperature (Tc) of the particular lipid, or above the Tc of the highest melting lipid in a mixture, liposomes form spontaneously. The structures which are formed are termed multilamellar vesicles (MLV's) where concentric lipid bilayer lamellae are separated by layers of aqueous phase and are likely to be several μm in diameter. The size distribution of dispersions of MLV's tends to be fairly wide, although this can be modified by altering the hydration time and degree of shaking for a given liposomal composition. Vigorous vortexing or longer gentle shaking of the dispersion produced vesicles with a greater number of lamellae (Szoka and Papahadjopoulos, 1980).

Many other techniques have been reported for the preparation of liposomes with differing sizes and aqueous entrapments. These include sonication of phospholipids to form small, homogeneous dispersion of liposomes (SUV's) which have a single lamella, a low aqueous entrapment and a size of 21.5nm to about 50nm (Huang, 1969; Huang and Thompson, 1974); ethanol injection, where a solution of the phospholipid in ethanol is slowly injected into a buffer solution producing a relatively dilute vesicular dispersion with entrapment volumes which are similar to sonicated samples and sizes between 30-110nm (Batzri and Korn, 1973); ether injection, where the lipids are dissolved in ether and injected into buffer to produce large

unilamellar liposomes (LUV's) with a size of between 150-250nm and a relatively high volume of entrapment (Deamer and Bangham, 1976); reverse phase evaporation, where liposomes are formed by the removal of the organic phase from a water-in-oil emulsion containing phospholipids and buffer in an excess of organic phase (Szoka and Papahadjopoulos, 1978). This results in the formation of large unilamellar liposomes, termed reverse-phase evaporation vesicles (REV's), which are capable of entrapping a large aqueous volume and may have sizes ranging from 80-900nm.

1.1.2 Liposomal Compositions

Liposomes have been prepared from a wide variety of phospholipids, both charged and uncharged and most contained a proportion of cholesterol.

1) Inclusion of Charged Species

A net surface charge may be imparted on the liposome by using lipids which were negatively charged over a wide pH range, such as phosphatidylserine, rather than using zwitterionic, neutral lipids such as phosphatidylcholine (Papahadjopoulos, 1968). Charged amphiphiles may also be incorporated into liposomal bilayers, up to a concentration of about 20mol%, with dicetyl phosphate or stearylamine producing negatively or positively charged vesicles respectively (Bangham et al, 1965; Papahadjopoulos and Watkins, 1967b). The inclusion of up to 10mol% of a charged molecule, either anionic or cationic, increased the distance between the lamellae of MLV's and hence the volume of aqueous phase entrapped (Bangham et al, 1967).

2) Inclusion of Cholesterol

Cholesterol may be incorporated into liposomal bilayers, up to a maximum of 50mol% (Ladbrooke et al, 1968) although it has been suggested by some workers that it could be included up to 2:1

cholesterol:phospholipid in highly sonicated systems (Horwitz et al. 1971). However, it seemed likely that when cholesterol was incorporated at these higher concentrations, some would be present as cholesterol crystals rather than being completely incorporated into the liposomal bilayer (Tyrrell et al, 1976).

The inclusion of cholesterol also had some important effects on the physical properties of liposomes. Addition of cholesterol reduced the permeability to solutes of the liposomes produced (Scarpa and de Gier, 1971) and this was thought to be caused by a reduction in the fluidity of the bilayer (Chapman and Penkett, 1966). For a given phospholipid, increasing the cholesterol content of SUV's appeared to increase their diameter (Johnson, 1973; Forge et al, 1978).

Cholesterol also affected the enthalpy of the measured phase transition of phospholipid vesicles. An increase in the cholesterol content of the vesicles reduced the enthalpy of the phase transition although the midpoint of the transition remained the same (Hinz and Sturtevant, 1972), while the addition of between 33mol% (Kimelberg and Mayhew, 1978) and 50mol% (Ladbrooke et al, 1968) cholesterol abolished the transition.

1.1.3 Entrapment of Molecules within Liposomes

Since liposomes contain both hydrophilic and hydrophobic regions, they are capable of entrapping both hydrophilic and lipophilic molecules. The hydrophilic molecules are entrapped in the aqueous spaces of the vesicle, the lipophilic molecules occupy the lipid areas and amphipathic molecules can insert their hydrophobic portions into the lipid bilayer and their hydrophilic portions into the aqueous phase (Gregoriadis, 1976).

1.1.4 Potential Uses of Liposomes

A wide variety of substances have been encapsulated in liposomes, with an even wider range of vesicular compositions used to encapsulate them. They have been used to entrap chelating agents to remove heavy metals from tissues (Jonah et al, 1975; Rahman et al, 1973; Rahman et al, 1974a), to deliver enzymes in storage diseases (Ryman et al, 1978; Gregoriadis and Buckland, 1973), to encapsulate anticancer drugs such as actinomycin D (Gregoriadis and Neerunjun, 1975; Rahman et al, 1974b), cytosine arabinoside (Mayhew et al, 1976; Mayhew et al, 1978), methotrexate (Kimelberg, 1976) and doxorubicin (Olson et al, 1982; Forssen and Tokes, 1979; Rahman et al, 1986; Sells et al, 1987), and cortisol to reduce inflammation of arthritic joints (Shaw et al, 1976). More recently, amphotericin B has been encapsulated in liposomes and administered as an intravenous infusion to cancer patients with fungal infections, with none of the common side-effects generally observed after infusion of the colloidal suspension of amphotericin B evident (Sculier et al, 1988). Cyclosporin and insulin have been co-encapsulated (Stuhne-Sekalec et al, 1986) and the effect of encapsulation of anti-inflammatory steroids on ocular delivery examined (Taniguchi et al, 1988). Other reported uses for liposomes include their ability to produce an immune response using surface coupled antigens (Latif and Bachhawat, 1987) and their effect on the stability of indomethacin suspensions (Law et al, 1987). It can thus be seen that the potential uses of liposomes is extensive and that the list is by no means exhausted thus far.

1.2 Liposomes *In Vivo*

1.2.1 *In Vivo* Fate of Administered Particles

The most common method of administration of liposomes and other particulate carriers is by intravenous injection, and after such administration their fate appears to be largely determined by their size and surface properties.

The type of capillary endothelial barrier found within various tissues often provides a barrier to the entry of vesicles. Continuous endothelial cells are the most widely distributed type found in skeletal, smooth and cardiac muscle, lung, gonads and the pancreas and contain tight junctions and a continuous basement membrane making it virtually impermeable to particulate material. Fenestrated endothelium contains pores which permit the passage of particles less than about 40nm. It contains a continuous basement membrane and is found in renal glomeruli, exocrine and endocrine glands and the gastro-intestinal tract. Sinusoidal endothelium has abundant pores with gaps of about 150nm and is found in liver, spleen and bone marrow. The basement membrane is absent in the liver, and is interrupted in the spleen and bone marrow (Tomlinson, 1987).

1.2.2 Liposomal Distribution after Intravenous Injection

Particulate carriers circulating in the cardiovascular system are generally cleared quickly by the mononuclear phagocyte system (MPS), although the rate of clearance is dependant on the particle size, dose, surface charge, the nature of the particle matrix and particle stability. The MPS includes the Kupffer cells of the liver and the phagocytic cells of the spleen and bone marrow. The macrophages of this system engulf a wide variety of particles through non-specific processes and after adsorption of opsonins onto the particle (Lehnert and Tech, 1985). These opsonins are bifunctional

serum factors which promote phagocytosis by binding to both the particles and the phagocytic cells (Chudwin et al, 1985). It has been shown that α_2-macroglobulin was the blood protein most strongly associated with liposomes in rats (Black and Gregoriadis, 1976).

When small liposomes with diameters much less than 100nm were injected intravenously, they were capable of reaching, and of being internalized by, the parenchymal cells of the liver i.e. the hepatocytes, showing their capacity to penetrate the fenestrations of the endothelial cells lining the liver sinusoids (Scherphof et al, 1987). Large liposomes with diameters well over 100nm were taken up exclusively by the Kupffer cells as far as hepatic uptake was concerned (Scherphof et al, 1987), and removal of MLV's and LUV's from the bloodstream was rapid, particularly by macrophages of the liver and spleen (Roerdink et al, 1981; Roerdink et al, 1984). Uptake by macrophages tended to be by endocytosis with the liposomes finally localising in the lysosomal compartments of the cells where they were degraded by lysosomal (phospho)lipases and the liposomally encapsulated contents released (Dijkstra et al, 1984; Dijkstra et al, 1985).

The clearance of liposomes from blood was shown to be inversely proportional to the vesicle size for six different vesicle sizes within the range 0.4-0.05μm (Magin et al, 1986). Blood clearance of heterogeneous multilamellar preparations showed complex kinetics with a rapid and slow phase of removal, while unilamellar liposomes were cleared by simple exponential clearance (Juliano and Stamp, 1975). These workers also found that uncharged and positively charged unilamellar liposomes were cleared less quickly from the bloodstream than negatively charged vesicles.

In general, intravenous injection of liposome preparations does

not result in extensive accumulation of the vesicles in the lungs. However, when the size of the liposomes was greater than 1μm, increased localization did occur compared with smaller diameter preparations (Sharma et al, 1977; Hunt et al, 1979: Fidler et al, 1980) and this has been attributed to the greater physical trapping of the vesicles in the capillary network of the lungs (Hunt et al, 1979). The accumulation in the lungs of liposomes bearing either a positive (Jonah et al, 1975) or negative (Fidler et al, 1980) surface charge appeared to be greater than that seen with uncharged liposomes of the same size.

The tissue distribution of multilamellar, negatively charged, ^{99m}Tc labelled liposomes in cancer patients was examined and it was found that the vesicles localized in the liver (44.5%), spleen (25.5%), lung (14.5%) and bone marrow (Lopez-Berestein et al, 1984). Although the liver took up a greater percentage of the radioactivity, it was found that for these liposomes, the spleen retained vesicles at a higher density. In all cases the injection of liposomes produced no side effects.

1.2.3 The Effect of the Liposomal Composition on Distribution

The composition of the injected liposomes also had some bearing on their ultimate fate. After intravenous injection of MLV's in rats, cholesterol-containing liposomes were found to be eliminated less rapidly and taken up to a lesser extent by liver macrophages than by cholesterol-free liposomes. Cholesterol-containing liposomes also appeared to be more resistant to intracellular degradation in vivo and to intracellular uptake by Kupffer cells and lysosomal degradation in vitro (Roerdink et al, 1989). Moghimi and Patel (1988) showed that in vitro and in the presence of serum,

Kupffer cells had a greater affinity for cholesterol-poor liposomes, whereas splenic phagocytic cells had a greater affinity for cholesterol-rich liposomes. They suggested that there were serum opsonins specific for hepatic and splenic phagocytic cells and that these opsonins had differing affinities for cholesterol-rich and cholesterol-poor vesicles.

The effect of a positive surface charge on the interaction of unilamellar liposomes, with diameters of 100nm and 160nm, with rat peritoneal macrophages was examined by Schwendener et al (1984). They found that increasing the amount of stearylamine from 0-25mol% increased the amount of vesicle-cell interaction and that although the number of smaller vesicles associated with cells was 100 times more than the larger vesicles, the larger vesicles introduced 10 times more aqueous phase to the cells. They also noted that at a concentration of 15mol% stearylamine there was 90% cell viability, but this was reduced to less than 50% when the stearylamine concentration was increased to greater than 20mol%. Cell damage induced by stearylamine has also been reported by Yoshihara and Nakae (1986) and Yoshihara et al (1987).

1.2.4 Methods of Avoiding the Mononuclear Phagocyte System (MPS)

Attempts have been made to increase the blood residence time of liposomes and to divert the vesicles away from the cells of the MPS.

1) Saturation of the MPS

One method of altering the tissue distribution of vesicles involved saturating the MPS with empty liposomes. However, Gregoriadis et al (1977) found that despite altering the tissue distribution of the liposomes and depressing the function of the MPS, liposomal delivery to tumour cells was not increased. Suppression of the MPS often led to particles being redirected to the lungs

(Bradfield, 1984) and although it was possible to reversibly block the MPS, repeated injection of liposomes did lead to its long-term paralysis (Allen et al, 1984).

2) Alterations in Opsonization

Other methods of changing the tissue distribution have centred on altering opsonization of the vesicles. Phagocytosis of liposomes and other particles is preceded by opsonization, adherence to cells followed by ingestion. The factors which enable opsonization to occur include electrostatic interactions, steric forces and hydration forces and a sufficiently low potential energy barrier between surfaces must also exist to allow them to approach sufficiently close to interact (Tomlinson, 1987). Thus, it has been shown that particles coated with positively or negatively charged macromolecules may have altered tissue distribution (Wilkins and Myers, 1966) and the formation of a high potential energy barrier could be created by forming a sterically stabilized surface using hydrated, hydrophilic polymers (Napper and Netschey, 1971).

The use of sterically stabilised coatings to reduce opsonization has been tried for both enzymes (Abuchowski and Davis, 1981) and particulate carriers.

Illum et al (1986) coated polystyrene microspheres (60nm in diameter) with a variety of hydrophilic materials such as secretory immunoglobulin A (SIgA), egg lecithin and the non-ionic surfactants, poloxamer 188 and 338 and found it was possible to divert the microspheres away from the liver after intravenous injection of the particles in rabbits. The most marked reduction in liver uptake was seen with a poloxamer 338 coating, and this reduction was accompanied by an increased uptake by the bone marrow. They also found that, in vitro and in the presence of serum, the poloxamer 338 coated

microspheres retained their ability to avoid phagocytosis, while those coated with poloxamer 188 did not. It was suggested that this difference was due to the the large anchoring hydrophobic group of the poloxamer 338 being resistant to displacement from the particle surface by plasma proteins. Both of the poloxamer surfactants contained identical hydrophilic groups and it was these groups which prevented cell adhesion and phagocytosis i.e. steric stabilization.

Further investigations by these workers have shown that 60nm polystyrene microspheres coated with the non-ionic block copolymer poloxamine 908 could be retained in the vascular compartment of rabbits after intravenous injection with little evidence of uptake by liver or bone marrow (Illum et l, 1987). Coating the polystyrene microspheres with poloxamer 407, it was possible to avoid liver and spleen uptake with much of the particulate material being localized in the bone marrow (Illum and Davis, 1987).

The uptake and distribution of 126nm poly(butyl 2-cyanoacrylate) nanoparticles coated with poloxamer 338 or poloxamine 908, after intravenous injection in rabbits, were found not to be significantly altered compared with uncoated nanoparticles (Douglas et al, 1986). Coating the nanoparticles with the poloxamer/poloxamine did produce a slight delay in the liver and spleen uptake, but the final uptake was not significantly different to that of the uncoated particles. Several reasons were postulated for this apparent failure to avoid uptake by the MPS. The surface of the uncoated nanoparticles provided a strong steric barrier due to the surface layer of covalently linked dextran and it was postulated that the addition of a further stabilizing agent may not increase further the overall stabilizing effect. The action of serum proteins on the surface of the adsorbed surfactant layer and the degradation of the

nanoparticles in vivo may also result in a loss of the surfactant coating at the particle surface.

The sorption of poloxamers onto liposomes has also been attempted (Jamshaid et al, 1988). These workers found that maximum sorption of the poloxamer onto the liposomes was achieved after 48h. incubation, and that the adsorbed layer thickness was less for liposomes than for polystyrene microspheres. This was thought to be due to some degree of penetration of the poloxamer into the outer bilayer of the liposomes. The electrophoretic mobility of the liposomes was reduced after addition of the polymer coating and it was suggested that this was due to the polyoxyethylene groups projecting from the liposomal surface. The efflux of a water soluble marker was increased by coating the particles with the polymer, and this increase was much greater with SUV's than MLV's. The inclusion of equimolar cholesterol in the liposomal bilayer was shown to reduce the efflux slightly.

3) Liposomal Phospholipids Used

It has also been found that the phospholipids used to prepare liposomes could influence the rate at which the vesicles were removed from the bloodstream by the MPS, using a constant vesicle size of approximately 100nm (Gabizon and Papahadjopoulos, 1988) and that this reduction in uptake by the MPS allowed greater accessibility to other body compartments such as implanted tumours. Interestingly, the three liposome compositions having the longest blood residence times were all prepared with negatively charged phospholipids, something which had previously been thought to reduce the length of time the liposomes circulated in the blood (Juliano and Stamp, 1975; Illum and Davis, 1983). The explanation postulated for this was that in the liposomes used by Gabizon and Papahadjopoulos (1988) the negative

charge of the phospholipid was shielded by a large, bulky hydrophilic group, whereas the negatively charged groups of phosphatidic acid and dicetyl phosphate molecules were exposed.

Large unilamellar liposomes, containing gangliosides and sphingomyelin, were also shown to have an increased blood residence time and a reduced uptake by macrophages in vivo (Allen and Chonn, 1987).

From the above work, it can be appreciated that it may be possible sometime in the near future to prepare liposomes capable of avoiding detection and removal by the MPS, thus opening up the possibility of directing the liposomes to specific tissues outwith the MPS.

1.2.5 Other Routes of Liposomal Administration

The intravenous route has been by far the most documented method of administering liposomes although other routes of administration have been reported for liposomes and other carrier systems.

Intraperitoneal injection of liposomes has been attempted and after such administration some liposomes were recovered from the liver and spleen. Liposomes were likely to end up in these tissues by initially being transported from the peritoneal cavity to the circulation via the lymphatic system and then to the liver and spleen (Dapergolas et al, 1976). Some degree of targeting of liposomes can be attained by this method of directly injecting the vesicles into the anatomical space of interest (Myers, 1984).

The fate of liposomes injected intramuscularly into the hind legs of rats appeared to be dependant on their sizes. Large liposomes were retained at the site of injection and slowly degraded, while after injection of small liposomes, some of the vesicles

entered the circulation and were transported to the liver and spleen (Dapergolas et al, 1976).

Injection of methotrexate-loaded liposomes into the cerebrospinal fluid of cynomolgus monkeys was carried out by Kimelberg et al (1978) who found that encapsulation of the drug produced increased drug levels in the central nervous system after one and four days, and also reduced drug metabolism.

A subcutaneous injection of liposomally entrapped cytarabine produced a slow drug release from the skin of mice and a single dose was often curative for mice with systemic leukaemia (Kim and Howell, 1987). When doxorubicin (DOX) in solution was inadvertantly extravasated i.e. given as a subcutaneous injection, the area quickly became irritated and ulcerated. If the drug was encapsulated in liposomes no ulceration was produced and the irritation was much less marked (Balazsovits et al, 1989).

Liposomes have also been injected intra-articularly in rabbits with experimental arthritis. It was found that liposomally entrapped steroids reduced the temperature and size of the joint to a greater degree than when the steroids were administered as free solutions (Dingle et al, 1978).

Oral administration of liposomal suspensions has also been attempted. In vitro, a substantial loss of entrapped glucose and carboxyfluorescein from liposomes was observed under conditions similar to those likely to be found in the gastro-intestinal tract. Intact liposomes did not appear to be taken up by epithelial cells and the liposomal entrapment of non-absorbable drugs did not enhance the uptake of the drug (Chiang and Weiner, 1987a). In rats, the oral administration of liposomes containing the orally non-absorbed marker PEG-4000, did not facilitate uptake of the marker (Chiang and

Weiner, 1987b). From these two studies it was suggested that liposomes showed little promise as oral drug delivery systems, and that the increased bioavailability of drugs noted in other studies may be due to factors other than liposomal uptake.

A method of maintaining renal DOX levels was reported by Kerr et al (1988b). These workers prepared DOX loaded microspheres of between 15-20μm diameter and after intrarenal arterial administration the microspheres became trapped within capillaries and arterioles of the kidney. This reduced the systemic exposure to the drug while maintaining intrarenal drug levels.

1.2.6 Problems Associated with the Storage and Handling of Phospholipids and Liposomes

The formation of liposomes can be easily accomplished by any of the methods previously described. However, to ensure that the batch to batch variation of the liposomal properties is kept to a minimum, several precautions in the handling and storage of the lipids and liposome suspensions must be taken. The purity of the lipids used to form liposomes must be determined since commercial sources of phospholipids vary in their purity and very small amounts of free fatty acids (Kantor and Prestegard, 1975) or lysophosphatides (Smolen and Shohet, 1974; Teige et al, 1974) can have a marked effect on the surface charge and permeability of the vesicles produced. In order to minimize the risk of oxidation of the phospholipids during storage and the preparation of liposomes, the lipids should always be stored and handled under an inert gas such as argon or nitrogen and powdered phospholipids should be stored at -20°C (Szoka and Papahadjopoulos, 1980). Furthermore, to obtain pure, powdered phospholipids from commercial sources is expensive and this may affect the commercial viability of some potentially useful liposomal preparations.

1.3 Other Vesicular Forming Compounds

1.3.1 Non-Phospholipid, Vesicle Forming Molecules

The problems which are likely to be encountered in the handling of phospholipid molecules and liposome samples prompted investigations into other types of molecules which have the ability to form vesicles with similar properties to liposomes, but can be produced easily in a pure form. These molecules would hopefully be less expensive to produce and less liable to degradation compared with the phospholipid molecules.

1.3.2 Properties Required for Molecules to form Vesicles

It was originally thought that the only difference between phospholipids that formed bilayers and those that formed micelles was that vesicle forming phospholipids contained two hydrocarbon chains while those that formed micelles had only one (Israelachvili *et al*, 1976), although some single chained lipids with very small uncharged headgroups were capable of forming vesicles (Israelachvili *et al*, 1980).

In a more detailed investigation into the factors affecting amphiphile aggregation into larger structures, Israelachvili *et al* (1980) proposed a parameter, the "critical packing parameter" (CPP), the value of which indicated the structures likely to be formed. The CPP was based on the physical dimensions of the molecule used, and was defined as;

$$CPP = v/a_o.l$$

where v = volume of the hydrocarbon chain

a_o = optimum surface area occupied by the head group

l = length of the hydrocarbon chain

The values likely to be obtained for the CPP are shown in Table 1.1 and from this it can be seen that molecules giving CPP values of $^1/_3$–$^1/_2$ are most likely to produce micelles, while a CPP of $^1/_2$–1 should result in the formation of vesicles.

Table 1.1. The relationship between the CPP calculated for molecules, their most probable molecular shape and the type of structure formed for different types of lipid molecules.

Lipids	CPP	Critical packing shape	Structures formed
Single chained lipids with large headgroup areas.	$<^1/_3$	Cone	Spherical micelles
Single chained lipids with small headgroup areas.	$^1/_3$–$^1/_2$	Truncated cone or wedge	Globular or cylindrical micelles
Double chained lipids with large headgroup areas. Some single chained lipids with very small uncharged headgroups.	$^1/_2$–1	Truncated cone	Flexible bilayers or vesicles
Double chained lipids with small headgroup areas.	~1	Cylinder	Planar bilayers
Double chained lipids with small headgroup areas. Cholesterol.	>1	Inverted truncated cone	Inverted micelles

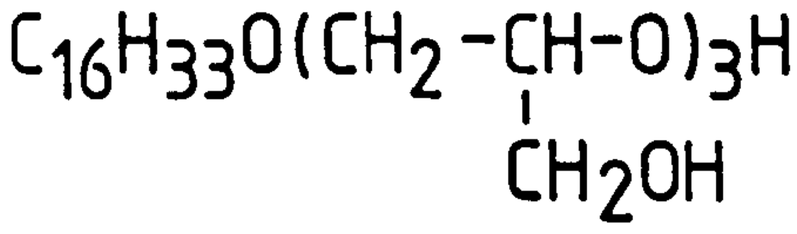
– from Israelachvili et al (1980).

1.3.3 Mono/Dialkyl Glycerols

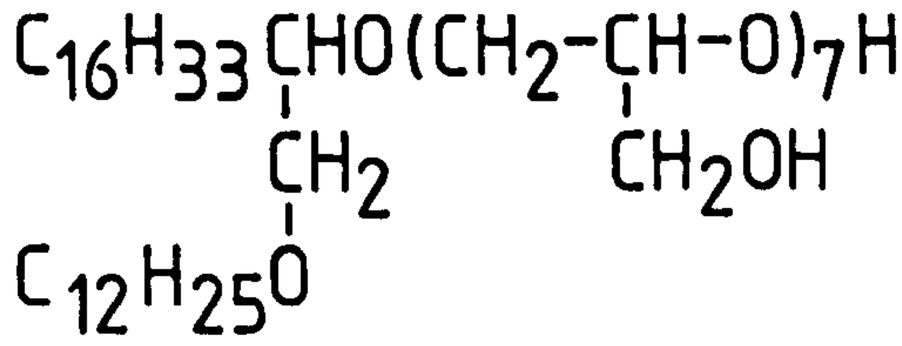
One of the earliest reports of vesicles being formed from non-phospholipid molecules was made by Vanlerberghe et al (1972) using non-ionic surfactants. They noted that alkylglycerol molecules, both monoalkyl and dialkyl, were capable of forming vesicular structures when subjected to similar methods of preparation to those used for liposomes. The physical characteristics of the surfactants used (Fig. 1.1) correspond well with the description given by Israelachvili et al (1980) for phospholipids likely to form vesicles. Surfactant I has a monoalkyl chain and has a fairly small uncharged headgroup, while Surfactant II is a dialkyl moleccle with a much larger, uncharged headgroup.

Since these initial findings by Vanlerberghe et al (1972; 1978), there have been several reports of uses for vesicles prepared from these surfactants. Handjani-Vila et al (1979) reported a simple method of producing creams which incorporated vesicles prepared from these non-ionic surfactants.

Non-ionic surfactant vesicles (NSV's), were prepared by several different methods and their in vitro behaviour examined by Baillie et al (1985). They found that these NSV's behaved in a similar way to liposomes with regard to the entrapment volumes obtained and ease of preparation. The release of 5(6)-carboxyfluorescein from these vesicles was found to have a fast initial phase over the first 4h followed by a slower release, with the inclusion of cholesterol reducing the rate of release in both phases. Electron micrographs taken of the NSV's prepared by the hand shaking, ether injection and sonication methods showed that the vesicles appeared similar to liposomes prepared by similar methods. Hand shaking NSV's were large and had many lamellae, ether injection vesicles were generally



Surfactant I



Surfactant II

Fig. 1.1 The chemical structure of two of the vesicle forming, non-ionic surfactants reported by Vanlerberghe et al (1972; 1978). The number of glycerol units in the hydrophilic headgroup is an average of 3 or 7 in Surfactants I and II respectively.

unilamellar and were relatively large, with large aqueous entrapment, and sonicated vesicles were small and unilamellar.

These NSV's have also been used *in vivo*, encapsulating several different drugs, and the results obtained suggest that these vesicles behaved similarly to liposomes *in vivo*.

Encapsulation of methotrexate resulted in a prolonged drug plasma level and reduced evidence of metabolism, when compared with free drug (Azmin *et al*, 1985; Azmin *et al*, 1986) and was similar to the picture seen after injection of drug loaded liposomes (Kimelberg and Atchison, 1978).

Another anticancer agent, doxorubicin (DOX), has also been encapsulated in NSV's and examined both *in vitro* and *in vivo*. Rogerson *et al* (1987) found that encapsulation of DOX reduced the light induced degradation of the drug, that increasing the proportion of cholesterol in the bilayer of the vesicle reduced the efflux of the drug and that only chemically intact drug was released from the NSV's. When drug loaded NSV's were injected intravenously *in vivo*, the distribution of the drug was altered (Rogerson *et al*, 1986; Kerr *et al*, 1988a; Rogerson *et al*, 1988). In all cases, the peak plasma concentration measured was increased, the plasma half-life was extended and the drug metabolism profile altered. The antitumour effect of the encapsulated drug was maintained and cardiac uptake of the drug was reduced by encapsulation (Kerr *et al*, 1988a; Rogerson *et al*, 1988).

Another area in which these vesicles have been used was in the treatment of experimental models of the parasitic disease, Leishmaniasis, by encapsulation of the antimony containing compound sodium stibogluconate (Hunter *et al*, 1988; Carter *et al*, 1989). These workers found that the NSV's behaved in a similar manner to

liposomes in suppressing the parasite burden in the liver, spleen and bone marrow, although there were subtle differences between the actual effects of the NSV's and liposomes at the various sites of infection.

1.3.4 Quaternary Ammonium Compounds

Many other synthetic amphiphiles have now been shown capable of producing vesicular structures.

Quaternary ammonium salts were shown to form bilayers by Kunitake and Okahata (1977). Using double chained ammonium salts (Fig. 1.2) as purely synthetic analogues of the natural phospholipids they found that bilayers were formed spontaneously in water if the alkyl chain length was between C_{10} and C_{20}. Double chained amphiphiles containing anionic groups such as sulphonate, phosphate and carboxylate have also been shown to produce bilayers (Kunitake and Okahata, 1978; Mortara et al, 1978).

The preparation and characterization of two double chained surfactants was reported by Jaeger and Golich (1987). They found that these surfactants (Fig. 1.3) were capable of forming multilamellar and small unilamellar vesicles which could entrap 5(6)-carboxyfluorescein. The release of the entrapped contents was shown to occur rapidly at 20°C, with about 20% of the entrapped contents released within 5min. The interesting point about these vesicles was that the surfactants were hydrolysed in both acidic and alkaline conditions which may be of potential use where a pH controlled delivery of the vesicles was required.

1.3.5 Cholesterol Based Surfactants

Several groups have attached hydrophilic moieties to cholesterol, through the -OH group at position 3, and have shown that

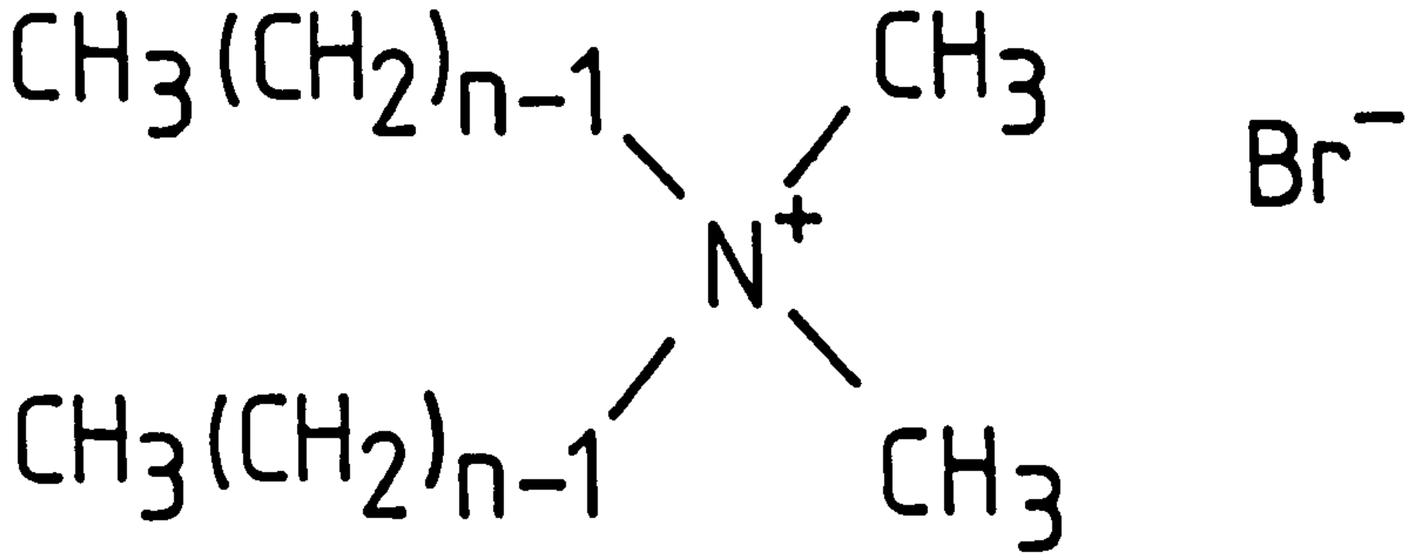
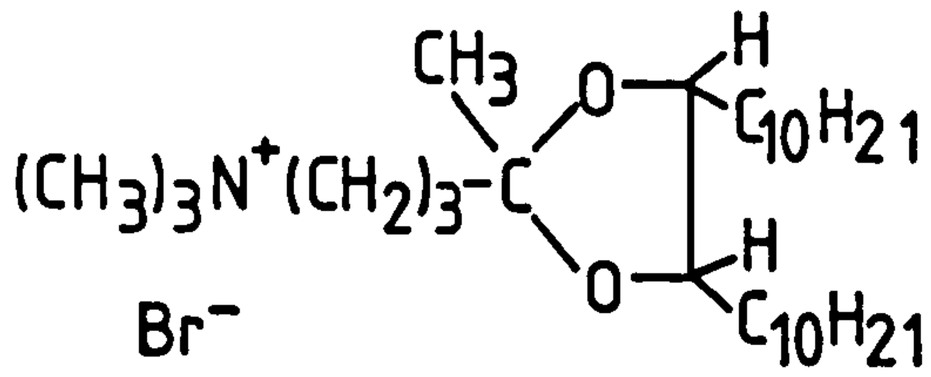


Fig. 1.2 An example of the class of quaternary ammonium compounds which were shown to be vesicle forming by Kunitake and Okahata (1977) when the hydrocarbon chain length was between C_{10} and C_{20}.



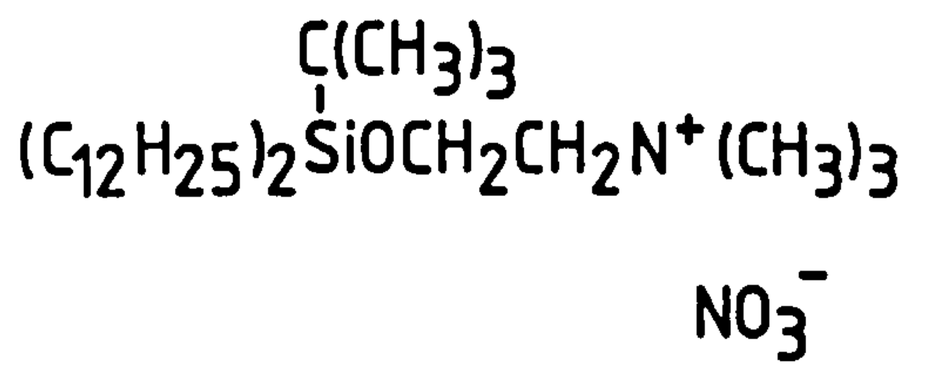


Fig. 1.3 The chemical structure of the two double chained, destructible surfactants prepared by Jaeger and Golich (1987) which were capable of forming vesicular systems.

vesicles can be produced which have a hydrophobic region which is entirely composed of cholesterol backbones.

Brockerhoff and Ramsammy (1982) were able to produce multilamellar vesicles with the cholesterol analogues, cholesterol-sulphate (cholesterol-SO_4) and 3-O-methoxyethoxyethoxyethylcholesterol (cholesterol-PEG) and small unilamellar vesicles by including equimolar cholesterol with cholesterol-PEG, cholesterol-SO_4 and cholesterolphosphocholine (cholesterol-PC). The multilamellar vesicles were prepared by sonication of lipid films for 10min and unilamellar vesicles by sonication of the film for 4h. The systems produced by the compounds alone were not predictable, due to the insolubility of the cholesterol-SO_4 and the formation of gels rather than vesicular dispersions when cholesterol-PC was used. All the multilamellar vesicles produced were shown to be osmotically sensitive and the sizes of the small unilamellar vesicles were similar to those measured for liposomes.

Patel et al (1984) also produced vesicles using a cholesterol derivative. In this case triethoxycholesterol (Fig. 1.4) was prepared and shown to form stable, rigid, bilayer-like structures when prepared by the hand shaking or sonication methods. However, unlike liposomes, the sonication of these vesicles did not result in a reduction in the vesicular size, with unsonicated vesicles measuring 103 ± 48nm and sonicated vesicles 165 ± 59nm. After intraperitoneal injection of methotrexate encapsulated in unsonicated vesicles into mice bearing hepatoma ascites tumour, a doubling of the survival time was achieved, compared with mice which received free drug or no treatment.

Another example of the formation of vesicles by non-ionic

surfactants was given by Echegoyen et al (1988), using neutral crown ether compounds (Fig. 1.5). Aqueous dispersions of vesicles were produced by sonication of the melted compound. The vesicles produced were approximately 30nm in diameter and were shown to have entrapment volumes which were very similar to those of liposomes of similar diameter. The vesicles were also shown to be insensitive to the addition of NaCl or KCl, with no signs of osmotic shrinkage, contrary to the behaviour of vesicles containing polyglyceryl ethers (Azmin et al, 1985). Preliminary studies into the bilayer fluidity of the vesicles produced from the crown ethers, indicated that they were very rigid. The delicate balance between the hydrophilic and hydrophobic portions of the molecule in determining the type of structure ultimately produced was noted, since an 18 membered, headgroup ring produced a micellar system rather than the vesicular system produced by the 15 membered ether ring.

1.3.6 Polyoxyethylene Alkyl Ethers

Vesicle formation from alkylethers with attached polyoxyethylene chains of varying length was studied by Hofland et al (1988; 1989). A dialkyl, C_{12} molecule with eight ethylene oxide (EO) groups [$(C_{12})_2EO_8$] was found to produce lamellar-like structures, which were not vesicular, when prepared by the hand shaking method, but the addition of 40mol% cholesterol resulted in vesicles being formed. A monoalkyl molecule ($C_{12}EO_3$) was capable of producing vesicles by the hand shaken and sonication methods both alone and when cholesterol was added. The release of Na-fluorescein has been shown to be influenced by the amount of cholesterol in the bilayer and also by whether the bilayers were in a liquid or gel state (Hofland et al, 1988) while the electrolyte concentration and pH were shown to have little effect on the structure of the vesicles. Vesicles prepared

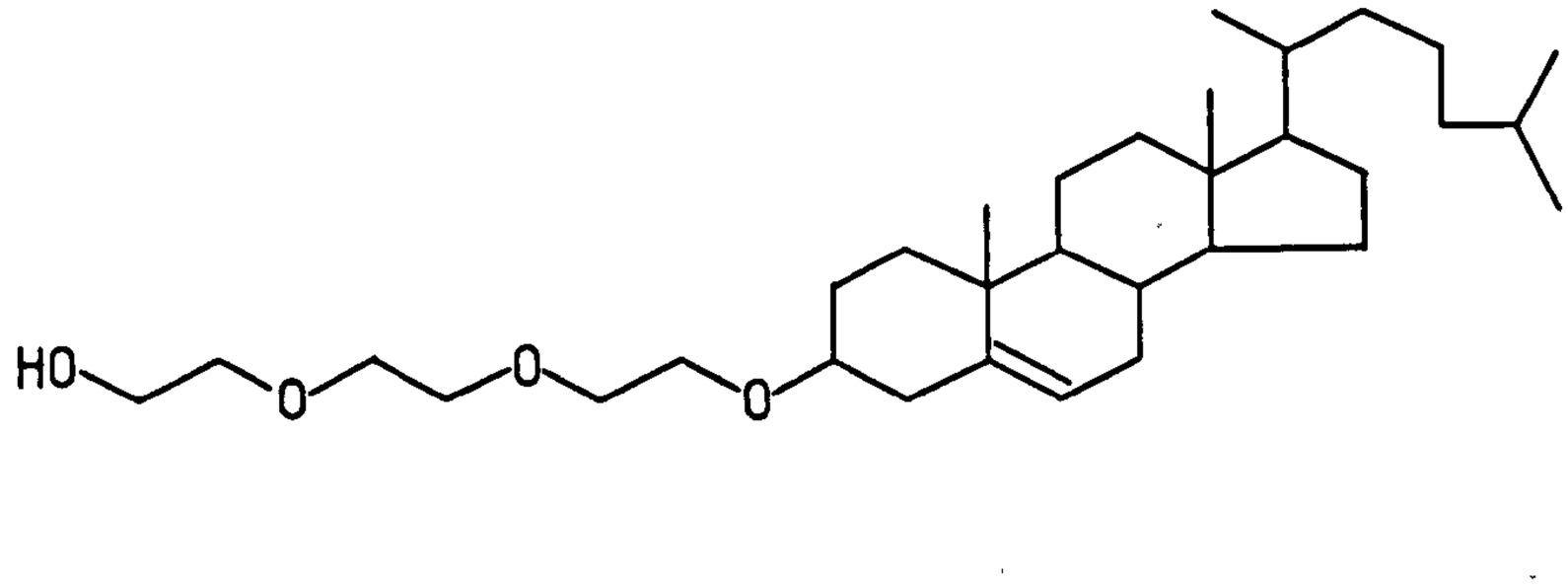


Fig. 1.4 A cholesterol based compound with an attached triethoxy group which can form vesicular systems as shown by Patel et al (1984).

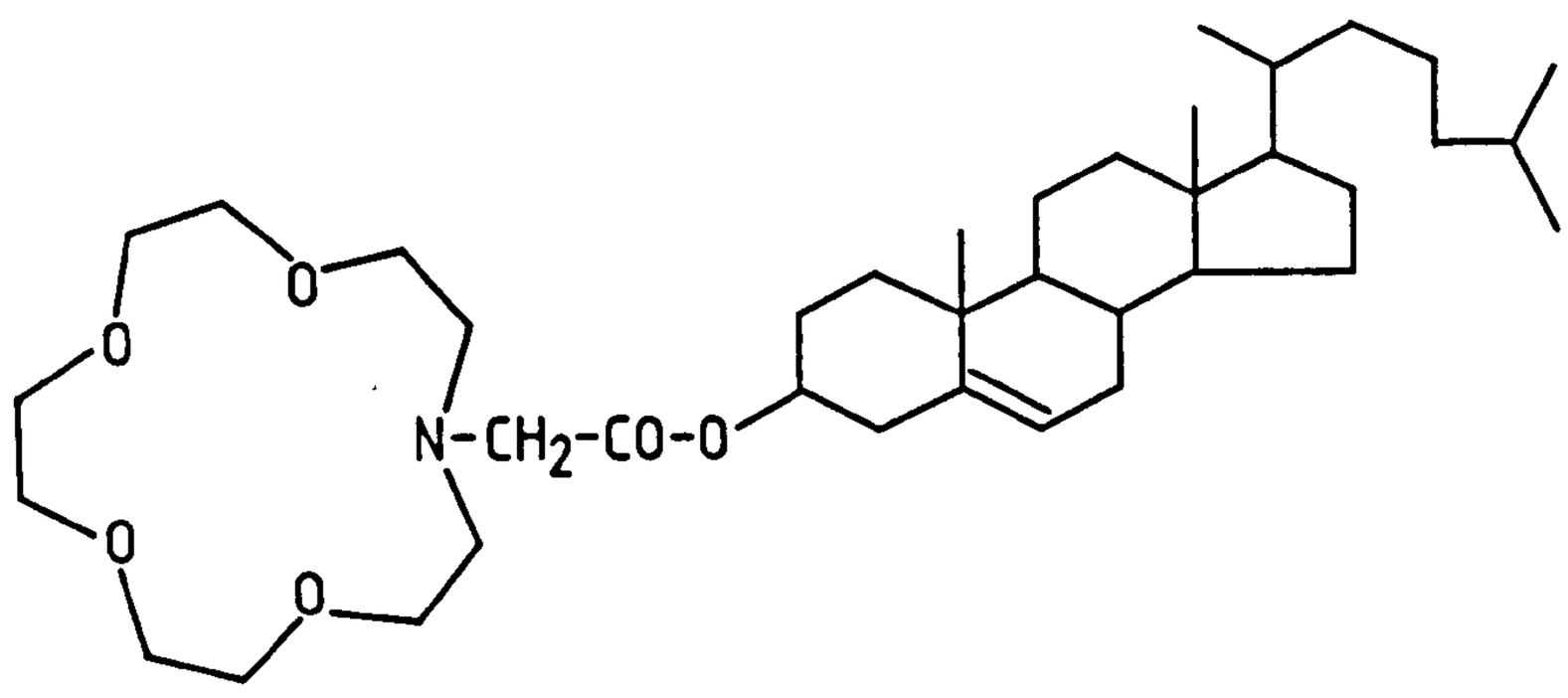


Fig. 1.5 The structure of one of the neutral crown ether compounds used by Echegoyen et al (1988) to prepare vesicular dispersions is shown. The molecule contains a cholesterol based, hydrophobic backbone with a hydrophilic crown ether headgroup.

from surfactants with low hydrophile-lipophile balance were shown to be less toxic to cultured keratinocytes and less ciliotoxic, these being used as models for transdermal and nasal delivery respectively (Hofland et al, 1989).

1.3.7 Future Potential of Non-Ionic Surfactant Vesicles

A wide range of non-ionic surfactants, with differing hydrophobic and hydrophilic portions, have now been shown capable of producing vesicular structures. These surfactants all appeared to be easier to handle than phospholipids and should be cheaper to produce. In almost all cases, the vesicles had similar properties to the much more extensively studied liposomes in that they were capable of entrapping and slowly releasing solutes and of forming multilamellar and unilamellar structures. The inclusion of cholesterol was essential for vesicular formation in some cases and reduced the rate of release of solutes from the vesicles produced. However, the use of non-ionic surfactants as vesicle forming compounds is still very much in its early stages and much still has to be done to examine the spectrum of physicochemical properties of the vesicles before their true potential as drug delivery vehicles can be fully determined.

1.4 Treatment of Tumours

1.4.1 Problems Associated with Treating Tumours

Many potential barriers to obtaining and maintaining an effective drug concentration in tumour cells exist, irrespective of whether the drug is delivered as a free drug solution, liposomally entrapped or administered by any other delivery system.

The aim of drug treatment in cancer chemotherapy is to treat malignant tumour cells with doses which allow the patient's vital cells e.g. bone marrow and gastro-intestinal cells to recover. In general, antitumour drugs are most useful against tumours with a high proportion of dividing cells i.e. they tend to be most effective against lymphomas and leukaemias. However, the most common types of tumours are the "solid" tumours e.g. colon, rectum, lung and breast tumours, all of which have a low proportion of dividing cells. Treatment of these tumours generally involves a combination of surgery, radiotherapy and chemotherapy, with surgery and radiotherapy being used to treat the primary or localised disease and chemotherapy used to control or eliminate metastatic disease (Pratt and Rudden, 1979).

Many of the characteristics of the tumour cells determine how it may most effectively be treated. The growth fraction of human tumours ranges from 20-70% depending on the tumour size and availability of oxygen and nutrients while the mass doubling time depends on the number of dividing cells rather than the total number of cells in the tumour and ranges from about 1 day in Burkitt's lymphoma to about 100 days in breast cancer.

The tumour size is another factor to consider, with large bulky tumours not generally being curable by chemotherapy alone, since 1) drugs may not be capable of penetrating into the tumour in sufficient

quantities to kill cells, 2) a larger percentage of the cells may be in their non-proliferative phase and may survive to re-establish the tumour mass, and 3) the larger the tumour, the longer the tumour is likely to have been present and the greater the chance it will have metastisized.

The position of the cell within the cell cycle is another important feature, especially for phase specific cytotoxic agents. The cell cycle is divided into a G_1 (intermitotic phase), S (DNA synthesis phase), G_2 (premitotic phase) and M (mitotic phase). Additionally, all normal adult cells and most tumour cells undergo a G_o phase (resting phase) in which there is a population of cells which are not proliferating (Fig. 1.6). Cell cycle dependant anticancer drugs act at one particular phase within the cycle, while cycle phase non-specific drugs are cytotoxic to cells at any point in the cycle (Table 1.2). Although some anticancer drugs are more effective against cells in one particular phase, all drugs are more effective against proliferating rather than non-proliferating cells (Pratt and Rudden, 1979).

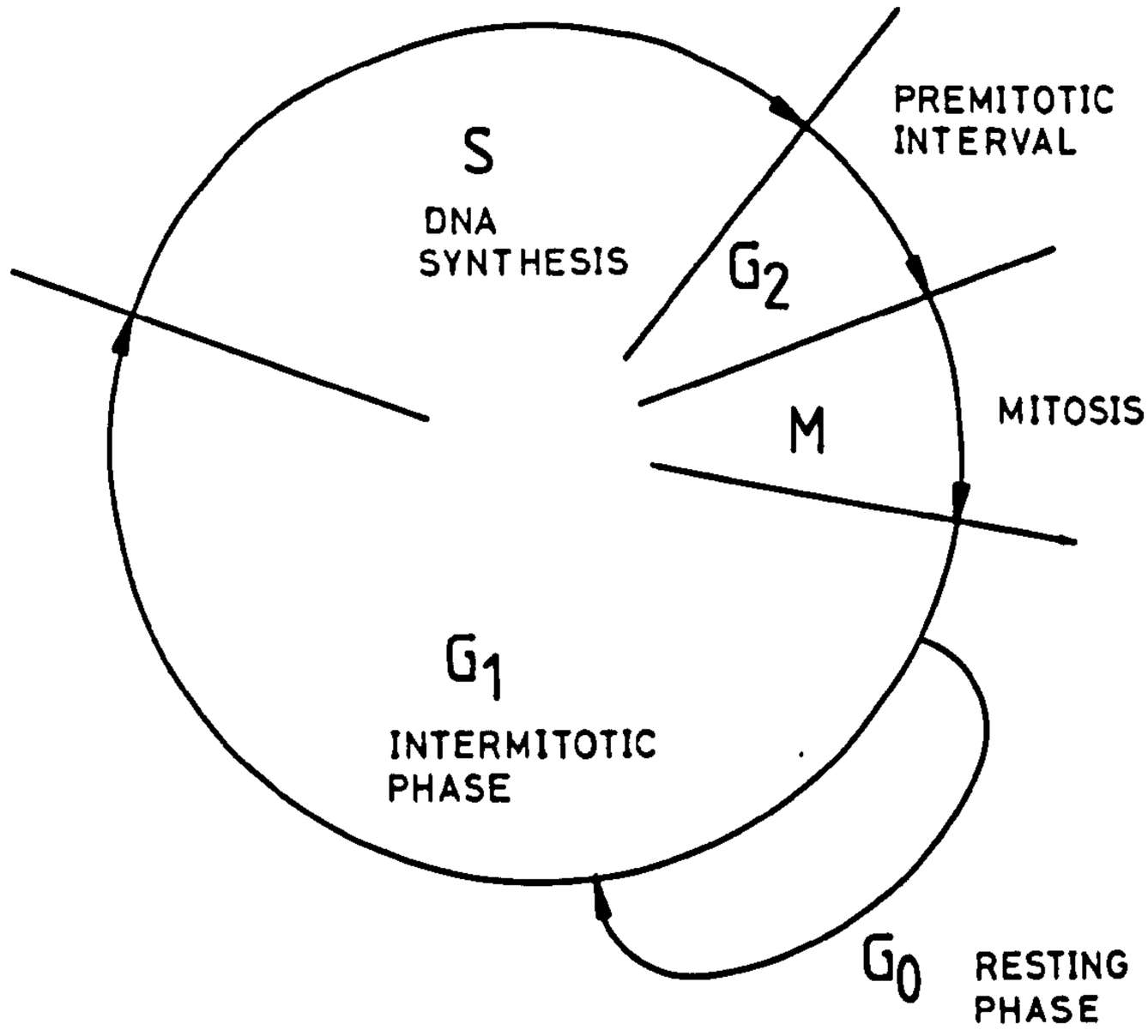


Fig. 1.6 The phases of the cell cycle are shown. The cycle is divided into G_1 (intermitotic phase), S (DNA synthesis phase), G_2 (premitotic phase) and M (mitotic phase). The population of cells not proliferating are in the G_o (resting phase) which may be a separate phase or a prolonged G_1 phase.

Table 1.2. Examples of anticancer agents which are specific for certain phases of the cell cycle and some of those which are phase non-specific.

S phase specific	- Cytosine Arabinoside
	- Hydroxyurea
S phase specific, self limiting	- 6-mercaptopurine
	- Methotrexate
M phase specific	- Vincristine
	- Vinblastine
Phase Non-specific	- Alkylating Agents
	- Nitrosoureas
	- Antitumour Antibiotics
	- Procarbazine
	- Cis-Platinum
	- Dacarbazine

1.4.2 Blood Perfusion of Tumours

The successful drug treatment of tumours is dependant on a drug concentration which is toxic to the tumours cells being attained within the tumour, and it is becoming increasingly apparent that poor penetration of drug into the tumour may be one aspect of cytotoxic drug resistance (Sutherland et al, 1979).

Tumours may obtain their vasculature from both newly formed vessels and by incorporation of vessels from the host tissue (Papadimitriou and Woods, 1974) although the arteriolar tumour vasculature tends to be deficient in smooth muscle and adrenergic innervation (Mattson and Peterson, 1981). Tumours release a substance called tumour angiogenesis factor (TAF), possibly in

response to hypoxia, which induces the growth of capillaries from the host tissue into the tumour and also initiates new growth which may continue until the host dies. Not all parts of a solid tumour become well vascularised and tumour growth may occur at a faster rate than capillary growth leading to a tumour with a necrotic centre of dead or dying cells surrounded by a layer of dormant cells and an outer rim of dividing cells (Pratt and Rudden, 1979).

1.4.3 Increasing the Penetration of Cytotoxic Drugs

In vitro studies of the effects of anticancer drugs on tumour cells have been undertaken using both monolayer and spheroid systems. Increasing the uptake of cytotoxic drugs into tumours has been attempted by using penetration enhancers and Kerr et al (1987) found that the addition of the non-ionic polyoxyethylene lauryl ether surfactant (Brij 30) increased the uptake of DOX into both monolayer and spheroid tumour cells.

When Tween 80 and DOX were coadministered into the peritoneal cavity of mice, increased plasma drug concentrations were obtained. This was suggested to be due to the Tween 80 causing an accumulation of fluid in the peritoneal cavity producing a transient reduction in the plasma volume (Harrison et al, 1981). Tween 80 has been shown to enhance the anticancer activity of DOX in selected murine models (Casazza et al, 1978).

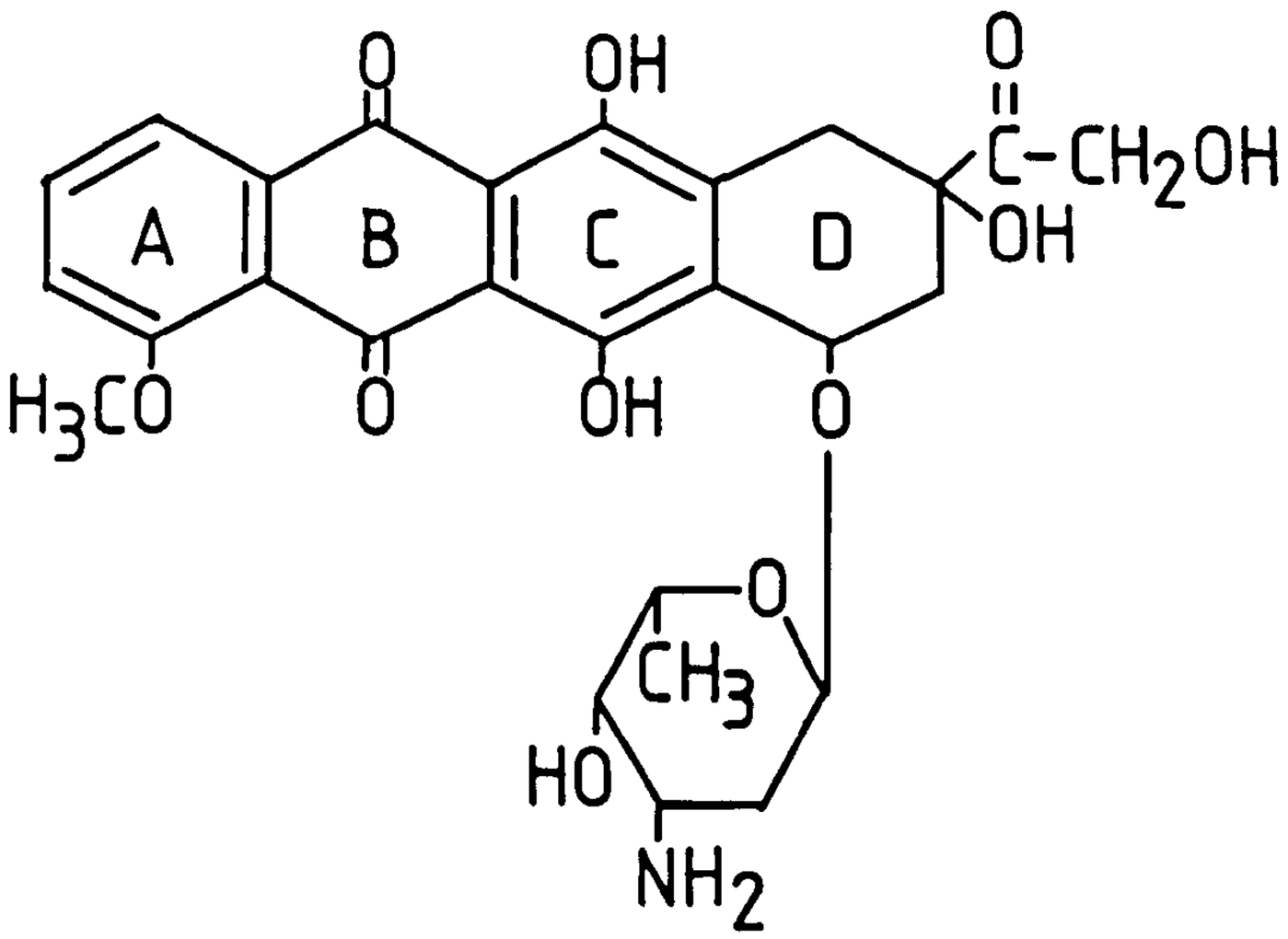
1.5 Doxorubicin and the Advantages of its Encapsulation in Vesicles

1.5.1 Properties of Doxorubicin

Doxorubicin is an anthracycline antibiotic produced by a mutant strain of Streptomyces peucetius and consists of a tetracyclic aglycone and a daunosamine sugar group (Fig. 1.7). The molecule has a hydrophobic portion consisting of the A,B and C rings and a hydrophilic portion consisting of the daunosamine sugar and the attached D ring. It is also an amphoteric molecule by virtue of the basic amino group and the acidic, phenolic hydroxyls of the anthracycline ring. These properties enable the molecule to react with a wide variety of cellular components such as nucleic acids, proteins and lipids. DOX cannot be given orally due to the susceptibility of the glycosidic bond joining the anthracycline nucleus to the sugar group to undergo chemical and enzymatic cleavage, resulting in inactivation of the compound, and must, therefore, be given intravenously, (Henry, 1976).

1.5.2 Mechanism of Action of Doxorubicin

DOX exerts its cytotoxic action by intercalating with DNA in the cell nucleus with subsequent inhibition of DNA and RNA synthesis (Di Marco, 1975). In vitro, DOX forms a stable complex with native DNA, with the aglycone portion of the DOX molecule intercalating between the base pairs of the DNA helix in the complex (Henry, 1976). X-ray diffraction studies have shown that 1) the amino sugar of the antibiotic was located in the major groove of the DNA, and the hydrophobic faces of the base pairs and drug interacted extensively, and 2) the ionized amino group of the daunosamine sugar lay close to the deoxyribose-phosphate chain, permitting a strong interaction between the drug and DNA phosphate away from the intercalation site (Pratt and Rudden, 1979). Synchronised cells in the G1 or G2 phase



Doxorubicin

Fig. 1.7 The chemical structure of doxorubicin showing the A, B and C rings which form the hydrophobic portion of the molecule, and the daunosamine sugar with the attached D ring which form the hydrophilic portion.

of the cell cycle were less sensitive to DOX than cells in the S phase, and similarly, plateau phase cells were less sensitive than cells from the log phase of growth (Wilkinson, 1978).

Some of the more recently developed anthracyclines do not appear to have a mode of action which involves intercalation with DNA (Chuang et al, 1983) and there is growing evidence that anthracyclines also exert actions on cell surfaces (Crane et al, 1980; Murphree et al, 1976; Wheeler et al, 1982).

1.5.3 Toxic Effects Observed after Administration of Doxorubicin

As with all drugs used to treat cancers, DOX is associated with a wide range of side-effects including alopecia, nausea, vomiting and bone marrow depression (Wilkinson, 1978). However, the major treatment limiting toxicity is an irreversible cardiotoxicity and this limits the cumulative dose administered to 550mg/m^2 (Green et al, 1984).

The formation of semi-quinone free radicals and other unstable one and two electron reduced products have been implicated in the production of cardiotoxicity (Land et al, 1985) and the formation of hydroxyl radicals was shown to be stimulated by DOX in isolated rat heart (Rajagopalan et al, 1988).

The DOX metabolite, doxorubicinol, was shown to be more effective than parent DOX at compromising both systolic and diastolic cardiac function and was also found to be produced and possibly accumulated in heart tissue and may, therefore, contribute to the overall cardiotoxicity of DOX (Olson et al, 1988).

One method of attempting to overcome this problem of cardiotoxicity has been to prepare analogues of DOX which retain the cytotoxicity of DOX but have reduced other toxicities (Israel et al,

1987). Cardiolipin, a component of the inner membrane of mitochondria, is abundant in cardiac muscle and effectively concentrates DOX. One of the mechanisms of this interaction was shown to be an electrostatic interaction between the negatively charged phosphate of the cardiolipin and the positive charge borne by the anthracycline molecule at physiological pH. This was shown to be the case for both DOX and one of its analogues 4'-epidoxorubicin (Nicolay et al, 1988). It has been suggested that one reason why anthracycline analogues of DOX that do not bind to DNA are not toxic to cardiac muscle was that they do not bind to cardiolipin (Wilkinson, 1978). However, more recent work has suggested that although DOX and two of its analogues interacted with both proteins and phospholipid molecules, the cardiotoxicity produced by DOX was unlikely to be related to the amount of drug bound to heart mitochondria (Griffin et al, 1986).

Since the formation of free radicals has been implicated in the cardiotoxicity of DOX, radical scavengers such as alpha-tocopherol (Wang et al, 1980; Legha et al, 1982), ascorbate (Fujita et al, 1982), ICRF-187 (Herman et al, 1981) and coenzyme Q (Domae et al, 1981) have been given in conjunction with DOX in an attempt to reduce their formation.

1.5.4 Interaction of Doxorubicin with Membranes

Several reports have been published which discuss the ability of anthracyclines, particularly DOX, to interact with negatively charged membranes. As discussed above, much work has centred on the interaction between cardiolipin and DOX and its analogues since this was thought to be one of the mechanisms of the potentially fatal cardiotoxicity caused by an electrostatic interaction between the negatively charged cardiolipin and positively charged drug molecule

(Nicolay et al, 1988).

Other negatively charged phospholipids were also found to be capable of interacting with DOX, Two moles of DOX were found to bind to one mole of cardiolipin, while only one mole of DOX bound to one mole of phosphatidylserine and phosphatidic acid and no adsorption was measured with neutral lipids. The binding of DOX to cardiolipin was thought to be due to an electrostatic interaction and also an interaction between adjacent anthraquinone chromophores (Goormaghtigh et al, 1980). Burke et al (1988) showed that in addition to the electrostatic interaction, hydrophobic considerations were important. They also showed that the steric bulk of the amino group of the anthracycline was an important factor in the binding, since substitution of the amino group produced a reduction in the affinity for the bilayer, even if the substituted group increased the ionization of the amine.

DOX also interacted with erythrocytes and it was shown that the interaction between DOX and carboxylate groups was very weak, with the negatively charged phosphate groups or sulphate groups being responsible for the main interactions between DOX and these systems (Garnier-Suillerot and Gattengo, 1988).

1.5.5 Potential Benefits of Doxorubicin Encapsulation

Analysis of the in vivo results obtained to date, after intravenous injection of liposomes showed that they had two main features. Firstly, they were generally directed, along with any associated drugs, to the organs of the MPS, particularly the liver and spleen and secondly, they reduced the uptake of drugs to the heart, kidney and gastro-intestinal tract. These features have been exploited, in that encapsulated drugs can be delivered to parasitic

infections residing within cells of the MPS (Alving et al, 1978; New et al, 1981), liposomal encapsulation of amphotericin B can be used to reduce its nephrotoxicity (Graybill et al, 1982; Mehta et al, 1984) and the cardiotoxicity observed with doxorubicin can be reduced by liposomal encapsulation (Rahman et al, 1980; Olson et al, 1982). Liposomes with encapsulated doxorubicin are already undergoing phase I clinical trials to examine their effect in the treatment of hepatic metastatic disease (Sells et al, 1987).

Doxorubicin (DOX) has also been encapsulated in other vesicular systems. The cardiac peak drug level generally observed with free doxorubicin was reduced by encapsulation in non-ionic surfactant vesicles (Rogerson et al, 1988; Kerr et al, 1988a) and plasma levels of doxorubicin were altered by encapsulation in microspheres (Willmott et al, 1985).

Encapsulation of DOX in liposomes has also been shown to reduce the topical toxicity, i.e. vesicant toxicity, observed after subcutaneous injection in mice (Balazsovits et al, 1989). Injection of free DOX resulted in immediate erythema and oedema at the site of injection and progressed to produce an ulcerated area. After injection of the liposomal preparation slight erythema and oedema were produced, but no ulceration, and after 3 weeks all signs of irritation had subsided.

1.5.6 Preparation of Doxorubicin Loaded Liposomes

Encapsulation of DOX in liposomes has been attempted with a wide range of compositions. Crommelin et al (1983) suggested that the preparation of dispersions of liposomes was best carried out at pH 4.0–6.3 since this gave the best characterized and reproducible dispersions. Since DOX is an amphipathic compound, it may be added to the aqueous hydrating solution (Gabizon et al, 1982) or added to

the phospholipid film (Rahman et al, 1982) during preparation of the liposomes.

Cholesterol had a marked effect on the drug entrapment in liposomes, with entrapment falling as the cholesterol content was increased from 5mol% to 50mol% (Ganapathi and Krishan, 1984), although the rate of release of drug from the vesicles in the presence of serum did not appear to be dependant on the cholesterol content. This was contrary to the findings of Storm et al (1989) who showed that cholesterol was a key component for reducing the leakiness of DOX from liposomes both during storage at 4-6°C over 4 weeks and in the presence of serum.

The inclusion of cholesterol in non-ionic surfactant vesicles reduced the rate of release of DOX, with a maximum reduction seen with cholesterol concentrations of 25mol% and above (Khand et al, 1987).

More recently, entrapment efficiencies of up to 98% have been reported (Mayer et al, 1985; Mayer et al, 1986). The DOX was taken up into large unilamellar liposomes (LUV's), of average diameter 90nm, in response to a valinomycin-K^+ dependant diffusional potential across the membrane of the vesicle. The inclusion of equimolar ratios of cholesterol inhibited uptake of the drug at 20°C, but enhanced uptake at higher temperatures, while DOX retention in these actively loaded vesicles was significantly greater than passively loaded vesicles.

Liposomes containing DOX and which exhibited temperature sensitive release of the drug were prepared by Tomita et al (1989). In the absence of serum, the dipalmitoylphosphatidylcholine vesicles retained the majority of entrapped drug at 32°C, but released about 70% at 42°C. When serum was present, the vesicles were leaky even

at 32°C, and the inclusion of 20mol% cholesterol was required to produce the best temperature sensitive release of DOX in the presence of serum.

Many examples of the ability of DOX encapsulated in liposomes to reduce the cardiac exposure and toxicity of the drug while retaining the antitumour effect of the drug have been reported in the literature (van Hoesel et al, 1984; Rahman et al, 1982; Gabizon et al, 1982). Most of the evidence available thus far suggests that tumours do not take up intravenously injected liposomes to any substantial degree when the vesicles are injected in vivo, probably due to the vesicles inability to extravasate into poorly perfused tumour cells (Poste, 1985). Since liposomes are generally removed from the circulation by organs of the MPS, the suggestion has been that the phagocytosed drug loaded liposomes act as a reservoir for the drug. The drug is slowly released from these cells such that the peak cardiac drug levels are avoided while the tumour cells receive a sustained cytotoxic level of the drug. For this scheme to have potential, the DOX must be released and released as parent drug from the macrophages, and this was found to be the case by Storm et al (1988). They were able to show that after intraperitoneal injection of DOX loaded liposomes and harvesting of the peritoneal macrophages, DOX was released intact from the macrophages, as determined by an in vitro growth reduction of tumour cells in culture.

Alterations in macrophage properties have also been reported after exposure to DOX. Salazar and Cohen (1984) showed that intraperitoneally injected DOX was capable of stimulating the production of peritoneal exudate cells which were cytotoxic to tumour cells despite there being no measurable concentration of drug. It

has also been reported that *in vitro* macrophage mediated destruction of tumour cells was independent of tumour cell resistance to DOX (Giavazzi *et al*, 1984). The encapsulation of DOX in liposomes and injection into the peritoneal cavity of mice also resulted in the changes necessary for the macrophage mediated tumoricidal activity *in vivo* (Mace *et al*, 1988). The liposomal encapsulation also resulted in a longer activity in producing these cells than free drug.

The attachment of monoclonal antibodies to liposomes has been attempted. The use of antibodies attached to liposomes is potentially a convenient method of delivering drugs to sites since although only a few drug molecules can be directly attached to an antibody, an antibody conjugated liposome is capable of carrying hundreds of drug molecules. Konno *et al* (1987) found that preferential delivery of antibody conjugated liposomes to tumours did take place to some extent after intravenous injection and the reduced cardiac peak normally associated with liposomal encapsulation of DOX was also noted.

Cancer, as a disease, is still regarded with much dread in developed countries. Many of the treatments available for patients suffering from cancers, particularly solid tumours, are often thought of as being worse than the diseases themselves due to the wide range of side effects associated with the treatment, be it radiotherapy or chemotherapy. Some patients may actually suffer from anticipatory nausea as they prepare for another course of treatment.

Psychological difficulties can also be suffered by patients who have difficulty in coming to terms with their own condition as well as by relatives and friends who are uncertain how to react.

Anything which can be done to reduce the suffering felt by this increasingly large group of the community deserves support.

1.6 Aims of Work

There were two main aims of this work. Firstly, an investigation of the physicochemical properties of vesicles, such as vesicular size, aqueous entrapment volumes, surface charge, vesicle-vesicle interactions and vesicular leakiness and how these properties could be modified by the incorporation of molecules which alter the hydrophilicity or charge of the vesicular surface. Secondly, the biological effects of some of these modifications were investigated by encapsulating the anticancer drug, doxorubicin, and intravenously administering the vesicles to mice.

2 Materials and Methods

2.1 Materials

Non-Ionic Surfactants

Surfactants I, II and IV (Fig. 2.1) were a gift from L'Oreal, France, and were analogues of the original surfactants used by Vanlerberghe et al (1972; 1978) to form non-ionic surfactant vesicles.

Cholesterol, dicetyl phosphate and stearylamine were used to alter the permeability and charge of the vesicles, while sodium chloride was used to study vesicular aggregation. All were purchased from Sigma Chemicals, Dorset, U.K..

5(6)-Carboxyfluorescein

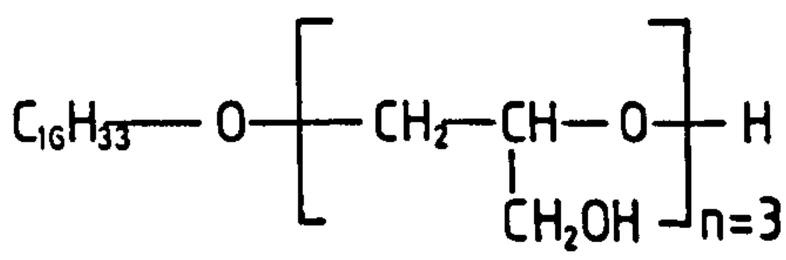
This was purchased from Eastman Kodak, Liverpool, U.K. and was partially purified before use as described in section 2.2.2.2.1.

Solulan Compounds

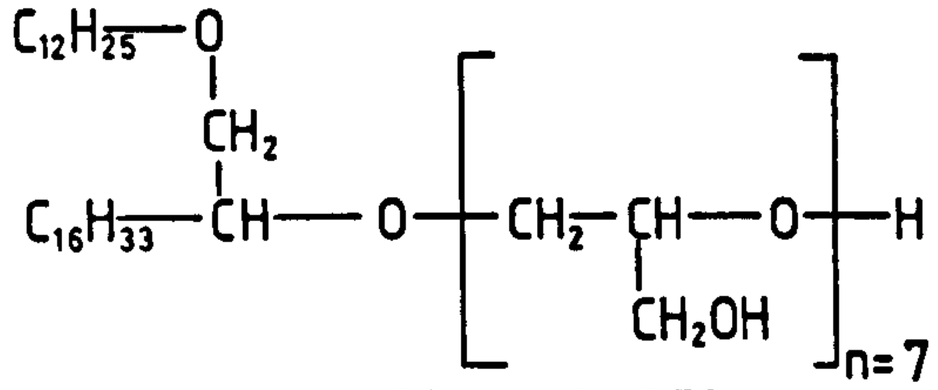
Solulan 5, Solulan 16 and Solulan C-24 were obtained from Anstead Ltd., Essex, U.K.. These compounds comprise a wide range of commercially available surfactants used mainly in the cosmetic industry as non-ionic solubilisers, emulsifiers and wetting agents.

Solulan 5, Solulan 16 and Solulan C-24 (Fig. 2.2) were obtained for incorporation into vesicular bilayers with the objective of making the non-ionic surfactant vesicle surface more hydrophilic and altering the way in which drugs and other molecules interacted with them.

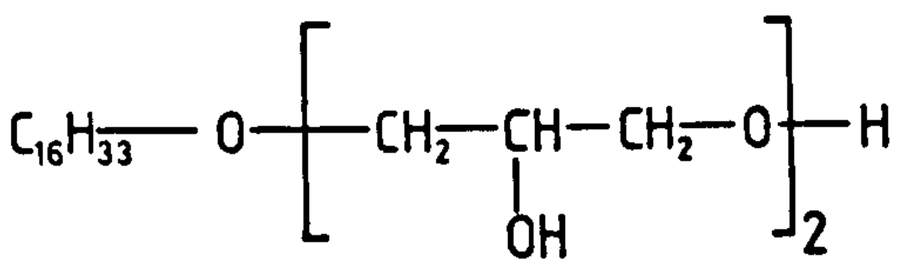
Solulan C-24 consisted of a cholesterol moiety, with an average of 24 polyoxyethylene (POE) groups attached through the OH at the three position of the cholesterol molecule; it had an HLB value of 14. The compound was slightly soluble in diethyl ether and freely



Surfactant I, m.wt., 473

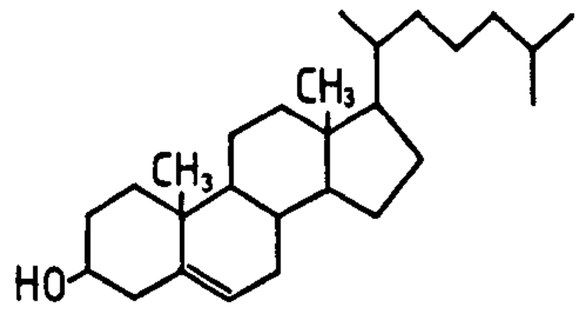


Surfactant II, m.wt., 972

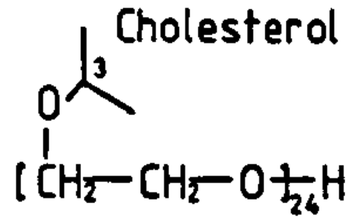


Surfactant IV, m.wt., 390

Fig. 2.1 The chemical structures of the non-ionic surfactants I. II and IV are shown. The average number of glycerol groups of Surfactant I and II are 3 and 7 respectively. Surfactant IV is a homogenous diglycerol compound which contains two diastereoisomers present in a 50:50 ratio.



Cholesterol, m.wt., 386·7



Average m.wt., 1443

HLB 14

Solulan C-24

Cholesterol —O—[—CH2—CH2—O—]16—H

Estimated m.wt., 1091 HLB 15

Solulan 16

Cholesterol —O—[—CH2—CH2—O—]5—H

Estimated m.wt., 607 HLB 8

Solulan 5

Fig. 2.2 The chemical structures, HLB values and molecular weights are shown for cholesterol, Solulan 5, Solulan 16 and Solulan C-24. The POE chain lengths are average values and since Solulan 5 and 16 contain compounds other than cholesterol as the backbone of the molecule, molecular weights are estimated.

soluble in chloroform and water.

Solulan 5 and Solulan 16 contained not only cholesterol but also lanolin alcohols and related fatty acids to which the POE was attached. The cholesterol based compound was present as about 30% of the total. Solulan 5 and Solulan 16 have, respectively, an average of 5 or 16 POE groups, and HLB values of 8 and 15.

Doxorubicin HCl

Doxorubicin HCl powder was obtained from Farmitalia Carlo Erba, Italy and was used as received.

Animals

AKR mice, weighing 25-30g, the animal Ridgway Osteogenic Sarcoma (ROS) model and doxorubicin metabolites were kindly supplied by the CRC Department of Medical Oncology, University of Glasgow.

Solvents

Chloroform and diethyl ether (anhydrous) were purchased from BDH Chemicals and all other chemicals and solvents were Analar or HPLC grade.

2.2 Methods

2.2.1 Descriptions of Vesicular Compositions

To avoid writing the composition of vesicles out in longhand, a system of abbreviations has been adopted. Table 2.1 gives the abbreviations for the various compounds used in the formation of vesicles. The vesicular compositions were notated by using the abbreviation for the compound followed by its concentration in mol% e.g. Surfactant II 50mol% with cholesterol 50mol% was abbreviated to II 50:CHOL 50; Surfactant IV 47.5mol% with cholesterol 47.5mol% and stearylamine 5.0mol% was abbreviated to IV 47.5:CHOL 47.5:SA 5.0; and Surfactant I 50mol% with cholesterol 25mol% and Solulan C-24 25mol% abbreviated to I 50:CHOL 25:SOL24 25.

Table 2.1 List of abbreviations used for vesicular components.

Compound Name	Abbreviation
Surfactant I	I
Surfactant II	II
Surfactant IV	IV
Cholesterol	CHOL
Dicetyl Phosphate	DCP
Stearylamine	SA
Solulan 5	SOL5
Solulan 16	SOL16
Solulan C-24	SOL24

2.2.2 Preparation and Characterization of Non-Ionic Surfactant Vesicles (NSV's) **In Vitro**

2.2.2.1 Methods of Preparation

2.2.2.1.1 Hand Shaken (H.S.) NSV's

The method used was based on that originally described by Bangham et al (1965) and described with respect to non-ionic surfactants by Baillie et al (1985). Surfactant and the other vesicular components (150μmoles) were dissolved in diethyl ether (10ml), or chloroform (10ml) in the case of Surfactant IV and the Solulan compounds, and transferred to a round bottomed flask (50ml). The solvent was removed under reduced pressure (Buchli Rotary Evaporator), at 50°C, leaving a thin film of material on the wall of the flask. The hydrating solution (5ml), at 50°C, was added and the flask was mechanically shaken for 1h in a water bath at 50-70°C. The heater of the water bath was disconnected and the multilamellar dispersion produced was allowed to return to room temperature while remaining in the water bath.

2.2.2.1.2 Ether Injection (E.I.) NSV's

This method was based on that described by Deamer and Bangham (1976). Surfactant mixtures (150μmoles) were dissolved in diethyl ether (20ml) and passed through a no.14 needle (0.25ml/min) into the hydrating solution (5ml) maintained at 60°C by a water jacket. Once injection of the solvent was complete, the solution was maintained at 60°C for a further 1h to ensure complete removal of the solvent.

2.2.2.1.3 Sonicated NSV's

Small unilamellar vesicles were produced by sonication of the corresponding multilamellar dispersions (Papahadjopoulos and Watkins,

1967a). The dispersions were sonicated (MSE PG100 probe sonicator 150W, with titanium probe) for 10x30s, with a 30s cooling period between bursts, at 15% of maximum power while maintaining the dispersion at 50°C.

2.2.2.1.4 Vesicular Compositions Prepared

Neutral vesicles were prepared containing surfactant alone or various ratios of sufactant and cholesterol. Charged vesicles were prepared by the addition of dicetyl phosphate or stearylamine, resulting in negatively or positively charged vesicles respectively. The Solulan molecules were added to the vesicular composition to alter the surface characteristics of the vesicles and were incorporated at varying concentrations (0.5-50mol%).

2.2.2.2 Preparation of 5(6)-Carboxyfluorescein (CF) Loaded NSV's and Calculation of Entrapment Volumes

2.2.2.2.1 Purification of CF

Prior to use, the commercially obtained CF (Fig. 2.3) was partially purified by a method based on that of Ralston et al (1981). This involved refluxing CF (25g) with ethanol (300ml) over activated charcoal (10g) for 30min. After cooling, the mixture was filtered through a Whatman 50 filter paper and the CF precipitated from ethanol:water (1:2, v/v) at -10°C followed by washing with distilled water (4x50ml) and drying at 40°C.

2.2.2.2.2 Preparation of 200mM CF Solution

Partially purified CF (3.763g) was dissolved in distilled water (30ml) and the solution adjusted to pH 7.4 by addition of small volumes of sodium hydroxide (4M). This solution was made up to 50ml with distilled water and the pH readjusted to pH 7.4.

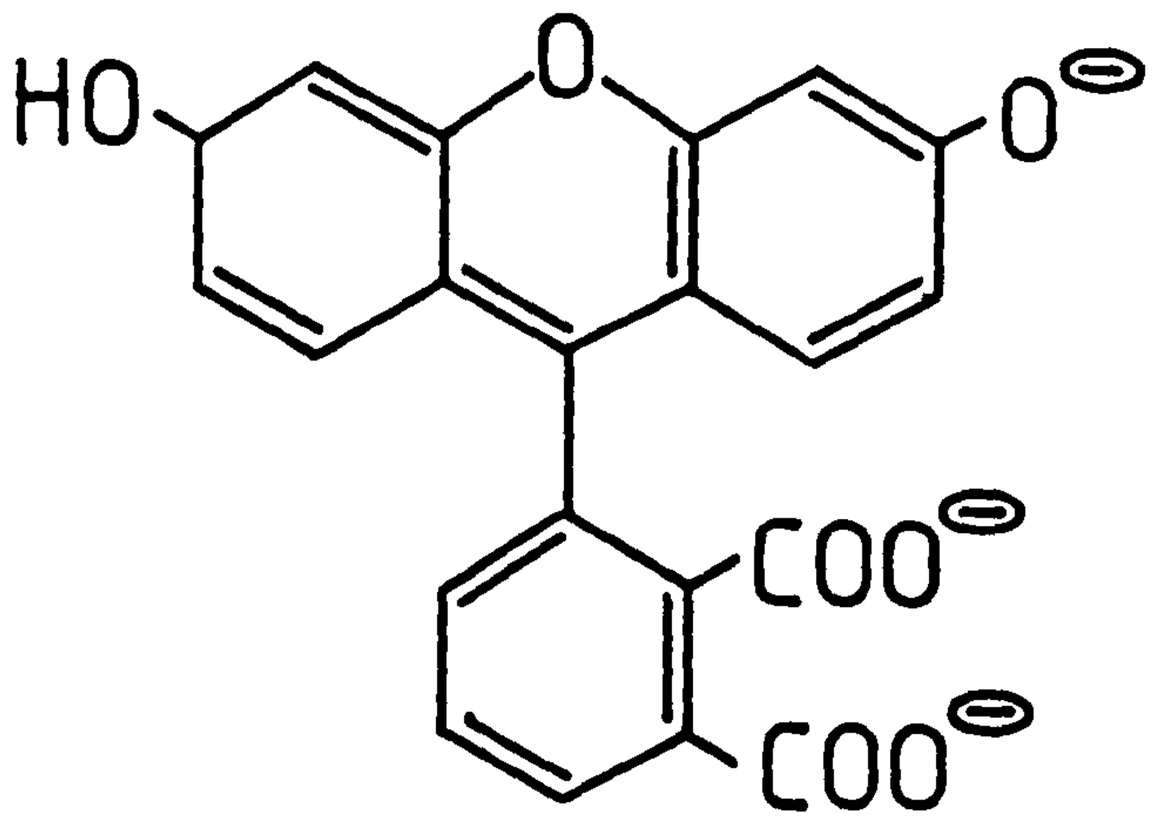


Fig. 2.3 The chemical structure of 5(6)-carboxyfluorescein (CF) at pH 7.4. (Molecular weight, 376).

2.2.2.2.3. Methods of Removal of Unentrapped Water-Soluble Molecules

Several techniques are available to remove unentrapped, water soluble molecules and include dialysis, centrifugation, gel chromatography and ultrafiltration, all of which have advantages and disadvantages.

Dialysis is a cheap and uncomplicated method of removal where greater than 95% of unentrapped drug can be removed. However, the process is slow and requires several changes of dialysing solution over 10-48h. The osmotic strength on both sides of the dialysis membrane must be balanced otherwise changes in the vesicle sample volume will become evident.

Centrifugation is a quick and efficient method of separation. On centrifugation, the vesicles form a pellet at the bottom of the centrifuge tube with the supernatant solution, containing the unentrapped drug, lying above the pellet. This supernatant is discarded and the pellet resuspended in fresh buffer solution. Two or more centrifugation stages are generally required to ensure effective removal of the majority of the unentrapped drug. There is also the possibility that smaller vesicles which are not spun down into the pellet may be discarded along with the supernatant. For small or charged vesicles, high centrifugation speeds are required which may lead to disruption of vesicles especially if the resuspending solution is not of the same osmotic strength as the original dispersion. The centrifuge speeds required to form a pellet range from 700xg for 10min for multilamellar liposomes (Johnson, 1975) to 150 000xg for 4h for very small vesicles (Roseman et al, 1975).

Gel chromatography is capable of not only removing unentrapped drugs from vesicles, but also separating the vesicle sample into fractions depending on their size. This method tends to be fairly slow and results in dilution of the original sample necessitating another concentration step, such as ultrafiltration or centrifugation.

Ultrafiltration has also been used as a method of removing unentrapped drug in its own right.

2.2.2.2.4 Removal of Unentrapped CF

Dialysis was the method chosen for removal of unentrapped CF. After formation, the vesicle sample was transferred into a dialysis sac (Cuprophane, 15mm diameter, molecular weight cut-off 12 000-14 000; Medicell International Ltd., London, England) and extensively dialysed against 200mM glucose solution. Dialysis was complete after 48h with 8-10 changes of dialysis solution (1000ml). The CF present in the dialysate was measured to ensure complete removal.

2.2.2.2.5 Measurement of CF Entrapment Volumes

Once dialysis was completed, a small aliquot (50μl) of the vesicle dispersion was disrupted with propan-2-ol (50μl) thereby liberating the entrapped CF. After suitable dilution with water the fluorescence was measured spectrophotometrically (Perkin Elmer Model 203 Fluorescence Spectrophotometer, exciter wavelength = 486nm, analyser wavelength = 514nm).

For calculation of the volume of entrapment, the concentration of the entrapped CF after dialysis was assumed to be the same as that of the hydrating solution i.e. 200mM, and entrapment volumes were expressed as litres of aqueous phase entrapped per mole of starting

material. This is in accordance with the recommendation of Tyrrell et al (1976) who suggested the use of a term relating the volume of aqueous phase entrapped to the amount of lipid used rather than the more misleading term relating the amount of material entrapped as a percentage of starting material which becomes liposomally associated.

2.2.2.3 Particle Size Analysis of NSV Preparations

The method of size analysis chosen was dependant on the size of the vesicles to be analysed. Sonicated or E.I. vesicles were measured by photon correlation spectroscopy (PCS), while H.S samples, generally containing multilamellar vesicles (MLV's), were analysed by a laser diffraction method or by PCS depending on their size.

Laser light scattering methods use a monochromatic and coherent laser beam which passes through a liquid suspension of particles, creating an interference pattern caused by constructive and destructive interference. The particles are moving due to Brownian motion, the rate of movement being size dependant, larger particles diffusing more slowly than smaller ones. This produces a constantly changing interference pattern, the rate of change being governed by the diameter of the particles.

2.2.2.3.1 Photon Correlation Spectroscopy

Photon correlation spectroscopy (PCS) involves observing a small part of the total diffraction pattern, using a photomultiplier, and passing the signals obtained to a correlator, where the frequency of the pattern or the fluctuation time constant, Tc, is derived.

Tc is related to the particle size by the following equations:

$$Tc = (DKs^2)^{-1}$$

$$D = \frac{Kb\ T}{6\pi VR} \qquad \text{(Stokes-Einstein Equation)}$$

$$\text{and,} \quad Ks = \frac{4\pi}{\lambda} .n \sin(\theta/2)$$

where D = diffusion coefficient of the particle

Kb = Boltzmanns constant

T = absolute temperature

V = liquid viscosity

R = hydrodynamic radius of the particle

n = refractive index of the liquid

λ = wavelength of the incident beam

θ = observation angle of scattered energy

All parameters except Tc and R are known, and with Tc being detected by the photomultiplier, D and hence R can be calculated. Monodisperse samples produce a single exponential decay for Tc, with smaller particles producing a faster decay, while for polydisperse samples, the function is a series of superimposed exponential decays of Tc, called the Z average.

2.2.2.3.2 Preparation of Samples for Photon Correlation Spectroscopy

Samples measured by PCS were generally less than 1μm in diameter. Vesicle samples were diluted with 0.2μm filtered solutions prior to analysis to avoid problems associated with multiple scattering. A helium-neon laser (40mW, wavelength = 632.8nm, Siemens, West Germany) producing a monochromatic beam of light was passed through the sample, maintained at 25°C. Scatter of the beam was detected by a photomultiplier set at an angle of 90° to the incident beam. Signals were passed to a Malvern Loglin

Correlator K7027 (Malvern Instruments Ltd., England), containing a digital correlator, a control microcomputer and a magnetic diskette storage unit, and mean particle diameters and polydispersity factors were obtained. Some of the potential problems with measuring vesicle size by PCS are discussed in section 3.2.3.2.

2.2.2.3.3 Laser Diffraction Particle Sizer

Size analysis of vesicles with sizes greater than 1μm was generally undertaken using a Malvern 2600 Series Laser Diffraction Particle Sizer (Malvern Instruments, England). A small volume of vesicle sample was added to the sample cell containing 0.2μm filtered distilled water. Light from a helium-neon laser (2mW, wavelength = 633nm) was passed through the sample cell and both the light scattered by the vesicles and the unscattered light were passed through a lens. Unscattered light was brought to a focus and passed through an aperture in the detector and out of the system. The scattered light was gathered on the detector which had a series of 31 concentric annular rings, with larger particles being detected on the inner rings and smaller particles on the outer rings. Size distributions were computed as a series of size ranges and undersize plots, from which 10%, 50% and 90% undersize values were obtained. For all size measurements obtained a number, rather than a volume, distribution was chosen.

2.2.2.4 Aggregation of Vesicles

Sonicated vesicles were prepared and added to solutions of 0.2μm filtered NaCl of varying concentrations and the aggregation or shrinkage of vesicles measured by PCS. Aggregation of vesicles will cause an increase in the apparent mean diameter of the sample since

the aggregated vesicles will move in solution as a single mass, with vesicles held together by electrostatic forces, and not as discrete particles. In an effort to determine whether the vesicles had aggregated or fused, the sample was vortexed and the size remeasured, by PCS, to note if the original vesicle size could be remeasured.

2.2.2.5 Particle Microelectrophoresis

Electrophoresis measures the motion of dissolved or suspended material under the influence of an applied electric field. Most substances acquire a surface charge when brought into contact with aqueous medium due to ionization, ion adsorption or ion dissolution. This surface charge influences the distribution of other ions in the medium as ions of opposite charge are attracted to the surface and ions of like charge are repelled. This leads to the formation of an electrical double layer with the charged surface being neutralized by counter ions. Electrophoretic measurements only give information about the mobile part of the electrical double layer and can, therefore, only be related to the zeta potential or the charge density at the surface of shear.

Electrophoretic mobilities (EM) were calculated using the following equations:

$$\mu = \frac{V}{E}$$

where μ = electrophoretic mobility ($cm^2\ V^{-1}\ s^{-1}$)

V = vesicle velocity ($cm\ s^{-1}$)

E = applied field strength ($V\ cm^{-1}$)

Hence:

$$\mu = \frac{\text{distance travelled (cm)/mean time taken (s)}}{\text{voltage applied (V)/distance between electrodes(cm)}}$$

$(cm^2\ V^{-1}\ s^{-1})$

Values of EM can be converted to the zeta potential using the following equation*:

$$\text{Zeta potential(mV)} = 12.83 \times 10^4\ \mu$$

where μ = electrophoretic mobility $(cm^2\ V^{-1}\ s^{-1})$

2.2.2.5.1 Stationary Levels

The need to determine stationary levels arises because the internal glass surfaces of the electrophoresis cell are generally charged. When an electric field is applied across the electrodes, this causes electro-osmosis of liquid near the wall of the cell and a corresponding flow in the opposite direction at the centre of the cell. Thus, the true electrophoretic mobility of particles is only measured where these two liquid flows cancel each other out and there is no net liquid flow i.e. at the stationary level.

2.2.2.5.2 Platinum Blacking of Electrodes

The electrodes were washed in distilled water then in concentrated nitric acid and finally in distilled water. A freshly prepared solution of chloroplatinic acid (2%) and lead acetate (0.2%) was put in a beaker (50ml) and the electrodes placed in the solution 5mm apart. The electrodes were blackened by passing a current of 100mA between the electrodes, and blackening was completed after a few minutes.

* Derived from the Smoluchowski equation.

2.2.2.5.3 Measurement of Vesicular Charge

H.S. vesicles were prepared containing 150μmoles material in $2x10^{-3}$M NaCl (2ml). Electrophoretic mobilities were measured using a Particle Microelectrophoresis Apparatus Mark II (Rank Brothers, Cambridge, England) with a flat cell. The flat cell used had stationary levels at 0.14mm from the walls and the distance between the electrodes was 5.17cm. The eyepiece graticule was calibrated such that one square was equivalent to 34.2μm.

Solutions of $2x10^{-3}$M NaCl were prepared and the pH adjusted to pH 4.5, 6.0, 7.4 and 9.5 by addition of HCl (1M) or NaOH (1M). Electrophoretic mobilities (EM) were measured by adding a few drops of the vesicle sample to the NaCl solution of desired pH in the flat cell and mixing. The cell was left in the water bath at 25°C for 20min to equilibrate. A voltage was applied across the electrodes and the time for particles to move across a chosen number of squares of the graticule at the stationary levels of both walls of the cell was measured. A time of between 5-15s was chosen since below 5s timing errors became significant, while above 15-20s errors due to drift of vesicles or by particles moving out of the stationary level became evident. Ten particles were timed at the front and back walls travelling in both directions of flow by changing the polarity of the electrodes.

2.2.2.6 Differential Scanning Calorimetry

Thermal analysis measures changes in the chemical/physical properties of a sample as a function of temperature. It can now be used to measure thermal decomposition of solids and liquids, solid-solid and solid-gas chemical reactions, material specification, purity and identification, inorganic solid material adsorption and

phase transitions. Many instruments are available for carrying out thermal analysis and include differential thermal analysis (DTA), differential scanning calorimetry (DSC) and thermogravimetric analysis (TG). DSC measures the difference in energy inputs into a substance and a reference material as a function of temperature while both are subjected to a controlled temperature program.

The DSC instrumentation used was a Du Pont 910 Differential Scanning Calorimeter with a Du Pont Thermal Analysis System 9900 (Du Pont. Wilmington). A small, accurately weighed sample was placed in a hermetically sealed pan and was scanned over the temperature range 10-95°C, at a heating rate of 5°/min. DSC scans were plotted as a change in heat flow of the sample against temperature, and area under the peak calculated to give the total enthalpy change associated with the peak.

2.2.3 Methods for In Vivo Studies

2.2.3.1 Preparation of Vesicles for In Vivo Studies

Sonicated NSV's were prepared with two different vesicular compositions, with both containing doxorubicin (DOX) as the entrapped drug.

2.2.3.1.1 NSV's Containing Solulan Molecules

Vesicles were prepared by the sonication method containing I 50:CHOL 25:SOL24 25. The material (75μmoles/ml) was hydrated with a solution containing DOX 3mg/ml in 300mM glucose (6ml) to produce drug loaded vesicles. Empty vesicles were produced by hydrating with 300mM glucose (6ml).

2.2.3.1.2 NSV's Containing Surfactant IV

The vesicular dispersions were produced by the sonication method, contained 75μmoles/ml material and were composed of IV 47.5:CHOL 47.5:SA 5.0. The material was hydrated with a solution containing DOX (5mg/ml in 300mM glucose, 5ml) to produce drug loaded vesicles or 300mM glucose (5ml) to produce empty vesicles.

2.2.3.1.3 Removal of Unentrapped Doxorubicin

After preparation, both drug loaded and empty vesicles were dialysed against 300mM glucose solution at 4°C in the dark. Dialysis was complete after 48h and with six changes of solution (6x800ml).

2.2.3.1.4 Determination of Vesicle Size

This was carried out for drug loaded and empty vesicles by PCS as previously described.

2.2.3.1.5 Measurement of Encapsulated Doxorubicin

After dialysis, a small volume of the drug loaded samples (50μl) was disrupted with propan-2-ol (50μl) and diluted with water. The fluorescence of the resulting solution was measured (Perkin Elmer Model 203 Fluorescence Spectrophotometer, exciter wavelength = 480nm, analyser wavelength = 560nm). The amount of DOX removed during dialysis was also measured giving an indication of when dialysis was complete and also of how much DOX was retained by the sample.

2.2.3.1.6 Measurement of Doxorubicin Release from Drug Loaded NSV's

A dialysis cell, containing two chambers each with a capacity of about 8ml, separated by a dialysis membrane (Cuprophane 15mm) and with removable stoppers was used. The drug loaded sample (5ml) was put in one chamber of the cell and 300mM glucose solution (5ml) in the other. The cell was rotated end over end at a speed of 30 revolutions per minute and was kept in the dark to minimize drug degradation. At time intervals a sample (50μl) was removed from the 300mM glucose side of the cell and analysed for DOX by HPLC.

2.2.3.1.7 Effect of Empty Vesicles on Release of Doxorubicin in Solution

Empty vesicle samples were prepared in 300mM glucose solution (4ml) and prior to commencing dialysis were mixed with 1ml of a solution of DOX (5mg/ml in 300mM glucose). A control solution containing 5mg DOX in 300mM glucose (5ml) was also prepared. Samples (50μl) were removed for DOX analysis by HPLC.

2.2.3.2 **In Vivo** Distribution Studies

AKR mice, weighing 25-30g, received food and water ad libitum, and were kept in a 12 hour light/dark cycle throughout the duration

of the experiment. Three groups of mice were injected intravenously, via the tail vein, and received either, 1) free DOX in solution, 2) DOX entrapped within sonicated NSV's or 3) a combination of empty sonicated NSV's together with free DOX. This third group was prepared by mixing empty vesicles with DOX powder prior to injection, such that the concentration of both DOX and vesicles was the same as the other samples. In all cases the volume injected was less than 0.2ml.

Five mice were sacrificed by cervical dislocation, at specified time points and they were exsanguinated from the inferior vena cava. Blood was collected from each mouse in lithium heparin tubes, centrifuged (100xg, 2min) and the plasma separated and stored at -20°C until analysis. Tissues were also removed, washed, frozen in liquid nitrogen and stored at -20°C prior to analysis.

2.2.3.2.1 Experimental Details for NSV's Containing Solulan Molecules

These vesicles were injected at a DOX dose of 5mg/kg, mice were sacrificed at 10min, 30min, 1h, 4h, 12h, 24h and 48h, and liver, heart, kidney and plasma removed.

2.2.3.2.2 Experimental Details for NSV's Containing Surfactant IV

Vesicles were injected at a DOX dose of 10mg/kg into mice bearing a Ridgway Osteogenic Sarcoma (ROS). A 100mg fragment of tumour was passaged into a surgically created subcutaneous pouch on the right flank of the animals and the distribution study was undertaken when the fragments had grown to 0.5-1.0g. Mice were sacrificed at 15min, 1h, 4h, 24h and 48h and liver, heart, lung, spleen, tumour and plasma retained for analysis.

2.2.3.2.3 Ridgway Osteogenic Sarcoma Model

The Ridgway Osteogenic Sarcoma (ROS) spontaneously arose as an inguinal mass in a male AKM mouse in 1948 in the laboratory of Dr. J.H. Burchenal at the Sloan-Kettering Institute in New York. This tumour has been shown to be responsive to a wide range of drugs, including anthracyclines, used to treat solid tumours in man. It generally exhibits an orderly and reproducible dose response to active drugs (Laster, 1975). The median life span of ROS-implanted mice is about 25-35 days.

2.2.3.2.4 Extraction of Doxorubicin and its Metabolites from Mouse Samples

DOX and its metabolites were extracted from plasma (Cummings, 1985) and tissues (Cummings and Morrison, 1986) and detected by a reverse-phase HPLC method (Cummings, 1985).

1) Tissues - the tissues were thawed, weighed and a volume (3-5ml) of phosphate buffered saline pH 7.2 (PBS) added before being finely minced (Ultra Turrex Rotary Cutter) and homogenised (Potter Elvehjem Homogeniser) to disrupt cells and release intracellular DOX and metabolites. 1ml of this homogenate was transferred to a glass bottle and internal standard (daunorubicin), and silver nitrate solution (33%w/v, 200μl) were added. The silver nitrate was added to remove intercalated drug from the sample. After 10min shaking (Buchler Vortex Evaporator, Gallenkamp, East Kilbride), 5ml extracting solvent was added (chloroform:propan-2-ol (2:1 v/v)) and the sample shaken for a further 20 min. After centrifugation (1000xg, 15min, 4°C) the upper aqueous phase was discarded, and the solvent layer decanted into a clean test-tube. The extraction solvent was evaporated to dryness (Buchler Vortex Evaporator, 40°C,

25mm Hg) and the dried samples were stored at -20°C prior to HPLC analysis.

2) Plasma - samples were thawed, volumes measured and internal standard and extraction solvent (5ml) added. Samples were shaken for 20min, centrifuged and the solvent layer recovered and evaporated to dryness. Samples were stored at -20°C prior to HPLC analysis.

2.2.3.2.5 HPLC Analysis for Doxorubicin and its Metabolites

Reverse-phase HPLC analysis was performed with an Altex Model 100A pump, an Altex Model 210 injection port and a 20µl loop (Beckmann RIIC, High Wycombe, U.K.). The column (250mm x 4.6mm) was packed with µ-bondapak C-18 (10µm particle size) in the CRC Department of Medical Oncology, University of Glasgow using a Shandon Column Packer (Shandon, Runcorn, U.K.). Plasma and tissue samples were reconstituted in methanol (50µl) and DOX and metabolites were measured by fluorimetry (Gilson Spectra-glo Filter Fluorimeter, exciter wavelength = 480nm, analyser wavelength = 560nm) with a 10µl quartz micro-flow cell (Gilson, Villers-le-Bel, France). Peak areas were calculated by a Shimadzu CR-1b computing integrator (Scotlab, Bellshill, U.K.). A mobile phase of propan-2-ol (130ml) in water (to 500ml) containing 5mM phosphoric acid was degassed by purging with helium for one minute and passed through the column at a rate of 1.2ml/min.

2.2.3.3 **In Vivo** Tumour Growth Delay Study

Sonicated NSV's composed of IV 47.5:CHOL 47.5:SA 5.0 were prepared. AKR mice weighing about 25g and bearing a ROS tumour were used. A 100mg fragment of ROS tumour was passaged into each mouse

on day zero and tumour volume was estimated by calliper measurement in two dimensions once the tumour became palpable. Tumour dimensions were measured every few days and tumour volumes calculated, assuming spherical geometry, using the following equation:

$$\text{Volume} = 4/3\pi([a+b]/4)^3$$

where a is the largest diameter and b is the diameter perpendicular to a.

Eight animals were used as a control group, being injected with only 300mM glucose (0.1ml). Four animals were used in the three other treatment groups, and were injected intravenously at a DOX dose of 10mg/kg on day ten i.e. ten days after tumour fragment passage. The groups were injected with either 1) free DOX in solution, 2) DOX entrapped within sonicated NSV's or 3) a combination of empty sonicated NSV's and free DOX in solution. Once the length of one axis of the tumour reached 30mm in one mouse, all the mice in that particular group were sacrificed.

2.2.3.4 Fluorescence Photomicrographs

AKR mice bearing an established ROS tumour were injected intravenously with drug loaded vesicles composed of IV 47.5:CHOL 47.5:SA 5.0 at a DOX dose of 5mg/kg. After 5min, 4h and 22h , two mice were sacrificed and liver, spleen, lung, kidney, heart and tumour were removed. The tissues were halved and fixed in either propan-2-ol:water (80%:20% v/v) or by instant freezing in liquid nitrogen. Tissues were sectioned and fluorescence photomicrographs taken where appropriate.

The same procedure was also carried out after injection of free DOX in solution at a dose of 5mg/kg.

3 *In Vitro* Results and Discussion

3.1 Introduction

Since the early report of a purely synthetic non-ionic surfactant vesicle being formed using a hexadecyl triglyceryl ether (Vanlerberghe *et al*, 1972; 1978), other groups of compounds have been shown to form vesicles, such as crown ethers (Echegoyen *et al*, 1988) and quaternary ammonium salts (Kunitake, 1986).

Vesicles containing the hexadecyl triglyceryl ether have been used as carriers for methotrexate (Azmin *et al*, 1985), doxorubicin (Rogerson *et al*, 1988) and sodium stibogluconate (Hunter *et al*, 1988) and as cosmetic systems (Handjani-Vila *et al*, 1979).

Surfactants I, II and IV used in this study are vesicle forming compounds based on the original surfactants used by Vanlerberghe *et al* (1972; 1978).

3.2 Vesicular Characterization

Characterization of vesicular systems is essential before each experiment since even a small change in the composition or method of preparation can result in marked differences in the resulting vesicular dispersion. The size distribution of the dispersions should be examined along with determination of the aqueous entrapment volume per mole of lipid material or the molar ratio of lipophilic compounds associated with the vesicular bilayer. For use as drug delivery systems, the effect of biological fluids on the size, composition and permeability of the vesicles should ideally be determined (Szoka and Papahadjopoulos, 1980).

In this part of the thesis, attempts were made to assess what effect changing some of the components of the vesicular composition had on the final properties of the vesicles produced. Factors which

were known to affect the fate of carriers in vivo included size and charge (Juliano and Stamp, 1975) and hydrophilicity (Illum et al, 1987). The above factors, plus the physicochemical properties of any drugs entrapped by the carrier system and the location of the drug in the vesicular structure may affect the release rate of the drug (Rahman et al, 1975).

Thus, in an attempt to gain a better understanding of the systems produced, the aqueous volume entrapped, the size of small unilamellar vesicles (SUV's) or the size or size distribution of multilamellar dispersions (MLV's) and the surface charge of the vesicles were measured. The effect of doxorubicin (DOX), the drug used later in the in vivo studies, was also examined. Aggregation of vesicles can be a problem during the preparation of vesicles and may affect the characteristics of the system. The aggregation induced by sodium chloride was examined and the effect of changes in vesicular composition on the aggregation determined. The addition of cholesterol and charged molecules can affect the phase transitions of vesicles (Lee, 1977b; Lee, 1977a) and thus the rates of release of drugs from these vesicles (Papahadjopoulos et al, 1973b). DSC scans were, therefore, obtained for vesicles and the effect of changes in composition on the scans noted.

3.2.1 Measurement of Vesicular Size

The size or size distribution of vesicular samples has a significant effect on the *in vivo* destination of the vesicles. Large multilamellar vesicles tended to become trapped within the lungs to a greater degree than small vesicles when administered intravenously (Abra *et al*, 1984). Small vesicles were removed from the bloodstream mainly by the liver and spleen when injected intravenously (Magin *et al*, 1986) and small unilamellar vesicles were cleared from the bloodstream more slowly than large multilamellar vesicles (Juliano and Stamp, 1975). Furthermore, heterogeneous populations of vesicles were cleared in a complex manner, with a fast initial phase followed by a slow phase, compared with the simple exponential clearance of a homogeneous vesicle sample (Juliano and Stamp, 1975).

The effect of changing the vesicular composition on the resulting size distribution was examined. The size of sonicated and some of the H.S. vesicle samples containing Solulan molecules were measured by PCS, while the size distribution of the larger, generally multilamellar vesicle dispersions were measured by the Malvern Particle Sizer.

3.2.1.1 Size Measurement by Photon Correlation Spectroscopy

Samples containing surfactant, cholesterol and charged molecules were prepared by the sonication method and the resulting mean hydrodynamic diameters and polydispersity factors are shown in Table 3.1. In all tables, values are shown with the standard deviation, with the number of measurements recorded shown in brackets. It appeared that slight changes in the structure of the surfactant could

lead to significant differences in the size of the vesicles produced. The addition of charged molecules also affected the size of the vesicle, with both positive and negative charges causing a reduction in diameter.

Table 3.1 Mean vesicle diameters (nm) for sonicated vesicles of various lipid compositions, with associated polydispersity.

Vesicle Composition	Mean Diameter(nm)	Polydispersity
I 50:CHOL 50 (29)	251.8 ± 25.07	0.20 ± 0.061
II 50:CHOL 50 (14)	143.6 ± 12.44	0.20 ± 0.027
IV 50:CHOL 50 (3)	280.5 ± 6.65	0.13 ± 0.012
IV 47.5:CHOL 47.5: DCP 5.0 (4)	174.4 ± 6.87	0.18 ± 0.019
IV 47.5:CHOL 47.5: SA 5.0 (12)	187.7 ± 11.01	0.20 ± 0.037

(The number of samples measured is shown in brackets.)

The sizes of vesicles containing equimolar concentrations of Surfactant I and Solulan, produced by both the H.S. and sonication methods are shown in Table 3.2. No obvious trend was apparent since three of the six compositions produced samples which could not be measured by PCS. The sample containing SOL5 appeared to disrupt on sonication but, on cooling, reformed to produce multilamellar vesicles, with small vesicles appearing to be entrapped within their core. The system containing SOL24 did not produce vesicles but appeared to form large, uniformly sized, disc-like aggregates which measured about 20-25μm in diameter (Plate 3.1). These aggregates did not become smaller on sonication, but were broken into smaller

fragments.

Table 3.2 Diameter (nm) and polydispersity of hand shaken (H.S.) and sonicated vesicles containing Surfactant I and Solulan compounds.

Vesicle Composition	Mean Diameter(nm)	Polydispersity
I 50:SOL5 50 H.S.(2)	420.9	0.2489
sonicated (2)	disrupted on sonication	
I 50:SOL16 50 H.S.(2)	740.2	0.2836
sonicated (2)	284.3	0.2383
I 50:SOL24 50 H.S.(2)	many large disc aggregates	
sonicated (2)	disc aggregates break up	

(The number of samples measured is shown in brackets.)

Samples prepared by the H.S. method, containing 25mol% Solulan along with Surfactant I and cholesterol, produced vesicles with all compositions examined (Table 3.3). As above, the SOL5 sample disrupted on sonication and produced multilamellar vesicles containing small vesicles within their core (Plate 3.2). No trend was obvious from the size measurements obtained with these compositions.

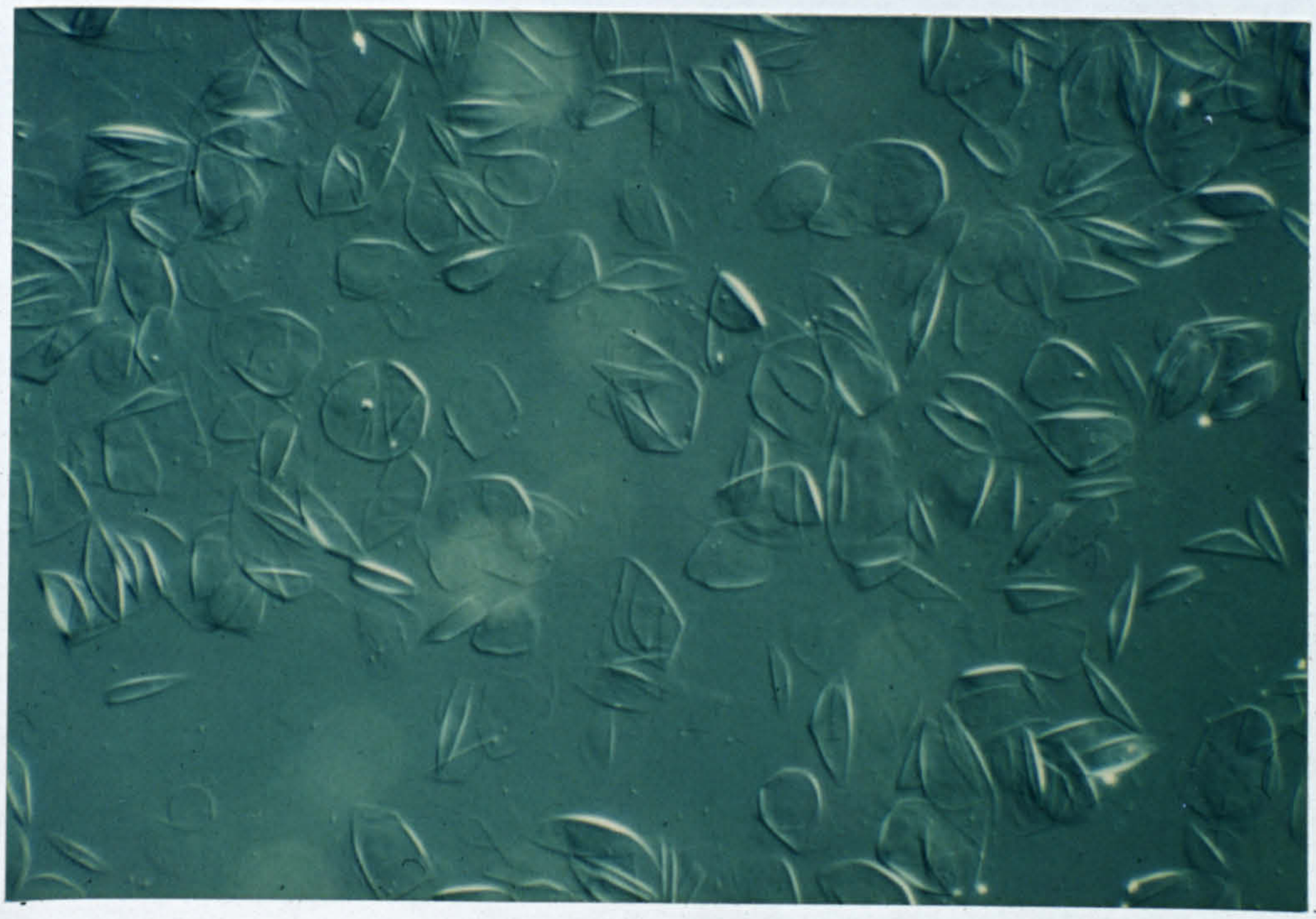


Plate 3.1 A photomicrograph of the disc-like structures obtained when attempting to form vesicles composed of I 50:SOL24 50 by the hand shaken method of preparation. (Magnification x200)

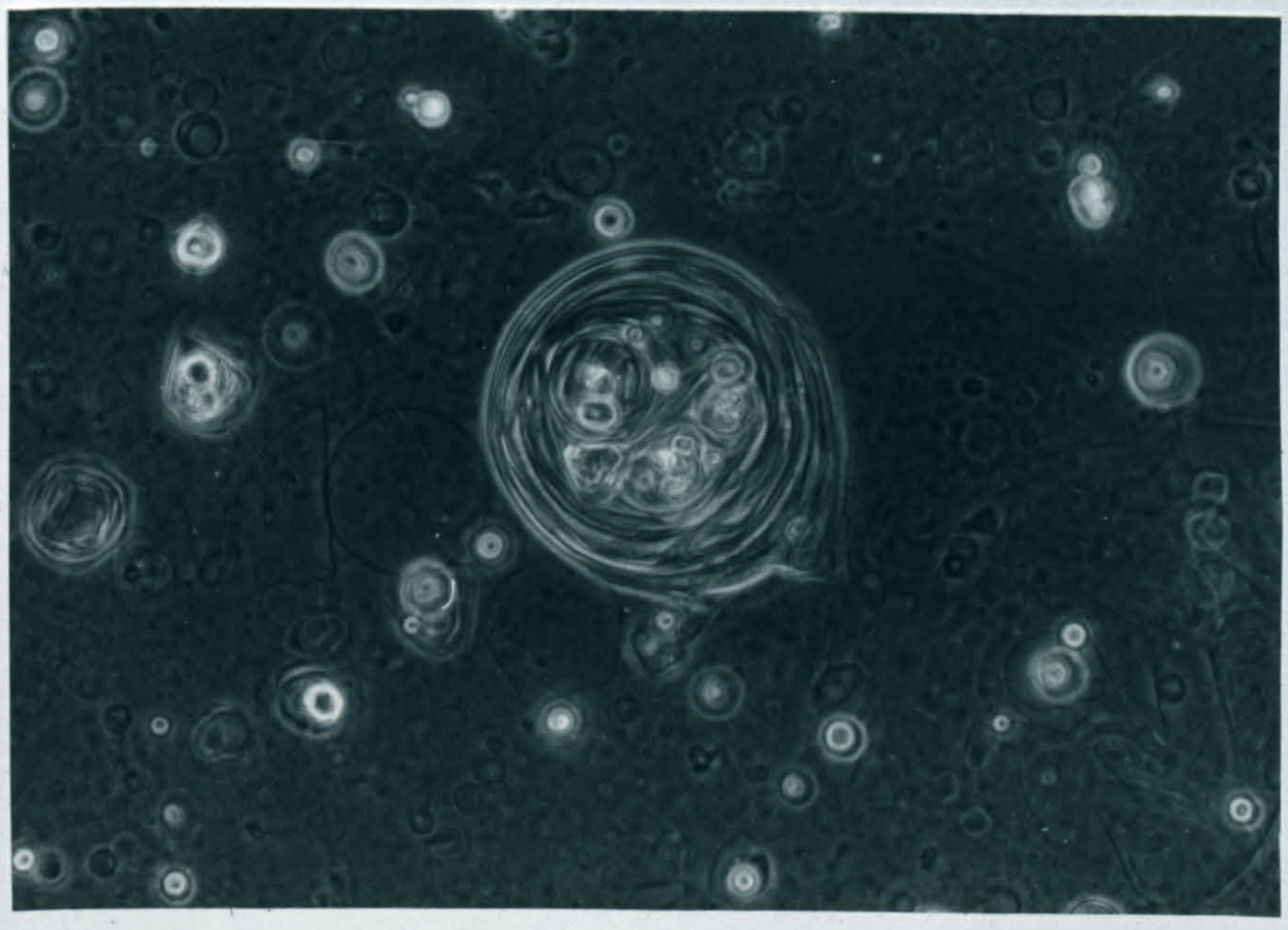


Plate 3.2 A photomicrograph of the multivesicular vesicles formed after sonication of multilamellar vesicles composed of I 50:CHOL 25:SOL5 25. (Magnification x400)

Table 3.3 Diameter (nm) and polydispersity of hand shaken (H.S.) and sonicated vesicle samples composed of Surfactant I 50mol%, cholesterol 25mol% and Solulan 25mol%.

Sample Composition	Mean Diameter (nm)	Polydispersity
I 50:CHOL 25:SOL5 25 H.S.(2)	971.8	0.29
sonicated (2)	disrupted on sonication	
I 50:CHOL 25:SOL16 25 H.S.(2)	953.1	0.29
sonicated (2)	630.4	0.25
I 50:CHOL 25:SOL24 25 H.S.(2)	1186	0.32
sonicated (12)	531.5 ± 23.96	0.27 ± 0.055

(The number of samples measured is shown in brackets.)

Using Surfactant II much more obvious trends were seen with respect to particle size. Vesicles containing equimolar concentrations of surfactant and Solulan molecules clearly showed that as the POE chain length increased the diameter of the vesicles fell, irrespective of the method of preparation (Table 3.4).

The same trend was noted when 25mol% cholesterol was included in the vesicular composition (Table 3.5) although larger vesicles tended to form for comparable vesicular compositions. Slowly increasing the mol% of SOL24 in the vesicles resulted in a gradual reduction of the vesicle size with no visible evidence of any disc-aggregates present (Table 3.6).

Table 3.4 Diameter (nm) and polydispersity of hand shaken (H.S.) and sonicated vesicle samples composed of Surfactant II 50mol% and Solulan 50mol%.

Sample Composition	Mean Diameter (nm)	Polydispersity
II 50:SOL5 50 H.S. (2)	338.1	0.23
sonicated (2)	251.5	0.17
II 50:SOL16 50 H.S.(2)	191.5	0.23
sonicated (2)	98.1	0.26
II 50:SOL24 50 H.S.(2)	168.6	0.22
sonicated (2)	55.2	0.53

(The number of samples measured is shown in brackets.)

Table 3.5 Vesicle size (nm) and polydispersity of hand shaken (H.S.) and sonicated vesicle samples composed of Surfactant II 50mol%, cholesterol 25mol% and Solulan 25mol%.

Sample Composition	Mean Diameter (nm)	Polydispersity
II 50:CHOL 25:SOL5 25 H.S.(2)	424.3	0.31
sonicated (2)	254.3	0.32
II 50:CHOL 25:SOL16 25 H.S.(2)	189.6	0.28
sonicated (2)	164.8	0.39
II 50:CHOL 50:SOL24 25 H.S.(2)	189.4	0.17
sonicated (2)	130.4	0.43

(The number of samples measured is shown in brackets.)

Table 3.6 Diameter (nm) and polydispersity of sonicated vesicle samples composed of Surfactant II 50mol%, cholesterol (50-X)mol% and Solulan C-24 X mol%.

Mol% Solulan C-24	Mean Diameter (nm)	Polydispersity
0 (14)	143.6 ± 12.44	0.19 ± 0.020
5 (2)	133.9	0.18
25 (2)	130.4	0.43
50 (2)	55.21	0.53

(The number of samples measured is shown in brackets.)

3.2.1.2 Determination of the Vesicular Size Distribution of Dispersions by Laser Diffraction

Results in this section are shown as 90%, 50% and 10% undersize values and the undersize plots obtained are also shown. Values quoted are the mean of at least three samples.

The effect of cholesterol and added charge on the size distribution of vesicles containing Surfactant I was examined.

The results (Table 3.7, Fig. 3.1) showed that the addition of cholesterol to the vesicular composition had little effect on the vesicular size distribution produced by the H.S. method. However, the addition of a positive or negative charge to the vesicle produced a size distribution which was larger than that obtained with the uncharged vesicles.

Table 3.7 90%, 50% and 10% undersize values are shown for hand shaken vesicle samples with the following compositions.

Vesicle Composition	90% Undersize	50% Undersize	10% Undersize
I 100	8.4μm	4.1μm	3.2μm
I 50:CHOL 50	8.4μm	4.0μm	3.1μm
I 47.5:CHOL 47.5:DCP 5.0	12.6μm	6.2μm	4.0μm
I 47.5:CHOL 47.5:SA 5.0	12.1μm	6.0μm	4.1μm

The sizes obtained for vesicles containing Surfactant IV, cholesterol and an added charge are shown in Table 3.8 and Fig. 3.2. The addition of cholesterol to Surfactant IV vesicles resulted in slightly larger vesicles forming compared with those formed from surfactant alone. However, the addition of charged molecules did not cause any further increase in size from the cholesterol containing vesicles.

Table 3.8 90%, 50% and 10% undersize values are shown for hand shaken vesicle samples with the following compositions.

Vesicle Composition	90% Undersize	50% Undersize	10% Undersize
IV 100	8.1μm	3.0μm	2.3μm
IV 50:CHOL 50	9.7μm	4.8μm	3.1μm
IV 47.5:CHOL 47.5:DCP 5.0	9.9μm	4.9μm	3.5μm
IV 47.5:CHOL 47.5:SA 5.0	9.6μm	4.7μm	3.4μm

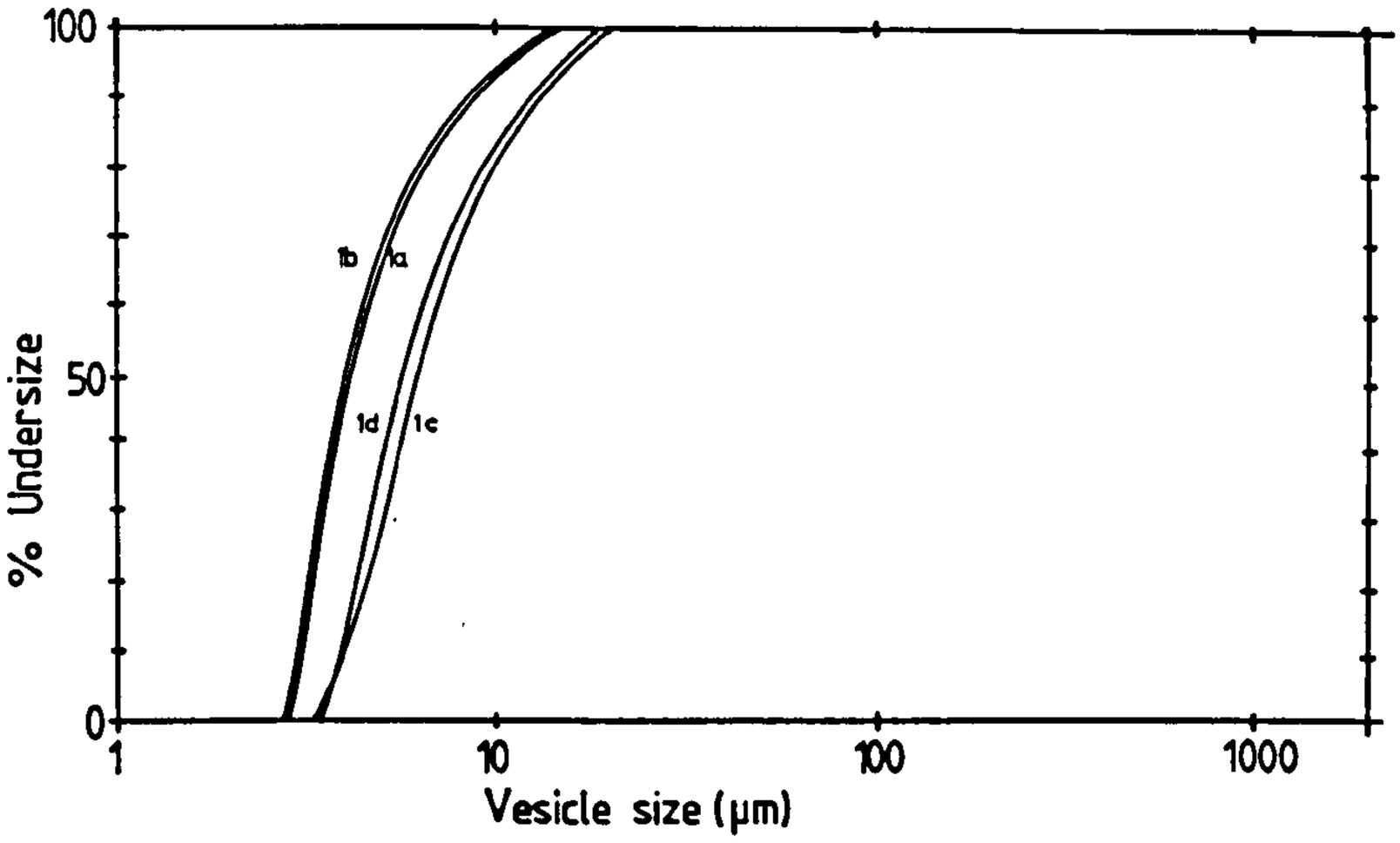


Fig. 3.1 % undersize curves are shown for vesicle compositions:

1a) I 100; 1b) I 50:CHOL 50; 1c) I 47.5:CHOL 47.5:DCP 5.0;

1d) I 47.5: CHOL 47.5:SA 5.0.

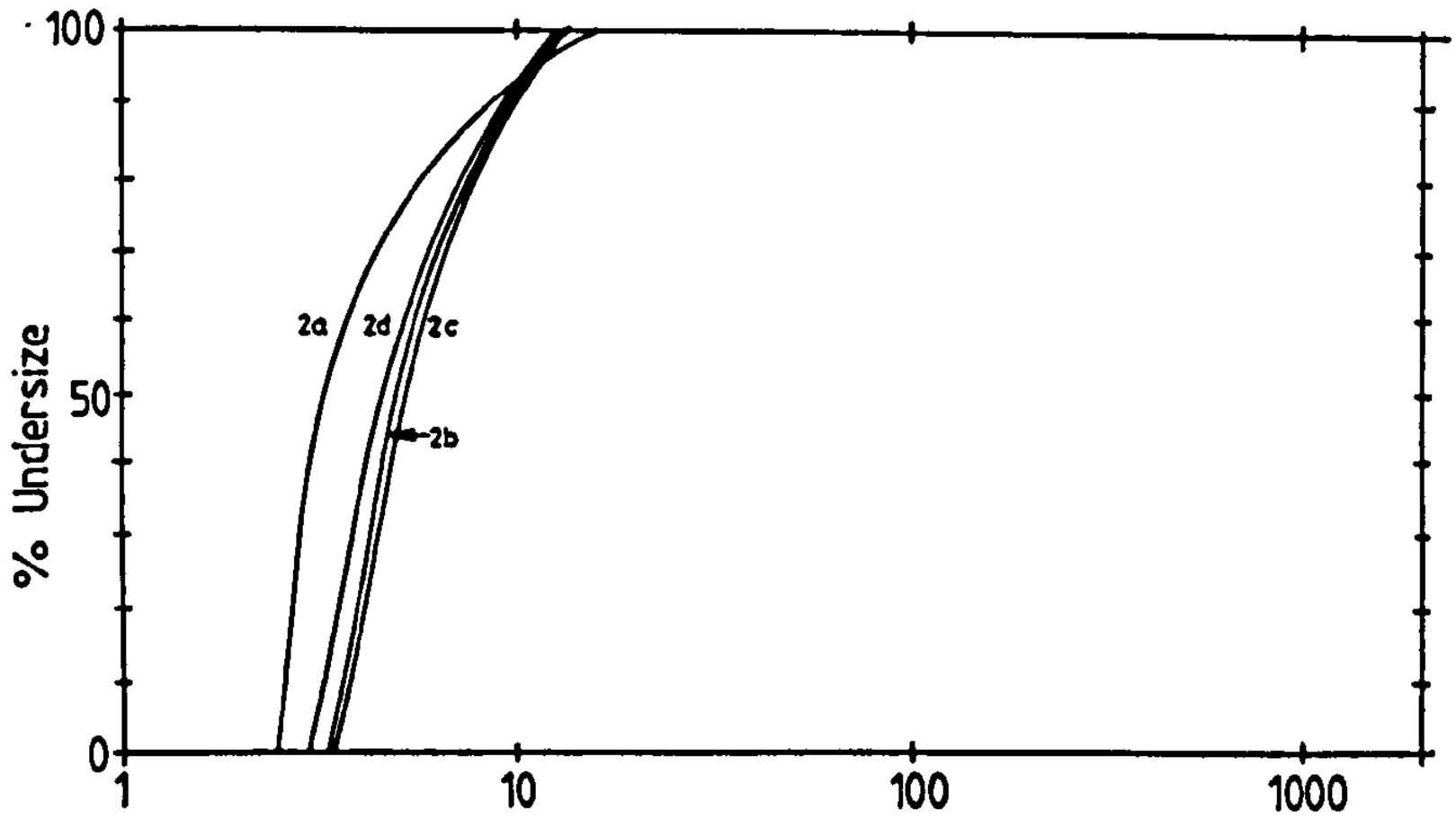


Fig. 3.2 % undersize curves are shown for vesicle compositions:

2a) IV 100; 2b) IV 50:CHOL 50; 2c) IV 47.5:CHOL 47.5:DCP 5.0;

2d) IV 47.5: CHOL 47.5:SA 5.0.

The addition of Solulan C-24 at various concentrations to vesicles had some interesting effects on the size distributions produced. The addition of SOL24 25mol% resulted in a reduction in size (Table 3.9 and Fig. 3.3). When 50mol% SOL24 was incorporated, a completely different size distribution was produced with some very large structures detected.

Table 3.9 90%, 50% and 10% undersize values are shown for hand shaken vesicle samples with the following compositions.

Vesicle Composition	90% Undersize	50% Undersize	10% Undersize
I 50:CHOL 50	8.4μm	4.0μm	3.1μm
I 50:CHOL 25:SOL24 25	1.6μm	1.3μm	1.1μm
I 50:SOL24 50	24.8μm	2.2μm	1.2μm

3.2.1.3 Discussion

Many groups have tried to produce theoretical models to explain how and why vesicles form and why vesicles have a particular size for a given composition (Lasic, 1988; Israelachvili and Mitchell, 1975; Israelachvili et al, 1977). Factors which are thought to affect the final vesicle size are the hydrocarbon chain volume, the length of the hydrocarbon chain and the surface area of the molecule at the hydrocarbon-water interface (Israelachvili et al, 1977). Gent and Prestegard (1974) also noted that increasing the mole fraction of cholesterol in egg yolk phosphatidylcholine vesicles resulted in an increase in the size of the unilamellar vesicles produced.

Israelachvili et al (1980) postulated that the hydrocarbon chain

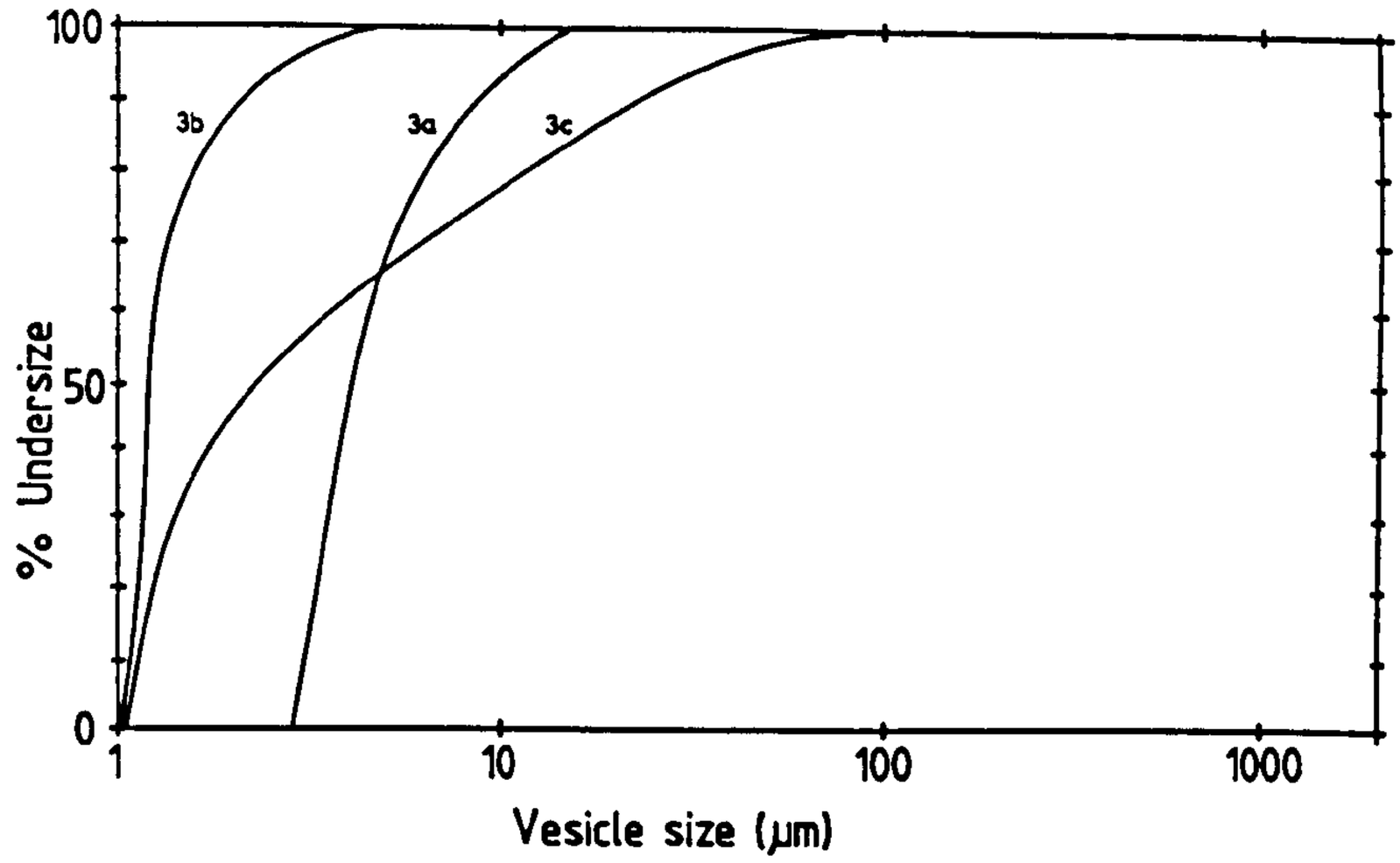


Fig. 3.3 % undersize curves are shown for vesicle compositions: 3a) I 50:CHOL 50; 3b) I 50:CHOL 25:SOL24 25; 3c) I 50:SOL24 50.

length (l), the hydrocarbon volume (v) and the minimum surface area occupied by the headgroup of the molecule (a_o) of molecules could be related to the critical packing parameter (CPP) as shown in equation 3.1. For molecules to form vesicles, the CPP should be within the range 0.5-1.

$$CPP = v/a_o.l \qquad (3.1)$$

For Surfactant I, a_o (33 Å^2) was measured and values for v (457.8 Å^3) and l (21.74 Å) calculated and the a_o of Surfactant II (59.7 Å^2) was also measured (Rogerson, 1986). Estimated values of v (875.5 Å^3) and l (23.1 Å) for Surfactant II were obtained from molecular models. The v and l values of Surfactant IV were the same as Surfactant I since they both contained identical hydrocarbon chains and a_o (30 Å^2) value was estimated from molecular models.

Given these values for each surfactant, the CPP's were 0.64, 0.63 and 0.70 for Surfactants I, II and IV respectively suggesting they were potential vesicle forming compounds. Israelachvili et al (1977) proposed an equation (3.2) whereby the minimum vesicular radius (R) could be calculated for molecules if l, v and a_o were known:

$$R = l/(1-v/a_o.l) \qquad (3.2)$$

Using equation 3.2, the minimum vesicular radius for Surfactant I was 60.4 Å, for Surfactant II was 62.4 Å and for Surfactant IV was 72.5 Å. The effect of including cholesterol in the vesicular composition on the minimum vesicular radius could also be shown using the above equations. Values for a_o (19 Å^2), v (400 Å^3) and l (17.5 Å) for cholesterol were determined by Carnie et al (1979). The inclusion of cholesterol in the vesicular composition at 50mol% led to larger vesicular radii being estimated, from equation 3.2, for

all the surfactants (Surfactant I = 122.6 Å; Surfactant II = 96.7Å; Surfactant IV = 178.4Å).

The size of unilamellar vesicles of all compositions measured was considerably larger than the minimum sizes calculated. The reasons for this are that other factors, in addition to the physical dimensions of the molecules, come into play when considering the actual formation of vesicles. However, these equations were useful in giving an indication of how the vesicular size may be affected by the incorporation of additional molecules.

Vesicles prepared by the sonication method and containing the monoalkyl Surfactants I and IV with cholesterol produced vesicles with a similar size despite the difference in the headgroup volume. This was presumably due to the fact that the hydrocarbon chain length and volume were the same and that the optimum surface area of the headgroup was very similar despite the differences in hydrophilic volume. Surfactant II, with a dialkyl chain and seven glycerol headgroups, produced vesicles much smaller than those with Surfactants I and V. Again this is probably due to an increased hydrocarbon volume, and possibly increased hydrocarbon length, and a change in the surface area of the molecule. The inclusion of a charged molecule in Surfactant IV vesicles markedly reduced the size of the vesicles produced. This decrease will most likely be caused by an increased surface area (a_o), due to the increased repulsion between the charged molecules within the bilayer, resulting in a reduced minimum vesicular radius (R) being calculated from equation 3.2.

The formation of multilamellar vesicles, by the H.S. method, appears on the surface to be a variable method of producing defined

dispersions with reproducible size distributions. Lasic (1988) found relatively well defined size distributions could be produced if the energy input, such as vigorous shaking for a defined time, as well as the vesicular composition and lipid concentrations were carefully controlled. The size distributions produced with the non-ionic surfactants appeared fairly reproducible, with little interbatch variations in the 90%, 50% and 10% undersize values. The size of multilamellar vesicles depends on the properties of the molecules in the bilayer as well as interactions between bilayers, with the ultimate size of the vesicle determined by the distance between the bilayers and the number of bilayers present. The inclusion of charged molecules in the composition increased the separation between bilayers of multilamellar vesicles (Bangham et al, 1967) although this separation was dependant on the effect the pH and the ionic strength of the hydrating solution had on the optimal surface area of the molecule (Israelachvili et al, 1976). In unilamellar vesicles, the final size is more closely determined by the properties of the molecules in the bilayer.

Multilamellar vesicles containing Surfactant I showed little change in size distribution when cholesterol was added although the addition of a charge produced an increase in the vesicular size distribution. Thus, when cholesterol was added, in order to maintain the same size distribution of vesicles, the number of bilayers and inter bilayer distances must either remain the same or change such that the net effect is to produce no change in the final vesicular size. The addition of a charge would increase the interbilayer distance, and although the number of bilayers may change, the net effect would be to produce larger vesicles.

Multilamellar vesicles containing only Surfactant IV, showed size distributions slightly smaller than that obtained with Surfactant I; this may be due to the slight differences in the headgroups of the surfactant molecules. The addition of cholesterol increased the size distribution of the vesicles to values similar to those produced by the addition of a charged molecule. The charged molecules would act on the size distribution as described previously by increasing the interbilayer separation while cholesterol would be more likely to increase the number of bilayers since cholesterol would have little effect on the charge at the bilayer surface.

The addition of the cholesteryl polyoxyethylene ethers to the vesicular composition had an interesting effect on the vesicle sizes produced. The inclusion, in the vesicular bilayer, of a molecule with a polyoxyethylene chain extending into the aqueous phase would most likely increase the average area (a_o) occupied by each molecule at the vesicle surface. The effect of increasing a_o would be to reduce the vesicle size, as predicted by equation 3.2.

It was found that the size of micelles, composed of surfactant molecules with POE chains, became smaller as the length of the POE chain was increased and it was suggested this was the result of increasing the hydrophilicity, thus causing a smaller number of monomers to form individual micellar units. An additional effect may be the enthalpic/entropic repulsion of the headgroups, similar to the repulsion produced by charged surfactants, which determined the maximum packing of monomers in the micelle and thus its size (Florence, 1969).

This effect was observed most notably with Surfactant II where increasing the POE chain length reduced the mean particle diameter of

vesicles produced by both the H.S. and sonication methods. Reducing the concentration of the Solulan compound in the vesicular bilayer would decrease the energy barrier effect and result in larger vesicles being formed.

When vesicles were formed with Surfactant I and the Solulan compounds, no clear picture emerged. Increasing the POE chain length appeared to reduce the size of sonicated vesicles but had little effect on the size of H.S. dispersions. Interesting structures were noted after sonication of vesicles containing Surfactant I and SOL5, both with and without cholesterol. Sonication appeared to disrupt the multilamellar, H.S. vesicles causing separation of the various components, but cooling the sample with slight agitation resulted in reformation of vesicles. These vesicles were large, multilamellar structures containing smaller vesicles (see Plate 3.2), similar to those reported to have been prepared with liposomes (Kim and Howell, 1987; Talsma et al, 1987).

The structures prepared with Surfactant I and SOL24 in equimolar proportions did not appear to be vesicular when viewed microscopically, but looked like discs which were 20-25μm in diameter. When the size distribution of these discs was examined, a 90% undersize value of 24.8μm was measured, corresponding well with the size estimated microscopically. However, the 50% and 10% undersize values were much smaller. This may be explained by considering the orientation of the disc in the laser beam and the way in which the instrument perceives this plane.

Smaller disc structures have been reported by several workers. Single chained phosphocholine molecules possessing a rigid segment in the hydrophobic chain were found to form disc-like aggregates when

dispersed in water (Okahata et al, 1980). During the preparation of vesicles by the detergent removal method, mixed micelles have been observed at one stage of vesicle formation (Ollivon et al, 1988; Ueno et al, 1989) and electron microscopy has shown discoid micelles transforming to vesicles (Fromherz et al, 1986). It therefore appeared likely that the discs formed by the addition of Surfactant I and SOL24 were some kind of mixed micelle system. These need further detailed investigation.

3.2.2 Measurement of Aqueous Entrapment of Vesicles

Powdered 5(6)-Carboxyfluorescein (CF) obtained from Eastman Kodak may be contaminated to such an extent that 8% of the total fluorescence is due to impurities. Partial purification removes some but not all of these impurities and those remaining can be removed by passing the CF solution over a hydrophobic column of LH-20 Sephadex. Separation of the 5 from the 6 isomer can be achieved by forming the diacetates followed by hydrolysis and precipitation at low pH (Ralston et al, 1981).

CF was purified because the impurities may affect vesicle stability and rates of transfer of CF into cells. Since this would not be a major drawback for the measurement of vesicular entrapment, partial purification of CF was deemed sufficient.

CF is a polar and highly water soluble fluorescent dye which is self quenching at the concentration used (200mM) and extensive dilution is required to overcome this quenching. The addition of propan-2-ol, vesicles or vesicles disrupted with propan-2-ol were found not to affect the fluorescence of CF solutions.

Vesicles were prepared by the H.S., E.I. and sonication methods, using a 200mM CF solution (5ml) as the hydrating solution and 150μmoles material. Samples were dialysed against 200mM glucose and entrapment volumes measured.

3.2.2.1 Results

Initially the effect of the cholesterol content on the entrapment volume was examined (Table 3.10) for the three surfactants. In all tables in this section results are shown as the mean value ± standard deviation, with the number of observations shown in brackets.

Table 3.10 Entrapment volumes (l mol^{-1}) for NSV's of different compositions and methods of preparation.

Vesicle Composition	Entrapment Volume (l mol^{-1})		
	H.S.	E.I.	Sonication
I 100	0.34±0.16(4)	0.83±0.085(3)	—
I 70:CHOL 30	0.22±0.092(2)	0.53±0.12(2)	—
I 50:CHOL 50	0.27±0.061(8)	0.31±0.089(3)	0.22±0.076(3)
I 95:DCP 5	0.80±0.16(2)	—	0.46±0.10(2)
II 50:CHOL50	0.15±0.084(7)	—	0.16±0.056(3)
IV 100	0.27±0.096(3)	—	—
IV 50:CHOL50	0.16±0.089(3)	—	0.19±0.04(3)

The greatest aqueous volume was entrapped when vesicles were prepared by the E.I. rather than the H.S. or sonication methods. Increasing the cholesterol content of the vesicles reduced this entrapment. Vesicles prepared from surfactant alone showed the greatest capacity for entrapping aqueous phase although this was only possible with the monoalkyl Surfactants I and IV and not with the dialkyl Surfactant II. Sonication of the pure surfactant systems resulted in disruption of the vesicles. The addition of a negative charge to the composition produced larger volumes of entrapment compared to the uncharged vesicles.

The inclusion of 25mol% or 50mol% Solulan had a marked effect on the entrapment volumes of vesicles produced.

Table 3.11 Entrapment volumes (l mol^{-1}) of hand shaken (H.S.) and sonicated NSV's with compositions containing 25mol% Solulan molecules.

Vesicle Composition	Entrapment Volume (l mol^{-1})	
	H.S.	Sonicated
I50:CHOL25:SOL5 25	0.16±0.073(2)	0.41±0.15(2)
I50:CHOL25:SOL16 25	0.42±0.10(2)	0.85±0.10(2)
I50:CHOL25:SOL24 25	0.86±0.11(2)	1.1±0.21(2)
II50:CHOL25:SOL5 25	0.23±0.064(2)	0.40±0.10 (2)
II50:CHOL25:SOL16 25	0.60±0.09(2)	0.74±0.12(2)
II50:CHOL25:SOL24 25	0.72±0.21(2)	0.99±0.16(2)
IV50:CHOL25:SOL5 25	0.14±0.09(2)	0.18±0.042(2)
IV50:CHOL25:SOL16 25	0.39±0.18(2)	0.54±0.11(2)
IV50:CHOL25:SOL24 25	0.60±0.095(2)	0.75±0.11(2)

The entrapments obtained with vesicles containing 25mol% Solulan molecules are shown in Table 3.11.

For all surfactants, increasing the POE chain length resulted in an increased entrapment and in all cases the sonicated samples produced a greater entrapment than the corresponding H.S. samples.

The inclusion of 50mol% Solulan resulted in similar trends to those seen above (Table 3.12). Increasing the POE chain length produced an increased aqueous entrapment, with sonicated vesicles again showing greater entrapment than the corresponding H.S. vesicles.

Table 3.12 Entrapment volumes (l mol^{-1}) of hand shaken (H.S.) and sonicated NSV's containing 50mol% Solulan.

Vesicle Composition	Entrapment Volume (l mol^{-1})	
	H.S.	Sonicated
I 50:SOL5 50	0.36±0.062(2)	0.34±0.095(2)
I 50:SOL16 50	0.40±0.089(2)	0.81±0.26(2)
I 50:SOL24 50	0.074±0.011(2)	0.054±0.034(2)
II 50:SOL5 50	0.31±0.056(2)	0.35±0.095(2)
II 50:SOL16 50	0.54±0.10(2)	0.80±0.11(2)
II 50:SOL24 50	0.73±0.083(2)	1.19±0.19(2)

The effect on entrapment volume of a gradual increase in the concentration of SOL24 of vesicles containing Surfactant I is shown in Table 3.13. As the mol% of SOL24 was increased, the entrapment volume increased.

Table 3.13 Entrapment volumes (l mol^{-1}) for hand shaken (H.S.) and sonicated NSV's containing increasing amounts of SOL24.

Vesicle Composition	Entrapment Volume (l mol^{-1})	
	H.S.	Sonicated
I50:CHOL 50	0.27±0.061(8)	0.22±0.076(3)
I50:CHOL 49:SOL24 1	0.26±0.082(2)	0.56±0.15(4)
I50:CHOL 45:SOL24 5	0.31±0.076(2)	0.80±0.10(2)
I50:CHOL 40:SOL24 10	0.39 (1)	0.71 (1)
I50:CHOL 25:SOL24 25	0.86±0.11(2)	1.13±0.21(2)

3.2.2.2 Discussion

CF has been widely used in the determination of vesicular properties. It has been used to follow liposome-cell (Weinstein et al, 1977) and liposome-liposome (Wilschut and Papahadjopoulos, 1979) interactions and as a marker to monitor enzymatic attack on intact liposomes (Chen, 1977). CF entrapment has also been reported for non-ionic surfactant vesicles (Baillie et al, 1985). The entrapment volumes measured for the systems used in this study compared favourably with those measured by Baillie et al (1985) for similar vesicular compositions.

It appeared that E.I. vesicles were the most efficient at entrapping aqueous phase since this method tended to produce essentially unilamellar vesicles which were larger than these obtained by sonication. Presumably H.S. vesicles made less efficient use of the available surfactant by producing multilamellar vesicles with aqueous phase trapped between the bilayers.

The addition of a charge to the vesicular composition increased the entrapment volume. The net distance between bilayers was determined by the repulsive forces between them, due to both electrostatic and hydration repulsion by the headgroups, and the attractive van der Waals forces between the hydrocarbon chains in adjacent bilayers (le Neveu et al, 1976). The introduction of a charged molecule to the bilayer caused the bilayers to repel one another and entrapment was thus increased, although the addition of more than 10mol% of a charged molecule did not further increase entrapment (Bangham et al, 1967).

When the Solulan molecules were incorporated into the vesicular composition, they would be incorporated into the bilayer in such a way that their POE chains would extend into the aqueous phase. These chains would form loose helices, in the shape of truncated cones, with water molecules becoming trapped both within and between the spirals. The proportion of water to number of oxyethylene groups would increase as the chain length increased (Elworthy and MacFarlane, 1965; Rawlins, 1977).

Thus it would be expected that as the POE chain length of the Solulan molecule increased, the aqueous entrapment volume would also increase. This effect was observed with all the vesicular compositions which contained 25mol% or 50mol% of Solulan compound. Fig. 3.4 shows the increased entrapment with increasing POE chain length for vesicles containing 50mol% surfactant, 25mol% cholesterol and 25mol% Solulan compound. It also clearly shows that increased entrapment volumes can be attained by preparing vesicles by the sonication rather than the hand shaken method for all the surfactants examined.

When the mol% of SOL24 was increased from zero, initially the entrapment would increase due to aqueous phase being trapped within the POE helices and as the concentration was increased, more aqueous phase would become trapped between the helices. This increase was observed when vesicles were formed by both the hand shaken and sonication methods of preparation (Fig. 3.5), although sonicated vesicles produced a much greater increase in entrapment volume after the addition of a few mol% of SOL24. Increasing the concentration of SOL24 in the vesicular bilayer would only increase the entrapment volume of the system if vesicles continued to be formed. Too high a concentration of the SOL24 would not produce a vesicular system, but one which contained the disc aggregates, with consequent poor entrapment, where the system was likely to have become solubilized.

The incorporation of SOL24 with monoalkyl surfactants, in equimolar ratios, did not produce vesicles, but disc-like aggregates were formed instead. These discs had very low aqueous entrapment, and appeared to be fairly rigid since they broke up when sonicated leading to a reduced entrapment volume being measured after sonication. The disc-like aggregates observed may be similar to those noted by Okahata et al (1980) using single chain phosphocholine amphiphiles possessing a rigid hydrophobic portion.

Sonication of multilamellar vesicles incorporating SOL5 and monoalkyl surfactants in equimolar ratios resulted in a breakdown of the system which spontaneously reformed on cooling. The vesicles which reformed were multilamellar and appeared to have entrapped smaller vesicles in the core. The resulting vesicles looked similar to the multivesicular liposomes reported by Talsma et al (1987) and Kim and Howell (1987).

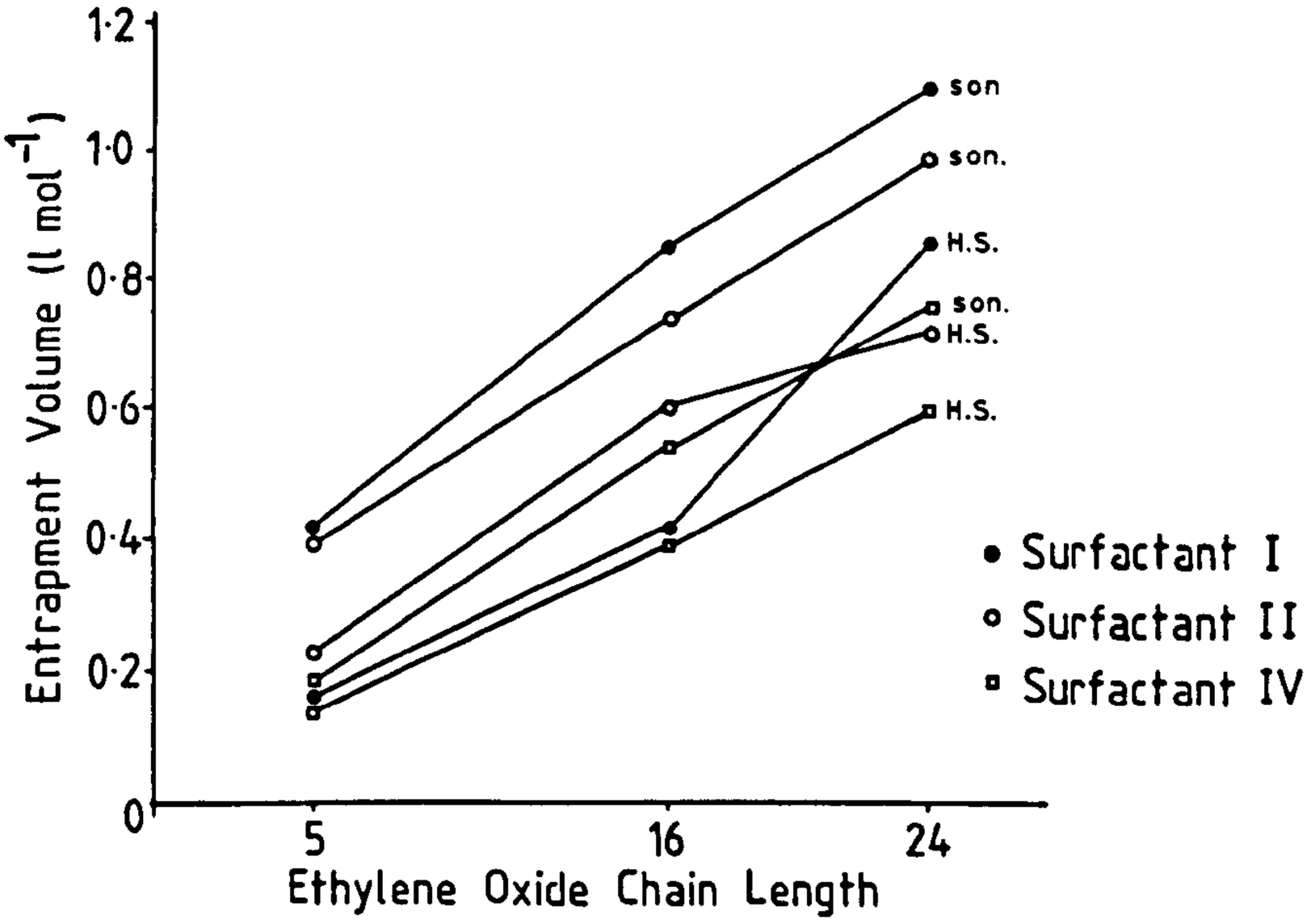


Fig. 3.4 The effect of increasing the POE chain length on the entrapment volume of vesicle samples prepared by the hand shaken (H.S.) and sonication (son.) methods is shown for vesicles composed of 50mol% surfactant. 25mol% cholesterol and 25mol% Solulan compound.

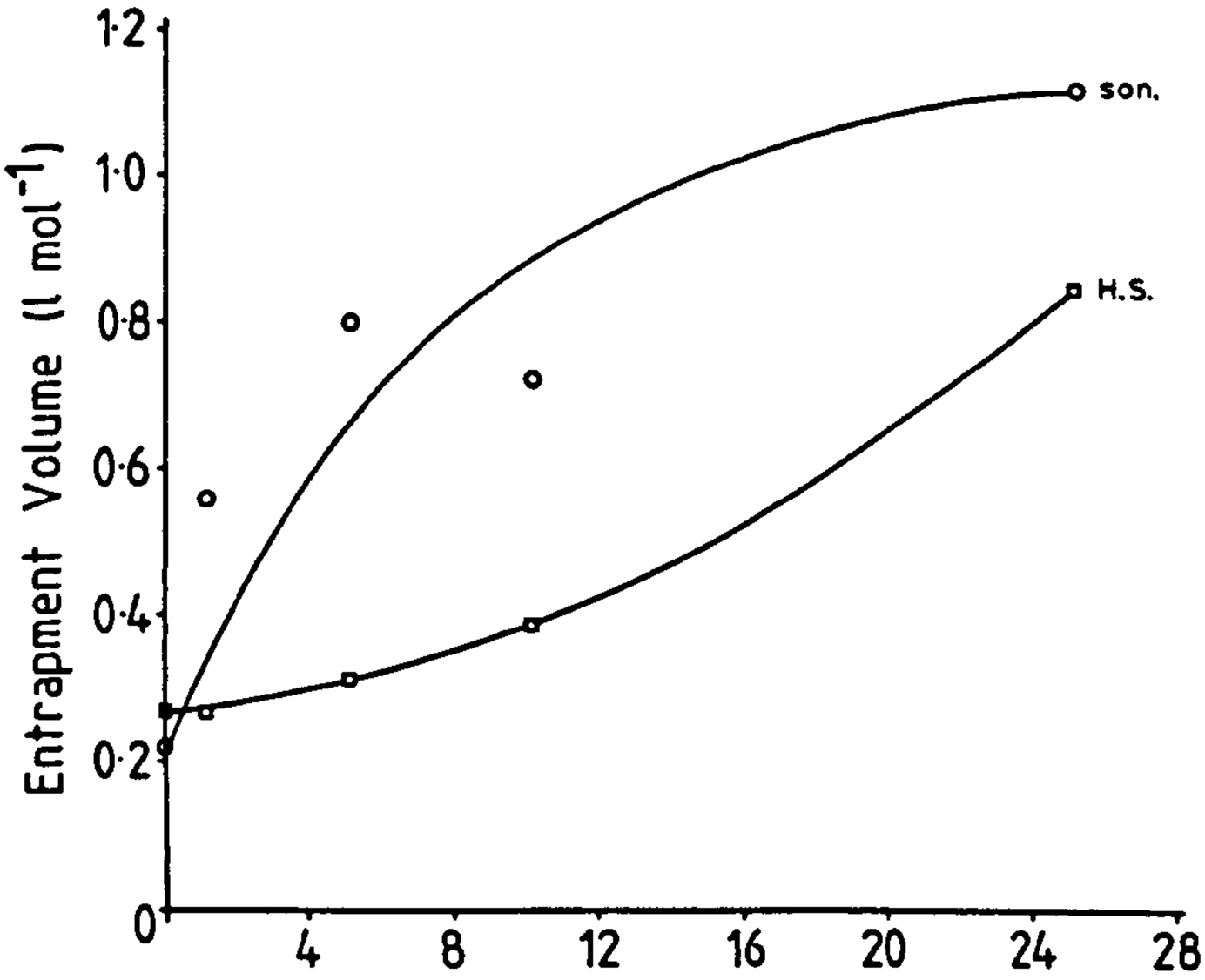


Fig. 3.5 The effect of increasing the concentration of Solulan C-24 on the entrapment volume of vesicles prepared by the hand shaken (H.S.) and sonication (son.) methods is shown. (Vesicular composition: I 50:CHOL (50-X):SOL24 X)

In an attempt to estimate the entrapment volumes likely to be obtained for samples with a given vesicle size, data from the previous two sections were combined. Plotting the vesicle size against the entrapment volume for both hand shaken (Fig. 3.6) and sonicated (Fig. 3.7) vesicle samples, irrespective of the composition of the vesicles involved, produced graphs which appeared to show similar trends. The best entrapment volumes were obtained at the extremes of the size ranges examined while the lowest entrapments were measured in the middle of the ranges. Interestingly, for sonicated vesicle samples the lowest entrapment volumes were obtained within the size range 200-350nm, the range where most of the sonicated vesicles containing surfactant and cholesterol was found.

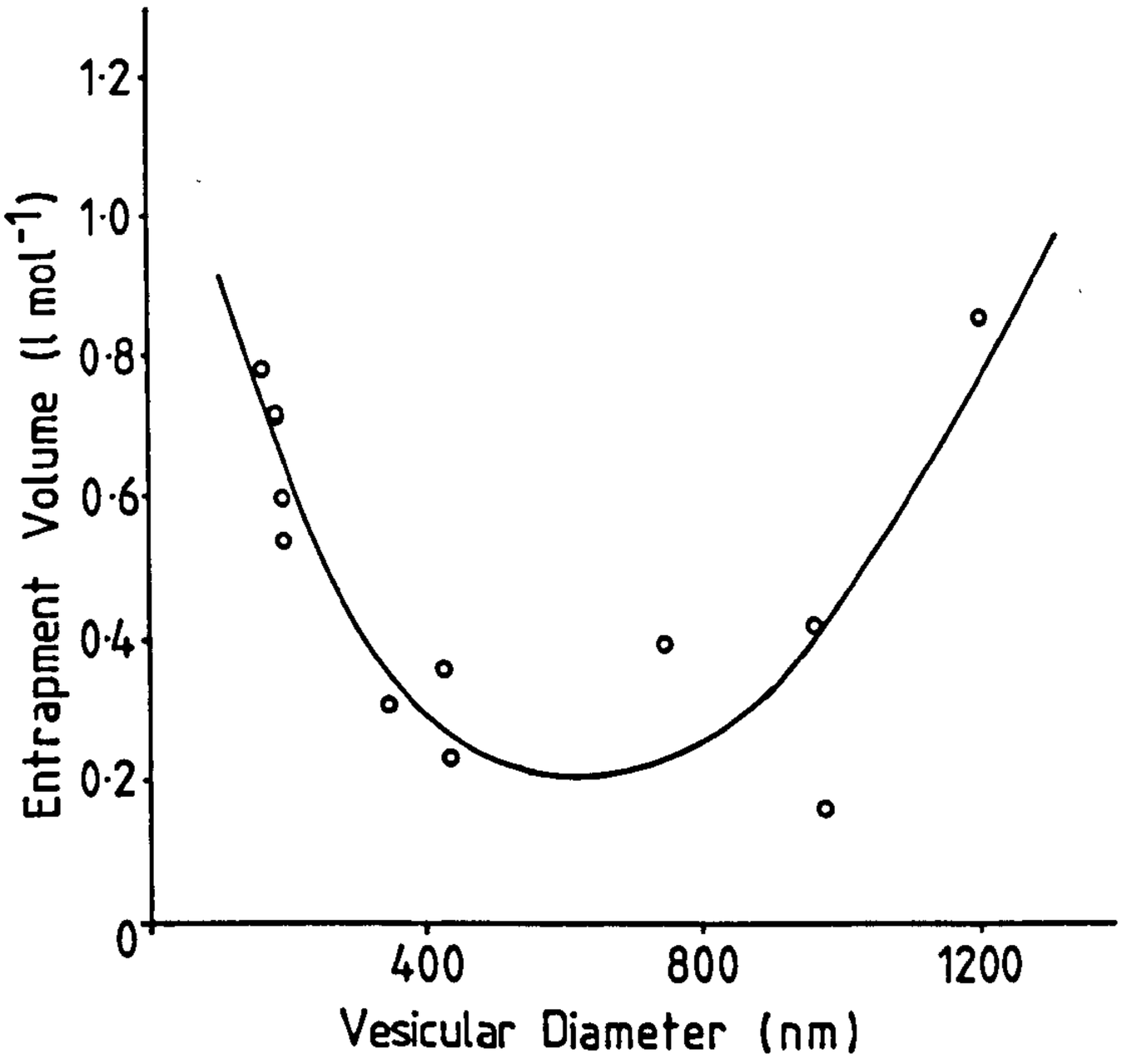


Fig. 3.6 The entrapment volumes of hand shaken vesicle samples against the vesicular diameter of the vesicles are shown.

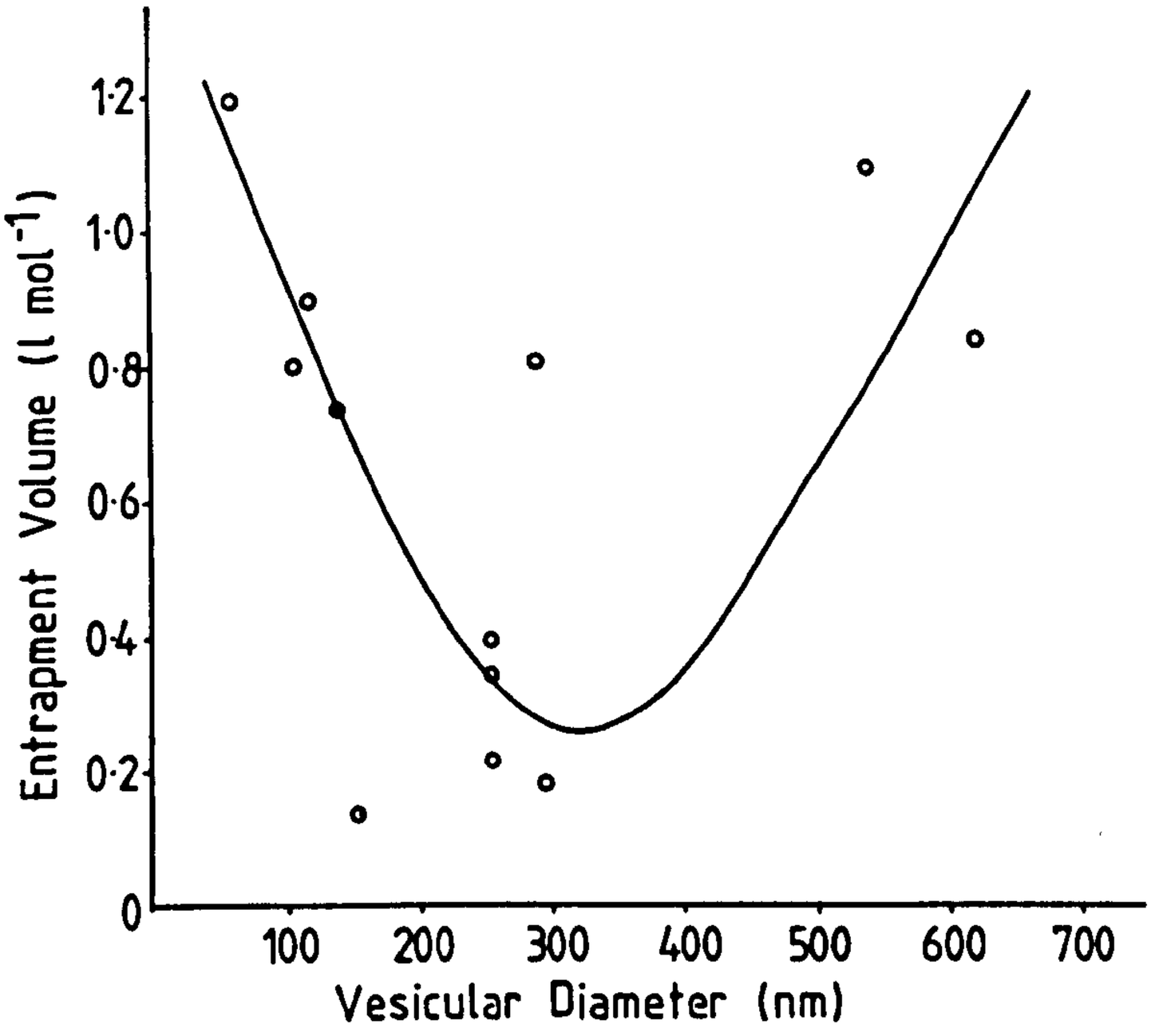


Fig. 3.7 The entrapment volumes of sonicated vesicle samples against the vesicular diameter obtained are shown.

3.2.3 Vesicular Aggregation Induced by NaCl

Aggregation and alterations in the appearance of vesicles have been identified by many workers as a problem, being induced by a variety of compounds and ionic species.

Aggregation *in vitro* has been reported by Storm *et al* (1989), when attempting to encapsulate DOX in negatively charged liposomes. Many examples of alterations in the structure of liposomes induced by divalent cations, particularly Ca^{2+}, have been reported. Abraham *et al* (1987) observed that small unilamellar liposomes were slowly transformed into large unilamellar liposomes and then to broad lamellar sheets by the addition of calcium ions. The formation of helical liposomes in the presence of Ca^{2+} has also been noted (Lin *et al*, 1982). Monovalent ions have also been shown to have an effect on vesicles, with NaCl shown capable of inducing either aggregation or fusion of vesicles depending on their composition (Carmona-Ribeiro and Chaimovich, 1986).

The aggregation of vesicles after injection *in vivo* may also affect their distribution, depending on the degree of aggregation.

The effect of NaCl on the aggregation of NSV's was examined since this had been previously identified as a potential problem with NSV's by Rogerson (1986).

3.2.3.1 Results

Sonicated vesicles were prepared containing 150μmoles material in water (2ml). A small aliquot (50μl) of this dispersion was diluted with 0.2μm filtered NaCl solution (to 2ml) and the change in vesicle size and polydispersity measured by PCS.

After each measurement, samples were vortexed and quickly

remeasured to indicate whether aggregation or fusion of vesicles had occurred.

In all samples examined, the mean measured size and the polydispersity followed the same trend i.e. an increase in the mean size corresponded with a rise in the polydispersity, and a fall in the measured size resulted in a reduced polydispersity. Samples produced aggregated rather than fused systems after addition of NaCl since the original vesicle size could be measured after vortexing the aggregated dispersions. For some vesicular compositions, size measurement had to be carried out very quickly after ending vortexing as the vesicle began to reaggregate immediately.

Vesicles containing I 50:CHOL 50 showed a maximum aggregation 12-26min after addition of the NaCl solution, and as the concentration was increased from 0.0435-0.2112% w/v, the maximum measured size also increased (Fig. 3.8). This maximum size was maintained for all but the two most concentrated solutions, where a fall in size was observed after about 27min.

Having seen that maximum aggregation was attained about 20 min after mixing the vesicles with the NaCl solution, several concentrations of NaCl were measured after this time and the size measured plotted against the NaCl concentration of the solution (Fig. 3.9). The measured size increased as the concentration of NaCl increased up to 0.17%w/v, then fell with further increases up to 1.0%w/v, and remained constant thereafter.

The inclusion of Solulan C-24 in the vesicular composition (i.e. I 50:CHOL 25:SOL24 25) resulted in an initial fall in the measured size when NaCl was added (Fig. 3.10). The addition of 0.0435%w/v and 0.08636%w/v NaCl produced a slight increase in the measured size

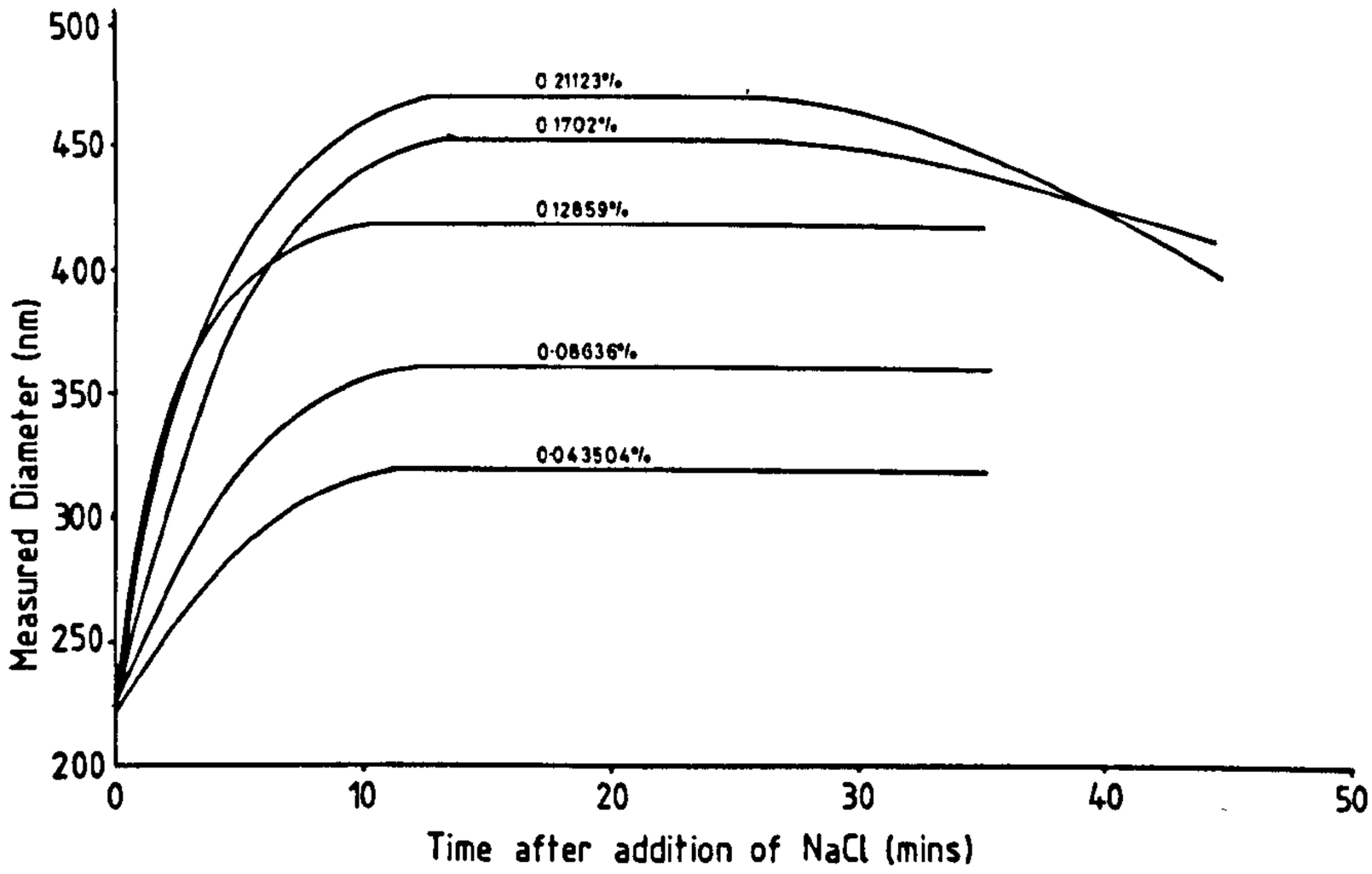


Fig. 3.8 The apparent diameter (nm) of vesicles composed of I 50:CHOL 50 after addition of the stated concentration of NaCl with time.

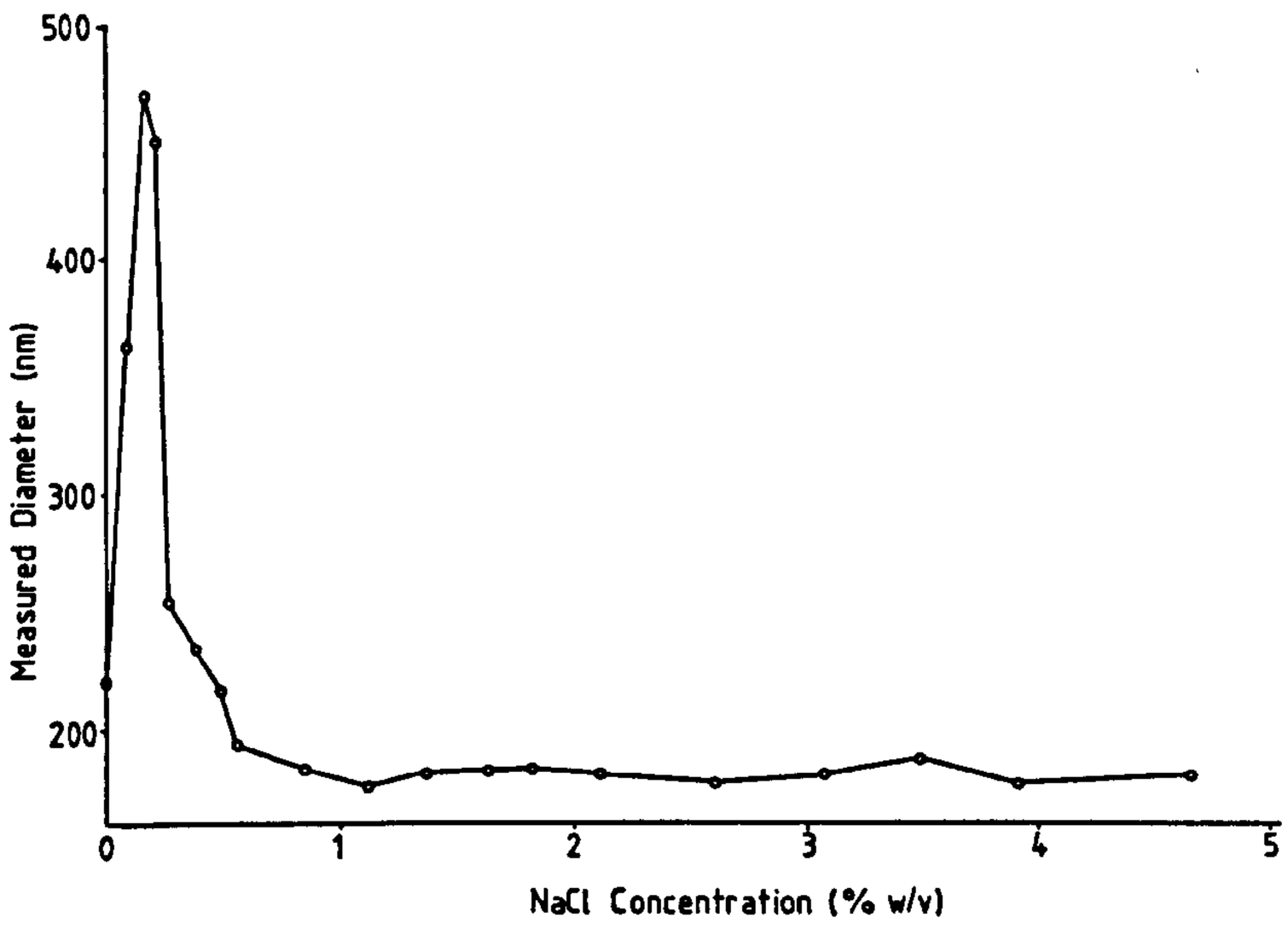


Fig. 3.9 The maximum measured apparent size produced by vesicles containing I 50:CHOL 50 after the addition of NaCl.

of the vesicles, while 0.13%$^{w}/_{v}$ NaCl caused a slight fall in the measured size.

The addition of 0.144%$^{w}/_{v}$ NaCl to vesicles containing IV 50:CHOL 50 resulted in a rapid and continual aggregation of the vesicles which was still occurring 3h after addition of NaCl (Fig. 3.11). The vesicles aggregated rapidly from the initial size of 280nm to about 6.5μm after 200min. Vesicles with Surfactant IV appeared to be much more sensitive to aggregation by NaCl than those containing Surfactant I.

When a charged molecule was added to vesicles prepared with Surfactant IV, aggregation could be prevented (Fig. 3.12). Increasing the amount of NaCl added to positively charged vesicles (IV 47.5:CHOL 47.5:SA 5.0) resulted in a fall in the size measured, while the addition of NaCl to negatively charged vesicles (IV 47.5:CHOL 47.5:DCP 5.0) caused a less marked reduction in size.

3.2.3.2 Discussion

Before discussing the results, it is important to outline some of the limitations and possible problems associated with measuring aggregation by PCS.

When the concentration of the NaCl solution was increased, there would be a slight change in the refractive index and viscosity of the solution compared with the values of water. These parameters were required by the PCS to calculate the mean diameter of the vesicles and any changes would affect the value calculated. Another potential problem, and one not limited to this series of experiments, was that extensive dilution of the original vesicle sample was required to prevent multiple scattering of the laser beam of the PCS. Thus, what was measured in this diluted system may not be representative of

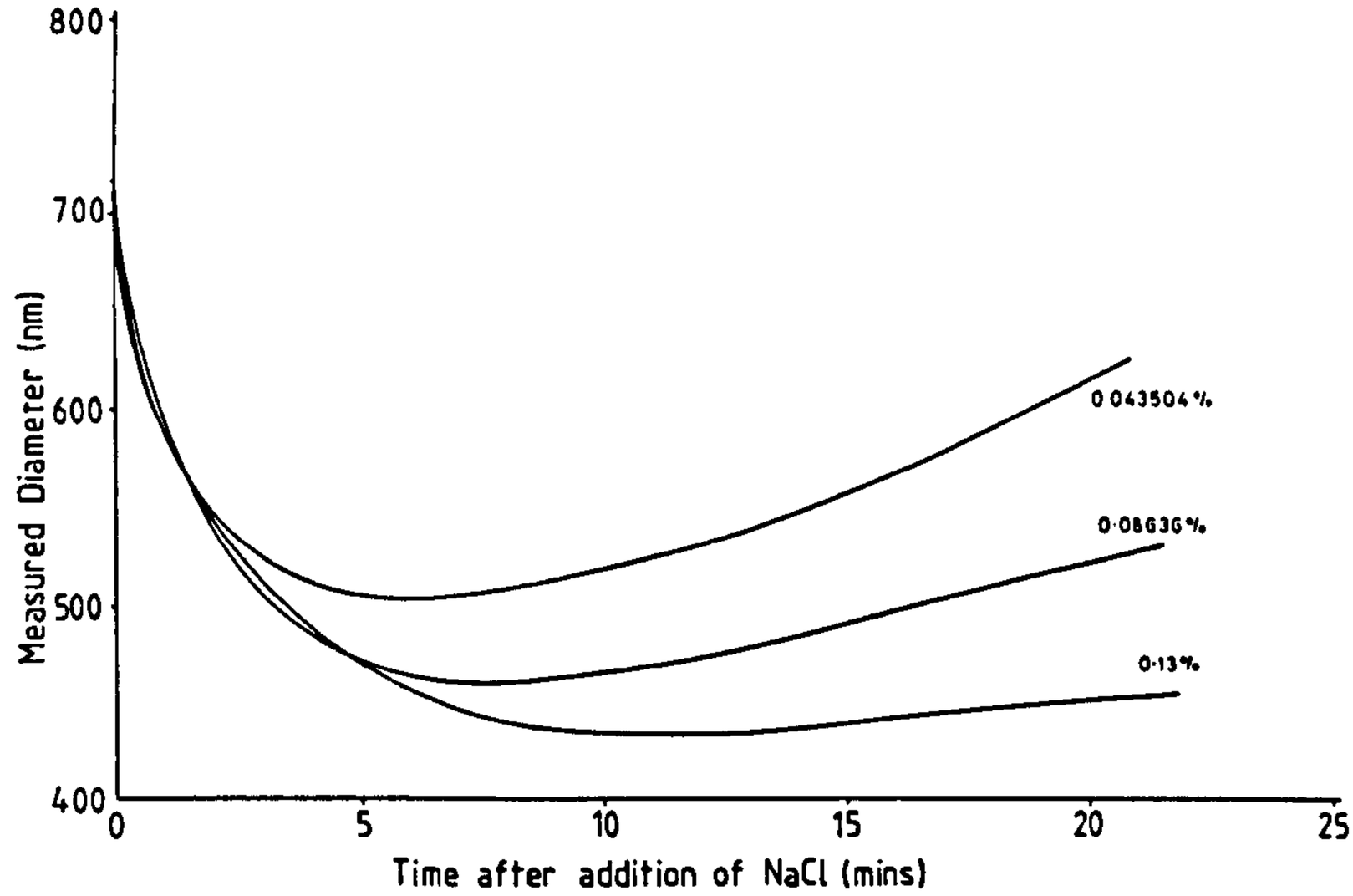


Fig. 3.10 The measured size of vesicles composed of I 50:CHOL 25: SOL24 25, after the addition of the concentrations of NaCl shown, with time.

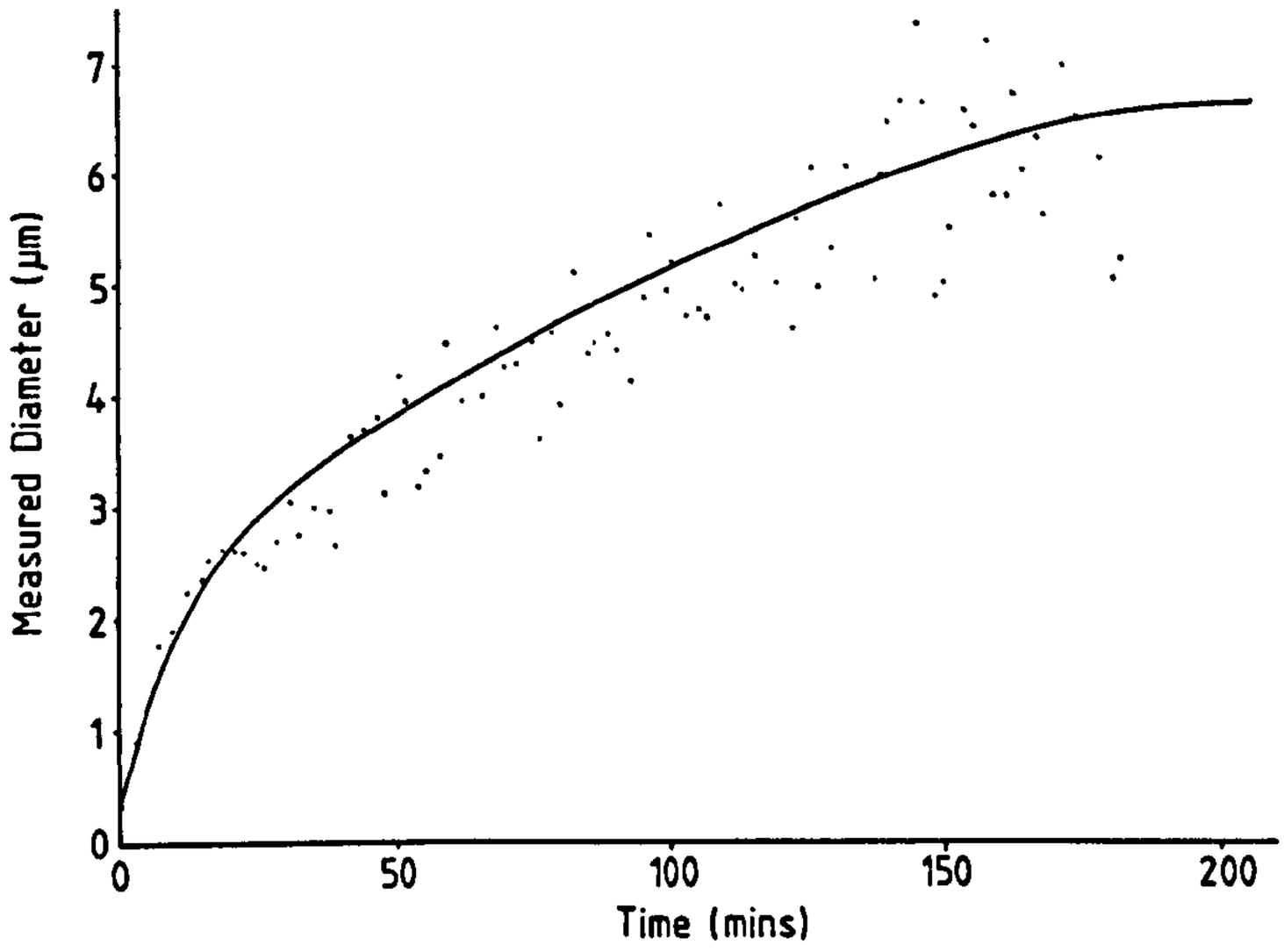


Fig. 3.11 The change in the apparent size of vesicles composed of IV 50:CHOL 50 with time, after the addition of 0.144%w/v NaCl.

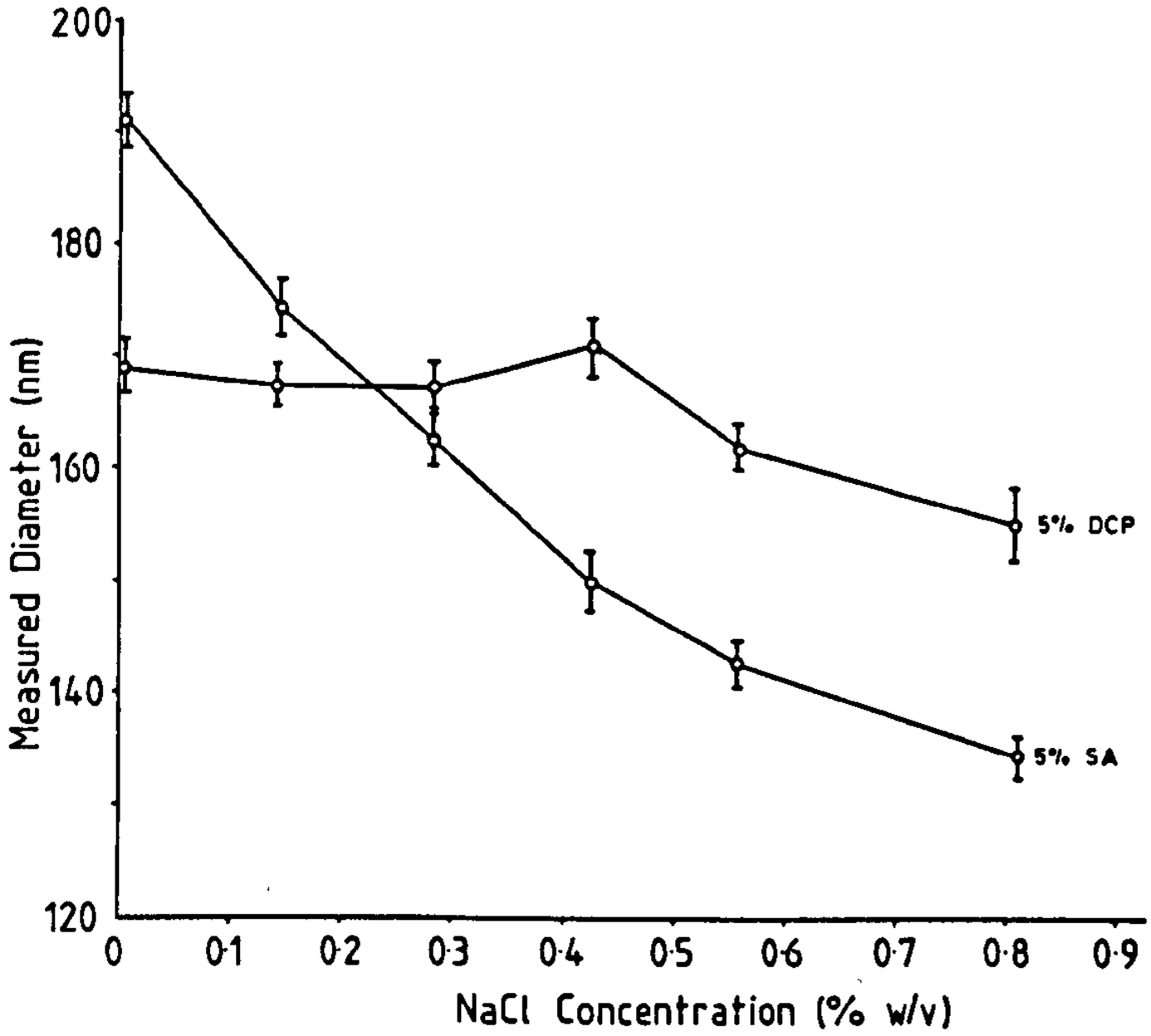


Fig. 3.12 The effect of NaCl on the apparent size of vesicles containing IV 47.5:CHOL 47.5:DCP 5.0 (5% DCP) and IV 47.5:CHOL 47.5:SA 5.0 (5% SA). Measurements were recorded 20min after the addition of NaCl and the points are plotted as mean ± standard deviation.

what was happening in the bulk dispersion. In this particular instance, it was likely that the presence of forty times more vesicles would alter the way in which the vesicles interacted with each other.

The principal interactions between phospholipid bilayers at long ranges were electrostatic, for charged vesicles, and electrodynamic (van der Waals). At short ranges, very strong repulsive forces, probably due to the structured ordering of water induced by the phospholipid headgroups were present (le Neveu et al, 1976; Marra and Israelachvili, 1985).

For vesicles to aggregate or fuse, there must be 1) mutual approach, 2) destabilization of the membranes, and 3) expansion of the area of membrane contact during adhesion or expansion of the hole during fusion (Dimitrov et al, 1985).

In the vesicle samples examined, the addition of NaCl did not appear to have caused vesicular fusion since the original vesicle size could be obtained after vigorous agitation of the sample.

Vesicles produced with dioctadecyldimethylammonium chloride (DODAC) and sodium dihexadecyl phosphate (DHP) were shown to interact at low concentrations of NaCl (Carmona-Ribeiro, 1989), while phospholipid vesicles appeared to be stable after addition of relatively high concentrations of NaCl (Ohki et al, 1984).

NaCl appeared to have a marked effect at low concentrations when added to vesicles containing Surfactant I, but the effect was only noted over a small concentration range with no evidence of aggregation at higher concentrations. At high concentrations, osmotic shrinkage of the vesicles may be occurring. This would be produced as the internal environment of the vesicle was water (the

hydrating solution) while the outside was the NaCl solution. Although small ions were capable of passing across membranes they were not as freely permeable as water (Papahadjopoulos, 1973c) and as a result the net flow of water would be out of the vesicle resulting in osmotic shrinkage.

At low NaCl concentrations and neutral pH, hydration forces were absent when amphiphile surfaces interacted (Carmona-Ribeiro, 1989) and hence vesicles would be more likely to approach closely enough, resulting in aggregation.

Comparing the aggregation observed with Surfactants I and IV, it was obvious that the latter were much more susceptible to aggregation under the influence of NaCl. This was most likely due to the differences in the headgroups of the surfactant molecules, since Surfactant IV had a much smaller and less bulky hydrophilic headgroup. The headgroup of Surfactant I had a greater potential to hydrogen-bond water with the ether oxygens and its greater bulk may also physically trap more water. Under the influence of NaCl the headgroups would become less hydrated due to the salting out effect of the NaCl. Surfactant IV was likely to have less associated water and become less stable by the salting out of water causing intervesicular hydrogen-bonding between the headgroups and, ultimately, aggregation.

The addition of 5mol% DCP or SA overcame this aggregation associated with Surfactant IV vesicles. Charged species were capable of overcoming the weak, short range van der Waals attractive forces by long range electrostatic repulsion (Evans and Needham, 1987) resulting in the vesicles remaining separate (see next section). Once again a slight reduction in size was measured and

may be due to osmotic shrinkage of the vesicles.

Incorporation of SOL24 into vesicles created a further hydrophilic area around the outside of the vesicle, which would be considerably hydrated, with water both hydrogen-bonded to the ether oxygen and physically trapped within and between the POE chains. The motion of the POE chains extending from the surface of the vesicle was likely to be restricted, and would form helices in the shape of truncated cones (Elworthy and MacFarlane, 1965; Rawlins, 1977).

Micelles formed from compounds with POE chains as the hydrophilic group were found to be less likely to coalesce due to the hydrophilic nature of the surface, and because the compacted, hydrated POE chains may act as an energy barrier (Florence, 1969). Oil-in-water emulsions containing non-ionic surfactants with POE chains were found to be more stable as the POE chain length increased.

Also as the POE chain length was increased the closest distance two particles may approach each other was also increased, leading to the suggestion that another entropic or steric stabilizing effect must also be important (Elworthy and Florence, 1969).

The addition of NaCl to systems containing these molecules caused an initial fall in the measured size, possibly due to dehydration of the POE chains and some degree of osmotic shrinkage. At low NaCl concentrations, it appeared that the vesicles began to regain their original size , while as the concentration was increased, this recovery took longer to start and occurred at a slower rate. At no stage did the vesicle size of any of the samples measured become greater than that of the original measured size

indicating that no aggregation had taken place. The reason why these vesicles return to their original size is not clear, but may involve equilibration of the added NaCl on both sides of the bilayer.

This series of experiments showed that uncharged vesicles tended to be unstable and liable to aggregate when exposed to NaCl, but that this instability could be overcome by the addition of a charged species or by increasing the hydrophilicity of the vesicular surface.

3.2.4 Aggregation of Vesicles with Time

Since uncharged vesicles containing Surfactant IV had been shown to be susceptible to aggregation, uncharged, positively and negatively charged sonicated vesicles were prepared containing 150μmoles in water(2ml). 50μl of each sample was diluted with 0.2μm filtered distilled water (to 2ml) and the mean diameter and polydispersity measured by PCS at 0, 1, 10 and 30 days (Table 3.14).

Table 3.14 Mean vesicle diameter (nm) and polydispersity as a function of time (days).

Vesicular Composition	Time (days)			
	0	1	10	30
IV 50: CHOL 50	281.0nm± 4.1/0.12	283.0nm± 3.6/0.13	288.2nm± 1.2/0.14	312.0nm± 4.0/0.21
IV 47.5: CHOL 47.5: SA 5.0	196.3nm± 2.7/0.17	203.3nm± 2.4/0.17	211.5nm± 2.1/0.23	190.8nm± 3.2/0.23
IV 47.5: CHOL 47.5: DCP 5.0	186.1nm± 3.4/0.21	186.2nm± 2.6/0.20	184.8nm± 4.3/0.19	179.0nm± 2.3/0.18

Uncharged or charged vesicles prepared in water appeared to be stable, with little evidence of aggregation after 30days. Similar results were obtained when 300mM glucose was used as the hydrating solution, but a gradual aggregation was observed when phosphate buffered saline was used to prepare uncharged vesicles. These

trends were also noted when Surfactant IV was substituted with Surfactant I.

3.2.5 Measurement of Vesicular Charge

Estimating the charge residing at the shear plane of a vesicle was important as this could affect its fate in vivo. It has been shown that blood clearance of neutrally and positively charged unilamellar liposomes occurred less rapidly than negatively charged ones (Juliano and Stamp, 1975).

H.S. samples were prepared using a hydrating solution of $2x10^{-3}M$ NaCl. Despite the fact that NaCl had previously been shown to cause vesicular aggregation, preparation in this low concentration of NaCl did not produce any microscopic evidence of aggregation. No obvious signs of osmotic shrinkage were observed i.e. microscopic examination showed no evidence of hexagonally shaped structures typically seen when multilamellar vesicles become dehydrated (Rogerson, 1986), probably because both sides of the bilayer were at the same ionic strength.

The electrophoretic mobility (EM) of each vesicular composition was measured at pH 4.5, 6.0, 7.4 and 9.5, values plotted against pH and the zeta potentials calculated.

3.2.5.1 Non-Ionic and Charged Vesicles

EM's were measured for vesicles composed of I 50:CHOL 50 and positively or negatively charged vesicles containing 5mol% SA or DCP respectively. The same compositions of vesicles were prepared using Surfactant IV.

The EM's obtained at the pH values stated are shown in Fig. 3.13 for vesicles containing Surfactant I, and Fig. 3.14 for those containing Surfactant IV. The calculated zeta potentials are shown in Table 3.14 and Table 3.15 respectively.

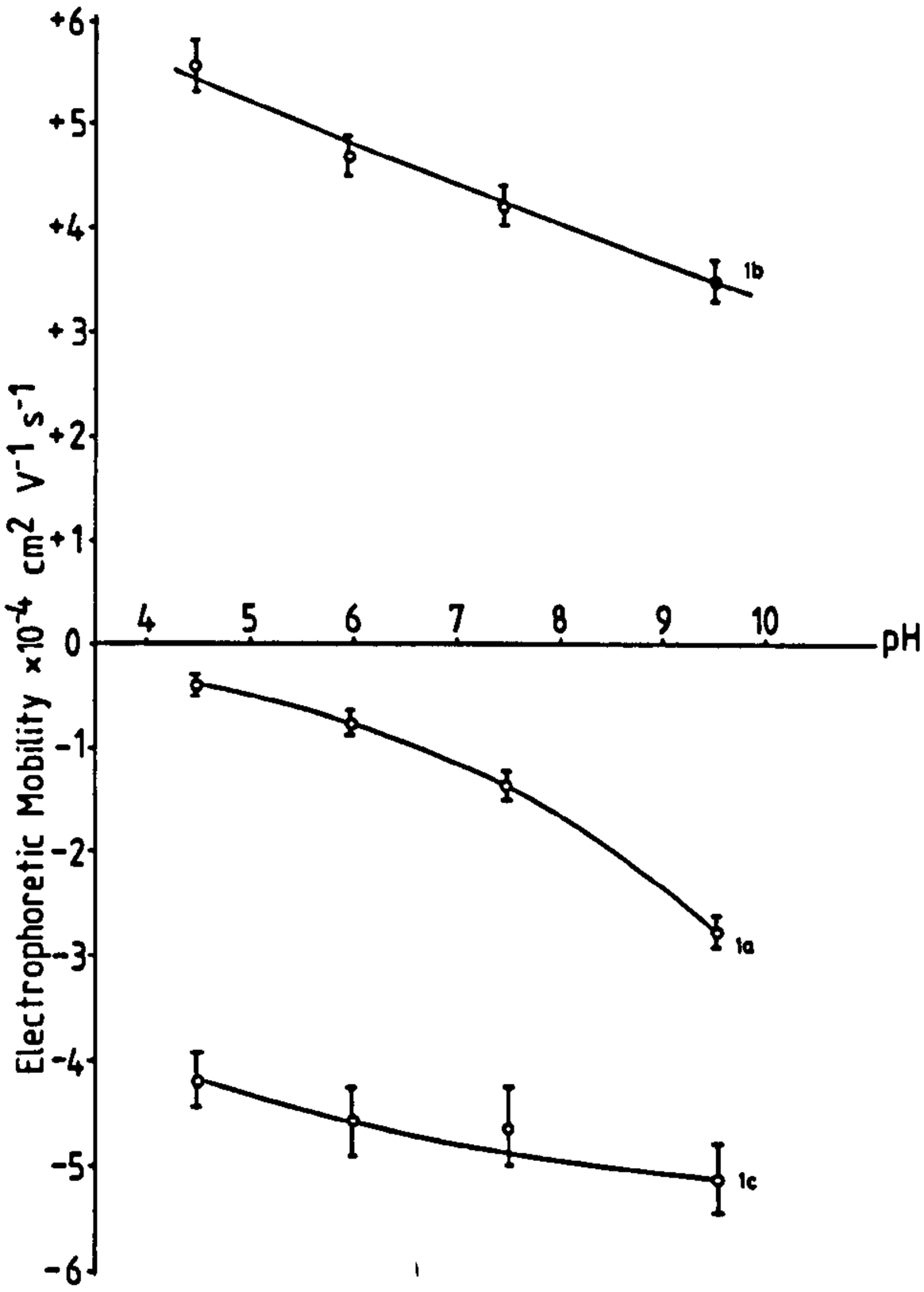


Fig. 3.13 Electrophoretic mobilities plotted against pH of solution for vesicles composed of: 1a) I 50:CHOL 50; 1b) I 47.5:CHOL 47.5:SA 5.0 and 1c) I 47.5:CHOL 47.5:DCP 5.0. Points are plotted as mean value ± standard deviation.

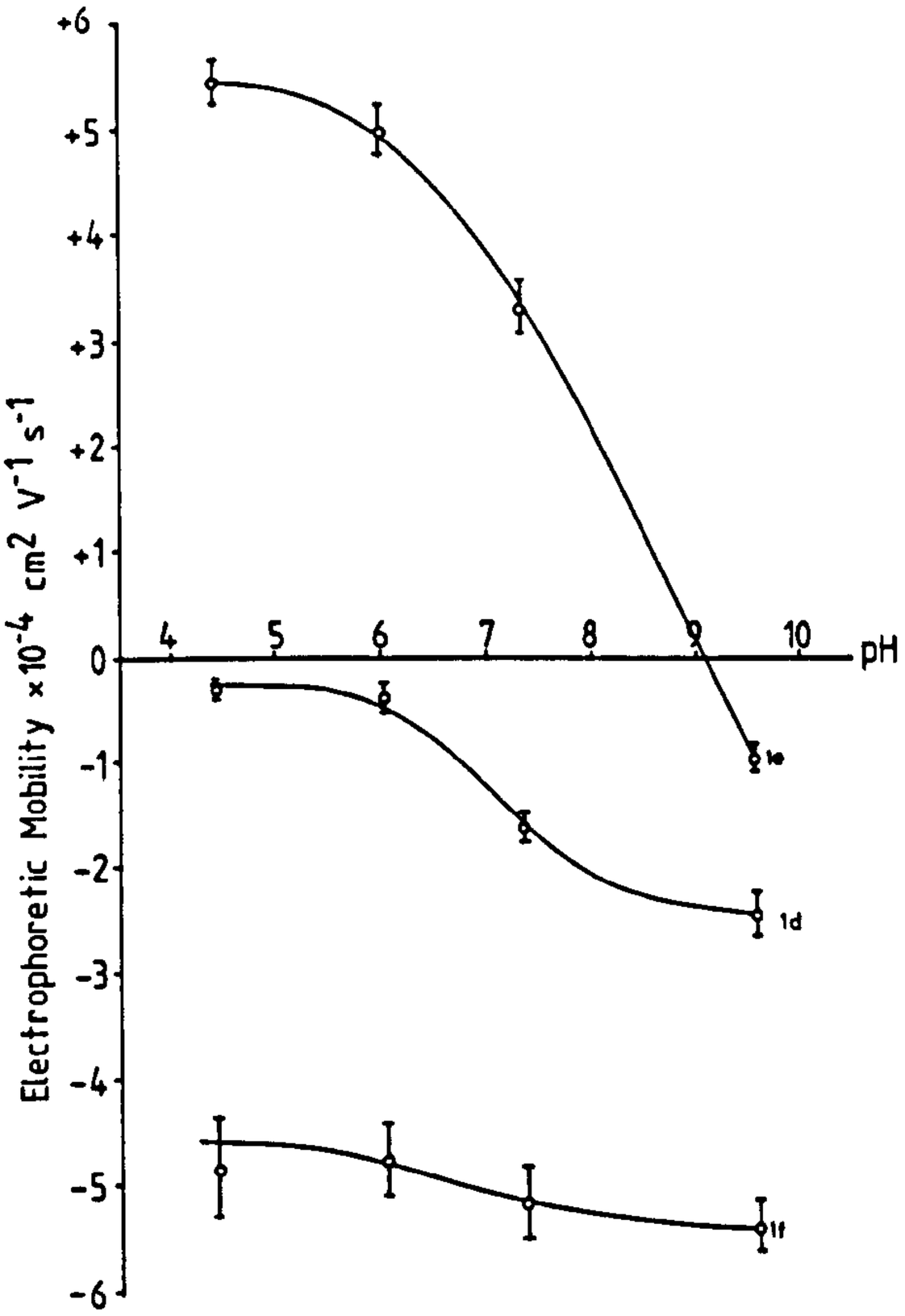


Fig. 3.14 Electrophoretic mobilities plotted against pH of solution for vesicles composed of: 1d) IV 50:CHOL 50; 1e) IV 47.5:CHOL 47.5:SA 5.0 and 1f) IV 47.5:CHOL 47.5:DCP 5.0. Points are plotted as mean value ± standard deviation.

As the pH was increased a more negative EM was measured for all compositions. Vesicles containing no added charge were found to have a negative EM. Positively charged vesicles containing Surfactant IV showed a rapid fall in EM as the pH was increased, and became negatively charged at pH 9.5.

Table 3.14 Zeta potentials for vesicles containing Surfactant I, as a function of pH.

Vesicle Composition	Zeta Potential (mV)			
	pH 4.5	pH 6.0	pH 7.4	pH 9.5
I 50: CHOL 50	-4.95	-9.68	-17.41	-35.15
I 47.5: CHOL 47.5: SA 5.0	+71.24	+60.51	+54.27	+45.55
I 47.5: CHOL 47.5: DCP 5.0	-53.63	-58.57	-58.93	-65.75

Table 3.15 Zeta potentials for vesicles containing Surfactant IV. as a function of pH.

Vesicle Composition	Zeta Potential (mV)			
	pH 4.5	pH 6.0	pH 7.4	pH 9.5
IV 50: CHOL 50	-3.535	-4.602	-20.57	-31.34
IV 47.5: CHOL 47.5: SA 5.0	+70.46	+64.28	+42.78	-12.22
IV 47.5: CHOL 47.5: DCP 5.0	-61.69	-60.62	-65.91	-69.10

3.2.5.2 Effect of Polyoxyethylene Chain Length on Electrophoretic Mobility

Uncharged and charged vesicles were prepared containing Surfactant I, cholesterol and Solulan molecules.

The EM's obtained from the vesicles with no added charge are shown in Fig. 3.15 and the zeta potentials calculated from these values in Table 3.16.

All vesicles examined were negatively charged at all pH values. Increasing the pH of the solution caused the vesicles to become more negatively charged. At low pH, all vesicle compositions had very similar EM's but as the pH was increased the differences in the EM's became greater. In general, as the POE chain length was increased the change in the EM with increasing pH was less.

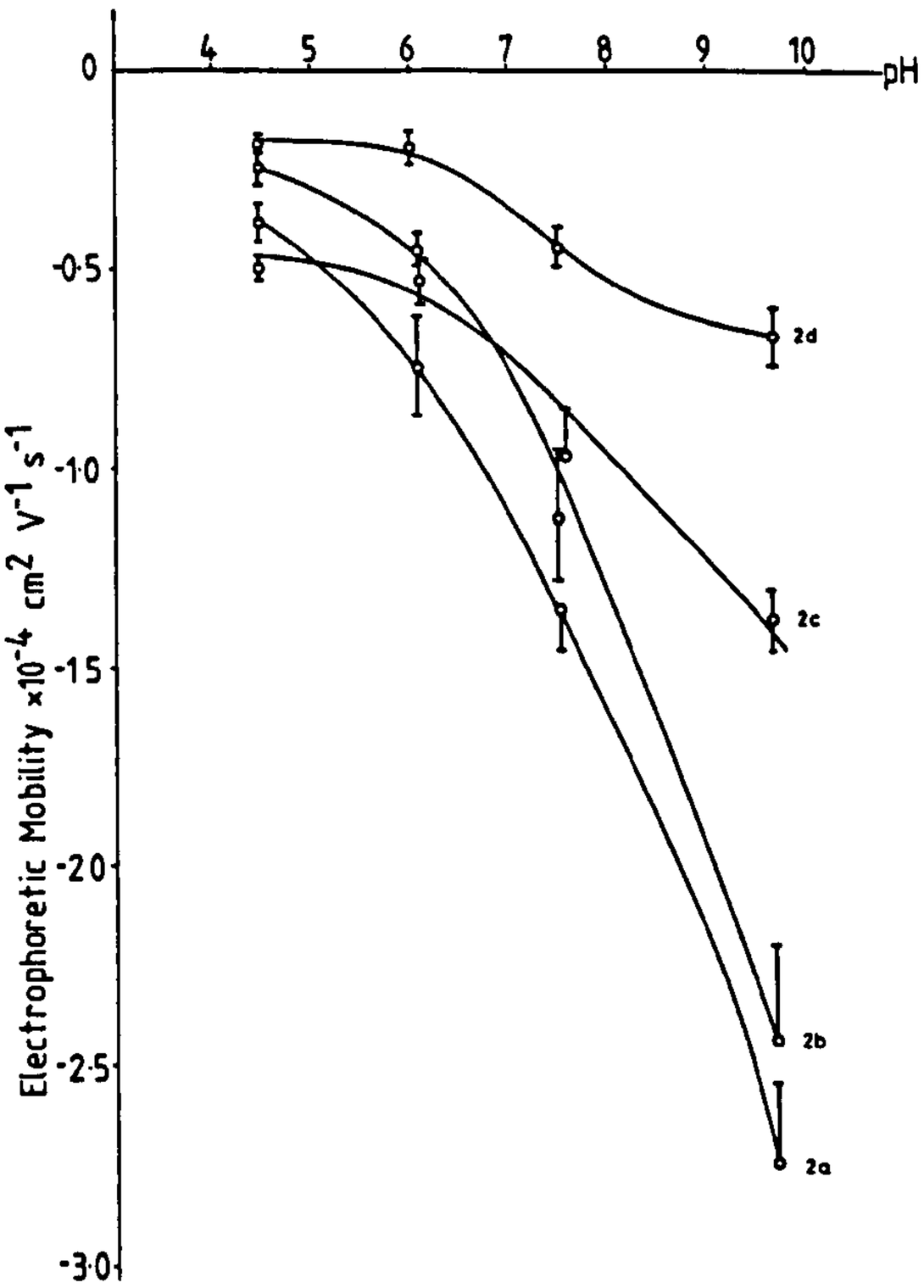


Fig. 3.15 Electrophoretic mobilities plotted against pH of solution for vesicles composed of: 2a) I 50:CHOL 50; 2b) I 50:CHOL 25:SOL5 25; 2c) I 50:CHOL 25:SOL16 25 and 2d) I 50:CHOL 25:SOL24 25. Points are plotted as mean value ± standard deviation.

Table 3.16 Zeta potentials for vesicles composed of Surfactant I with Solulan molecules.

Vesicle Composition	Zeta Potential (mV)			
	pH 4.5	pH 6.0	pH 7.4	pH 9.5
I 50: CHOL 50	-4.95	-9.68	-17.41	-35.15
I 50: CHOL 25: SOL5 25	-3.08	-5.86	-14.43	-31.25
I 50: CHOL 25: SOL16 25	-6.39	-6.77	-12.58	-17.80
I 50: CHOL 25: SOL24 25	-2.42	-2.48	-5.79	-8.67

The addition of 5mol% DCP to the vesicular composition resulted in the vesicles being much more negatively charged. Fig. 3.16 shows the EM's measured against the pH of the solution, and Table 3.17 the zeta potentials calculated.

Increasing the pH of the solution caused the vesicles to become slightly more negatively charged. Increasing the POE chain length reduced the EM at a given pH, although in this case vesicles containing SOL16 reduced the EM to a greater extent than those with SOL24.

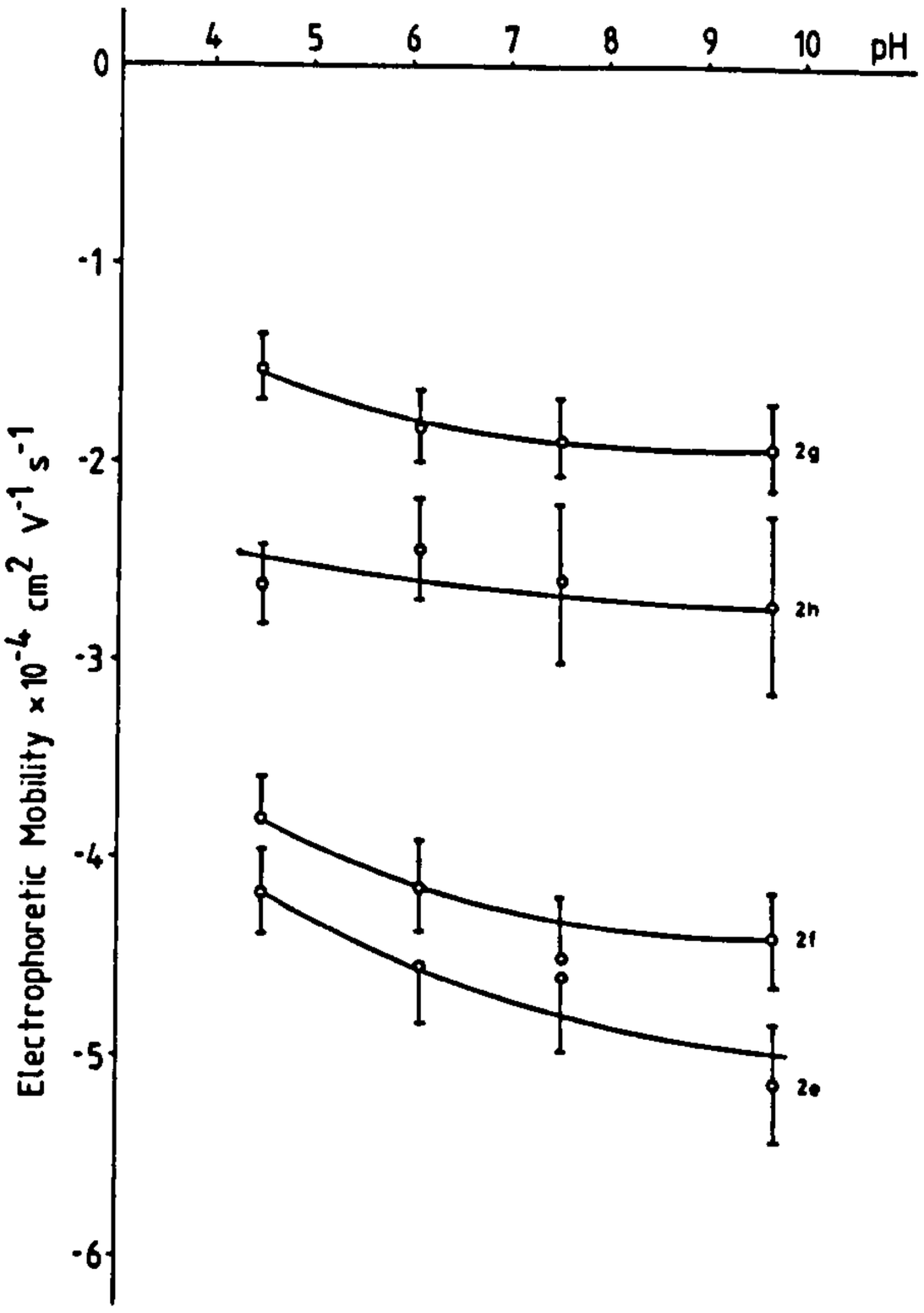


Fig. 3.16 Electrophoretic mobilities plotted against pH of solution for vesicles composed of: 2e) I 47.5:CHOL 47.5:DCP 5.0; 2f) I 47.5: CHOL 23.75:SOL5 23.75:DCP 5.0; 2g) I 47.5:CHOL 23.75:SOL16 23.75:DCP 5.0 and 2h) I 47.5:CHOL 23.75:SOL24 23.75:DCP 5.0. Points are plotted as mean values ± standard deviation.

Table 3.17 Zeta potentials for vesicles containing an added negative charge.

Vesicle Composition	Zeta Potential (mV)			
	pH 4.5	pH 6.0	pH 7.4	pH 9.5
I 47.5: CHOL 47.5: DCP 5.0	-53.63	-58.57	-58.93	-65.75
I 47.5: CHOL 23.75: SOL5 23.75: DCP 5.0	-48.84	-53.37	-58.07	-56.26
I 47.5: CHOL 23.75: SOL16 23.75: DCP 5.0	-19.54	-23.45	-24.24	-24.84
I 47.5: CHOL 23.75: SOL24 23.75: DCP 5.0	-33.67	-31.37	-33.47	-35.12

The addition of 5mol% SA to the vesicular composition resulted in vesicles which were positively charged. EM's measured at the stated pH are plotted in Fig. 3.17 and calculated zeta potentials are shown in Table 3.18. Increasing the pH of the solution resulted in vesicles of a given composition becoming less positively charged i.e. they become more negative. As the POE chain length was increased the vesicles became less positive at a given pH.

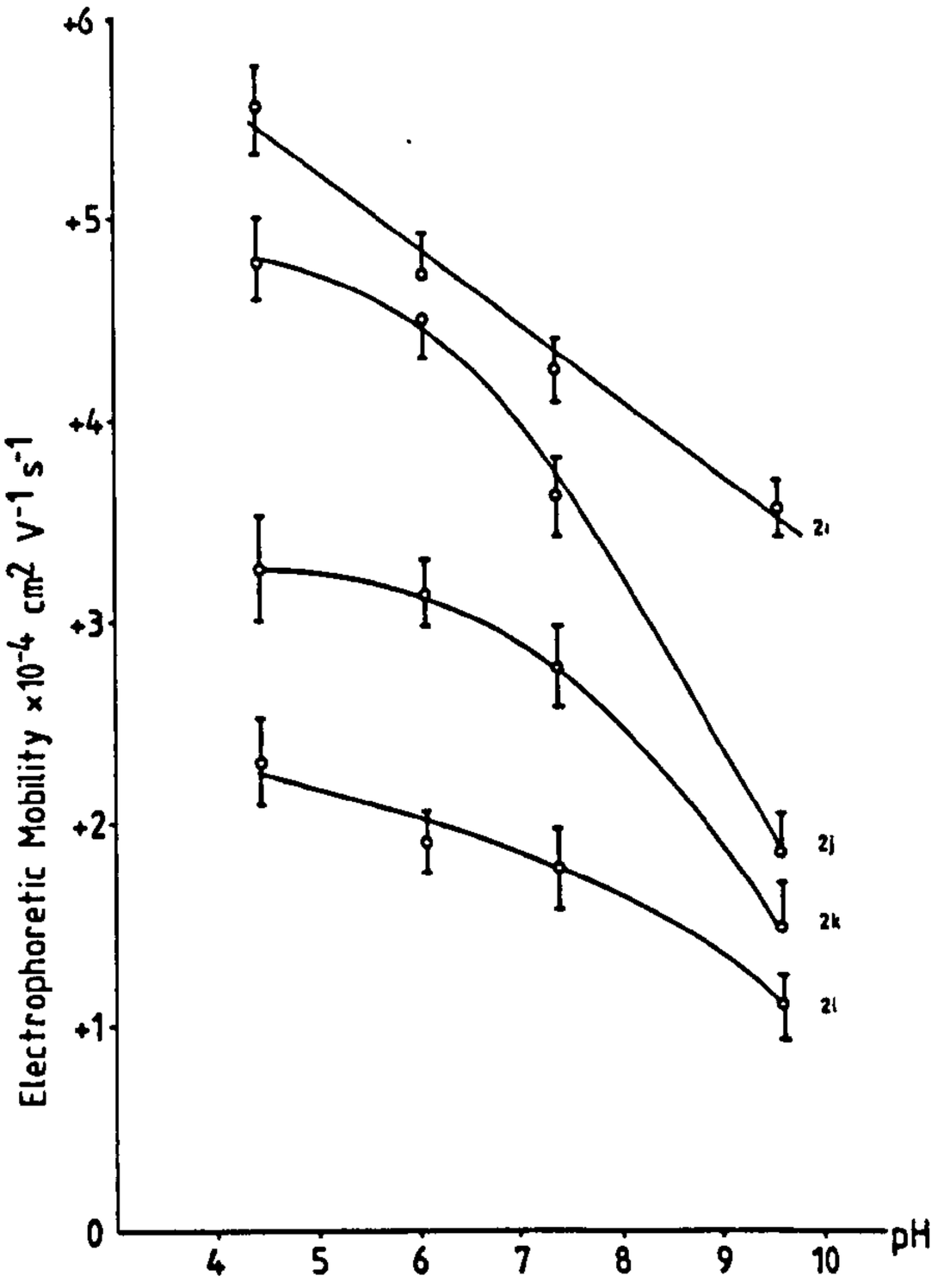


Fig. 3.17 Electrophoretic mobilities plotted against pH of solution for vesicles composed of: 2i) I 47.5:CHOL 47.5:SA 5.0; 2j) I 47.5: CHOL 23.75:SOL5 23.75:SA 5.0; 2k) I 47.5:CHOL 23.75:SOL 16 23.75:SA 5.0 and 2l) I 47.5:CHOL 23.75:SOL24 23.75:SA 5.0. Points are plotted as mean values ± standard deviation.

Table 3.18 Zeta potentials for vesicles containing an added positive charge.

Vesicle Composition	Zeta Potential (mV)			
	pH 4.5	pH 6.0	pH 7.4	pH 9.5
I 47.5: CHOL 47.5: SA 5.0	+71.24	+60.51	+54.27	+45.55
I 47.5: CHOL 23.75: SOL5 23.75: SA 5.0	+61.48	+58.21	+46.60	+23.63
I 47.5: CHOL 23.75: SOL16 23.75: SA 5.0	+41.94	+40.39	+35.37	+19.09
I 47.5: CHOL 23.75: SOL24 23.75: SA 5.0	+29.75	+24.61	+23.06	+14.36

3.2.5.3 Effect of Doxorubicin Encapsulation on Electrophoretic Mobility

In an attempt to assess the effect of vesicular entrapment of doxorubicin (DOX) on the EM, H.S. samples were prepared using a hydrating solution of DOX (5mg/ml) in NaCl ($2x10^{-3}M$). The resulting dispersion was dialysed against $2x10^{-3}M$ NaCl to remove unentrapped DOX. Drug loaded and empty vesicles of two different compositions were examined (I 50:CHOL 25:SOL24 25 and IV 47.5:CHOL 47.5:SA 5.0).

Drug loaded and empty vesicles containing SOL24 were negatively charged at all pH values examined (Fig. 3.18), both becoming more negative as the pH was increased. The shapes of the EM against pH curves were very similar with the drug loaded vesicles producing EM's

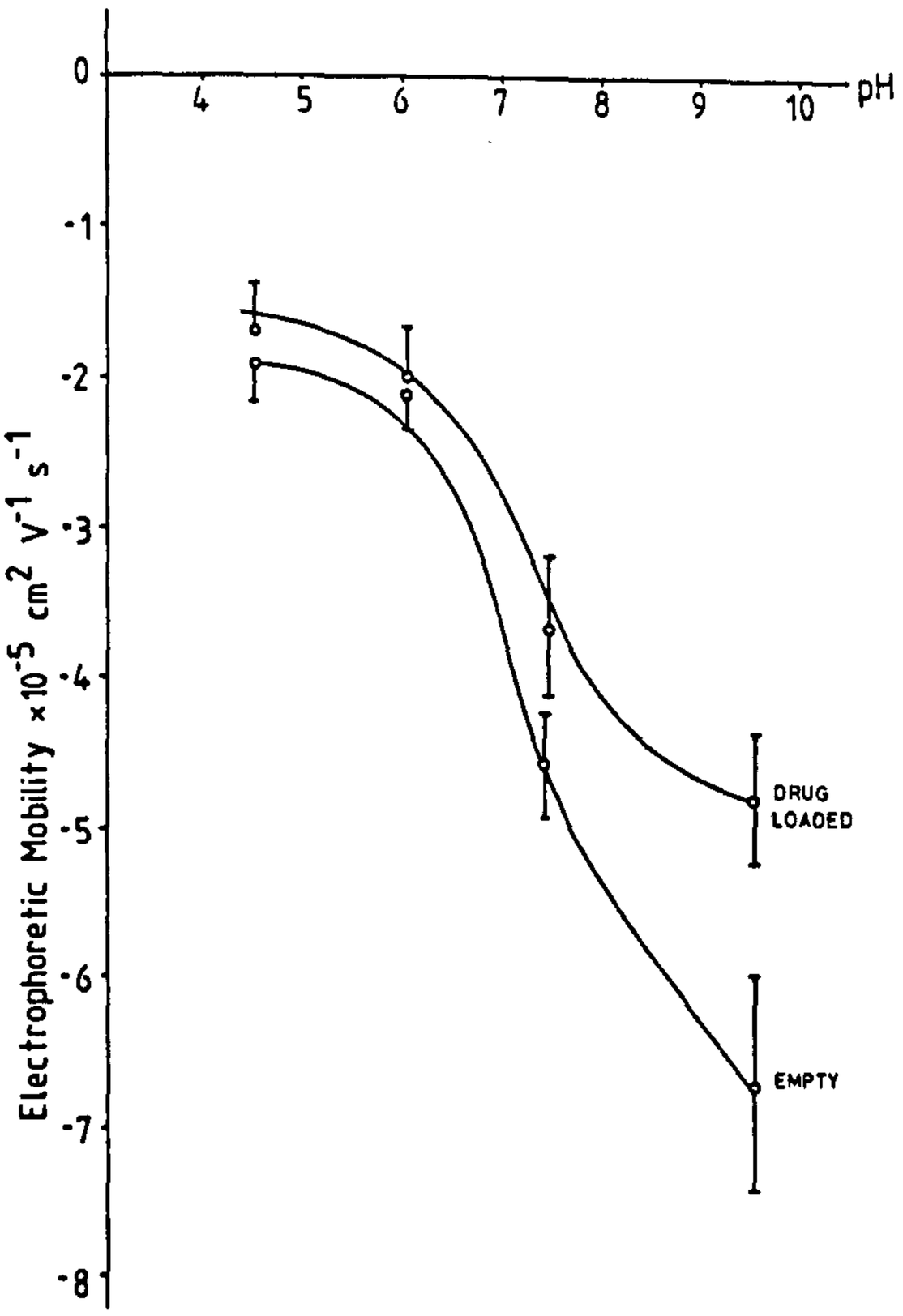


Fig. 3.18 Electrophoretic mobilities of drug (DOX) loaded and empty vesicles composed of I 50:CHOL 25:SOL24 25 are plotted against the pH of the solution. Points are plotted as mean value ± standard deviation.

which were less negative than the corresponding empty vesicles at all pH values. For most pH values this difference was minimal and was probably related to the additional effect the drug molecule had on the vesicles' mobility. Zeta potentials calculated for each sample are shown in Table 3.19.

Table 3.19 Calculated zeta potentials for empty and drug (DOX) loaded vesicles composed of I 50:CHOL 25:SOL24 25.

Vesicle Type	Zeta Potential (mV)			
	pH 4.5	pH 6.0	pH 7.4	pH 9.5
Empty Vesicles	-2.44	-2.64	-5.77	-8.67
Drug Loaded Vesicles	-2.13	-2.57	-4.70	-6.11

The encapsulation of DOX in vesicles containing 5mol% SA had a marked effect on the resultant EM's (Fig. 3.19) and zeta potentials (Table 3.20) when compared with empty vesicles. At low pH the empty vesicles were more positively charged than the drug loaded vesicles, but as the pH was increased, the charge on the drug loaded vesicles remained fairly constant while that on the empty vesicles fell rapidly, particularly at high pH.

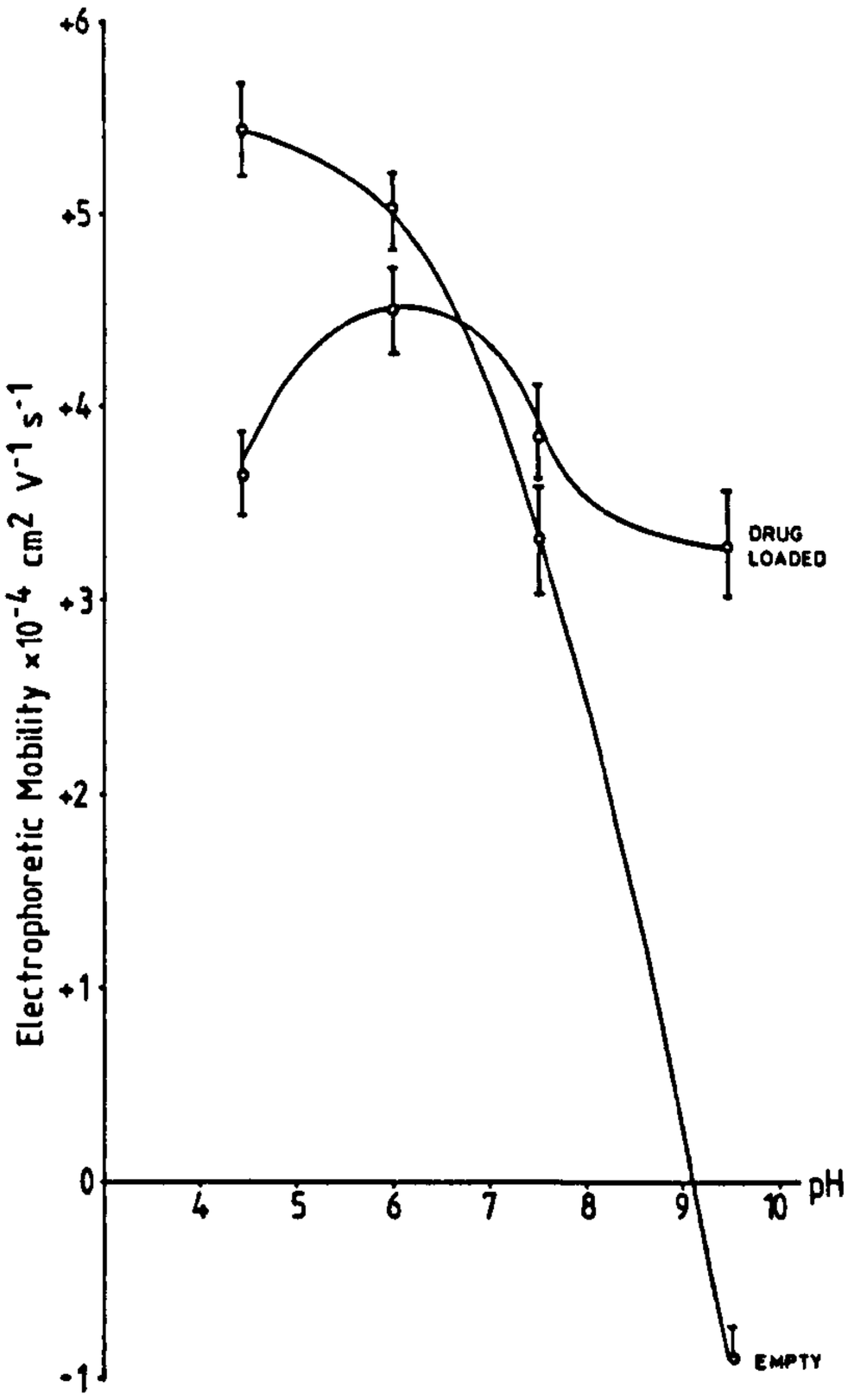


Fig. 3.19 Electrophoretic mobilities of drug (DOX) loaded and empty vesicles composed of IV 47.5:CHOL 47.5:SA 5.0 are plotted against the pH of the solution. Points are plotted as mean value ± standard deviation.

Table 3.20 Calculated zeta potentials for empty and drug (DOX) loaded vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.

Vesicle Type	Zeta Potential (mV)			
	pH 4.5	pH 6.0	pH 7.4	pH 9.5
Empty Vesicles	+69.92	+64.47	+42.66	-11.55
Drug Loaded Vesicles	+46.83	+57.74	+49.40	+42.08

3.2.5.4 Discussion

All the unionized vesicles examined above, which may have been expected to have zero electrophoretic mobility, actually showed a slight negative charge, due to the preferential negative adsorption of H^+ ions, compared with OH^- ions. It had also been shown that surfaces which were already charged had a tendancy to adsorb counterions, and that it was possible for counterion adsorption to cause reversal of charge (Shaw, 1969).

This was seen with positively charged vesicles containing Surfactant IV (IV 47.5:CHOL 47.5:SA 5.0) where at high pH the vesicles became negatively charged giving an iso-ionic point at about pH 9.1. This may be due in part to adsorption of OH^- ions onto the vesicle surface and also by a reduction in the number of ionized molecules of SA at the higher pH. However, there must also be some contribution to the final overall vesicular charge by the hydrophilic headgroups of the surfactant molecules, since this change in vesicular charge was not seen with positively charged vesicles

containing Surfactant I (I 47.5:CHOL 47.5:SA 5.0). This may be due to differences in the bulk of the hydrophilic headgroups and the way in which they interact with the other components of the vesicle. Surfactant I, with its average of three glycerol units, has a more bulky headgroup compared with Surfactant IV which is a diglycerol.

It could be suggested that the marked difference in the surface charge measured on positively charged Surfactant I and Surfactant IV vesicles at high pH was an artefact. However, the surface charge on both vesicle compositions was measured for five separately prepared samples and all produced vesicles which had a similar charge. It therefore seemed likely that the surface charges measured were true values and not artefacts.

Law et al (1988) measured the EM of uncharged, positively and negatively charged liposomes with similar compositions to those above, and similar EM against pH plots were obtained. However, after incubation with bovine serum albumin (BSA) the EM was altered and it was suggested the BSA was adsorbed onto the surface of the liposome, irrespective of their charge, resulting in all vesicle types giving an iso-ionic point at pH 5.0-5.5.

It has also been suggested that positive liposomes may initially interact with negative cells by electrostatic adsorption, before being internalized (Magee et al, 1974). However, since vesicles were likely to be coated with plasma proteins when injected in vivo resulting in a modified surface charge, this proposed electrostatic interaction may not be significant.

The addition of Solulan molecules, to all vesicle compositions, reduced the charge measured at the vesicular surface. As the POE chain length was increased, the EM fell, at constant pH. The one

exception to this was after the addition of DCP 5mol%, where SOL16 vesicles reduced the charge to a greater degree than the SOL24 vesicles. This was presumably caused by SOL5 and SOL16 containing wool fats and wool alcohols, in addition to cholesterol, with the corresponding attached POE chain. These additional compounds may further modify the surface charge of the vesicle.

The increased stability of oil-in-water emulsions found by increasing the length of POE chains of non-ionic surfactants was not caused by an increased charge on the emulsion globules. EM determinations showed that as the POE chain length increased the charge was reduced and led to the suggestion that other steric stabilizing effects must be important (Elworthy and Florence, 1969).

As the length of the POE chain of the Solulan molecule in the bilayer of the NSV was increased, a larger hydrated shell would exist around the vesicle. This would lead to an increased distance between the surface of shear and the outer surface of the hydrophobic portion of the bilayer, where more counterions may become associated, leading to a reduced EM.

The encapsulation of a drug molecule may further alter the charge on a vesicle depending the extent of ionization of the drug at the pH of the solution, and the effect of other vesicular components on this ionization. The location of the drug molecule within the vesicle is also likely to have an effect on the final vesicular charge.

Doxorubicin (DOX) was encapsulated in vesicles with two different compositions (I 50:CHOL 25:SOL24 25; IV 47.5:CHOL 47.5:SA 5.0). DOX in solution was known to be in its protonated, positively charged state below about pH 8.0 while above pH 10 the negatively

charged species dominated (Sturgeon and Schulman, 1977). At pH 7.2, two-thirds or more of the total DOX presented was found to partition into the hydrophilic phase of the two phase system of Folch (Duarte-Karim et al, 1976). The partitioning of DOX into a octanol:phosphate buffered saline (pH 7.2) system was examined by Goldman et al (1978) and drug was found in approximately equal proportions in both phases. This suggested that DOX would be found at the interface of the hydrophobic and hydrophilic regions of the NSV's.

The EM's measured with the SOL24 containing vesicles showed both empty and drug loaded vesicles produced EM against pH plots which were very similar, with the drug loaded vesicles being slightly less negatively charged than the empty vesicles at all pH values. It was shown previously that SOL24 containing vesicles were the most effective at reducing the charge at the shear surface. The incorporation of DOX in vesicles in solutions of pH less than pH 8.0 would be expected to make the vesicle surface more positively charged i.e. make it less negative. The exact charge of a DOX molecule in solution at pH 9.5 was not as clearly defined due to the presence of several moieties which may be ionized. However, it would appear from the EM data obtained that the molecule was still positively charged at pH 9.5 in this particular vesicular environment.

With the addition of DOX to positively charged vesicles containing Surfactant IV the picture was not so clear. The presence of two potentially positively charged molecules in the bilayer appeared to have some effect on the ionization of the molecules since the positive charges did not appear to be additive. At low pH the drug loaded vesicles were less charged than the empty vesicles

despite the fact that both the SA and DOX molecules would be highly ionized at this pH if in solution individually. As the pH was increased, little difference in the charges on the drug loaded and empty vesicles was seen. With a further increase in pH the drug loaded vesicles remained positively charged, while the empty vesicles became negatively charged. Encapsulation of DOX in positively charged vesicles had a dramatic effect on the resulting vesicular charge and appeared related to the pH of the solution, which in turn affected the cumulative degree of ionization of the DOX and SA molecules and the subtle interactions each of the molecules had on the ionization of the other.

3.2.6 Differential Scanning Calorimetry of NSV's

Multilamellar vesicles were prepared by the H.S. method containing 150μmoles of material in water (2ml). Scans were obtained over a temperature range of 10-95°C. and were plotted as heat flow against temperature, with the area under the endothermic peak also being calculated.

3.2.6.1 Effect of Cholesterol Content

NSV's were prepared containing Surfactant IV, with increasing cholesterol content, and the scans produced are shown in Fig. 3.20.

As the concentration of cholesterol was increased, the height of the endothermic peak was reduced and was ultimately abolished by 50mol% cholesterol. The onset temperature of the peak was also slightly lower as the cholesterol content increased.

When the area under the endothermic peak was calculated (J/g of sample), the reduction in the area was linear (Fig. 3.21). Extrapolation of the line to the x-axis produces a figure of 33mol% cholesterol for complete abolition of the endotherm. The incorporaton of SOL24 (IV 50:CHOL 25:SOL24 25) produced no peak i.e the thermogram was the same as that obtained for IV 50:CHOL 50.

3.2.6.2 Effect of Added Charge

Uncharged vesicles containing IV 90:CHOL 10, positively charged vesicles containing 5 or 10mol% stearylamine and negatively charged vesicles containing 5 or 10mol% dicetyl phosphate were prepared.

The addition of a charge had very little effect on the DSC scans produced (Fig. 3.22). DCP caused a very slight increase in the peak onset temperature but had very little effect on the area under the peak.

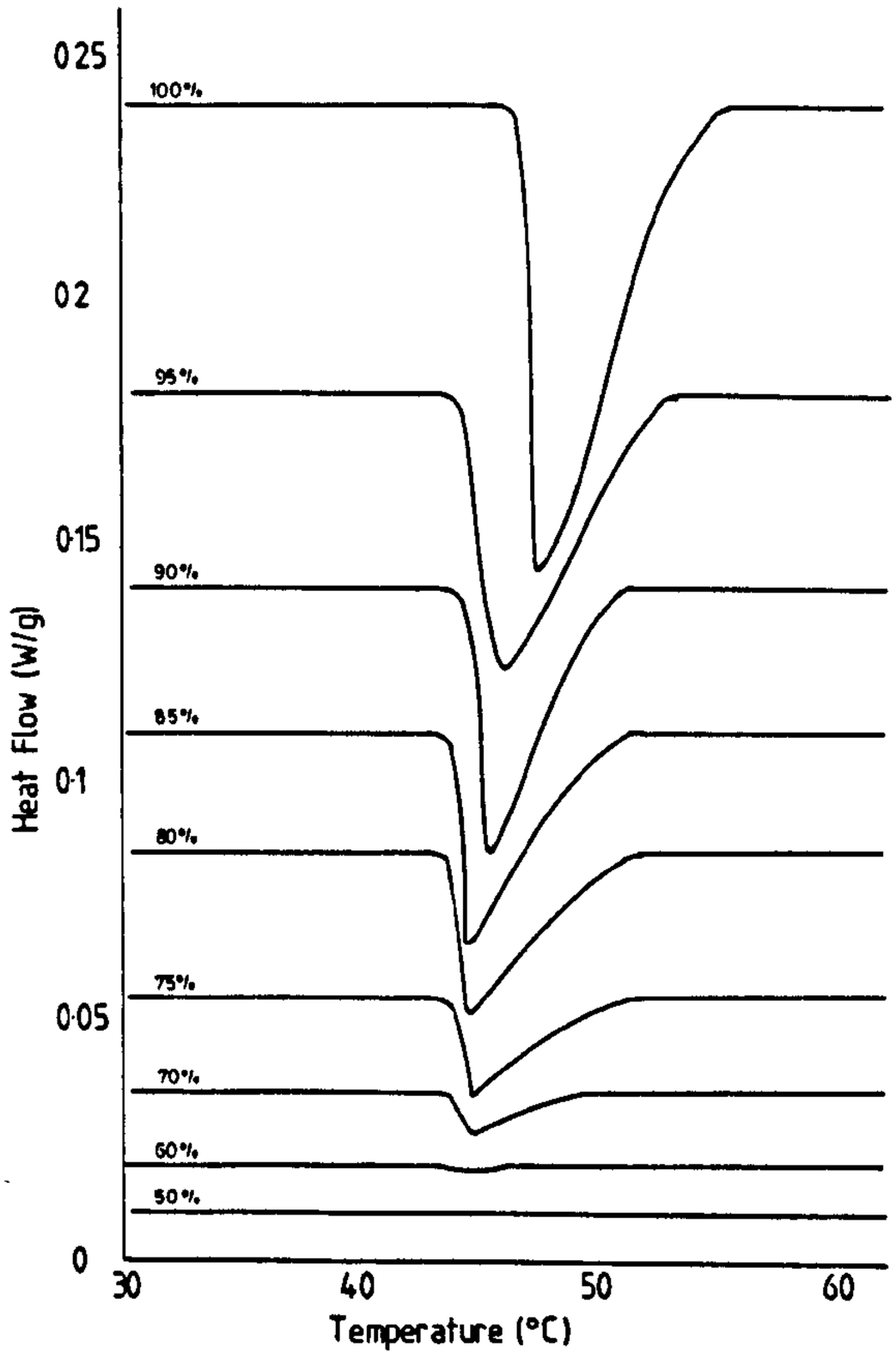


Fig. 3.20 DSC scans produced by vesicles containing the mol% of Surfactant IV shown with cholesterol over the temperature range of 30–60°C.

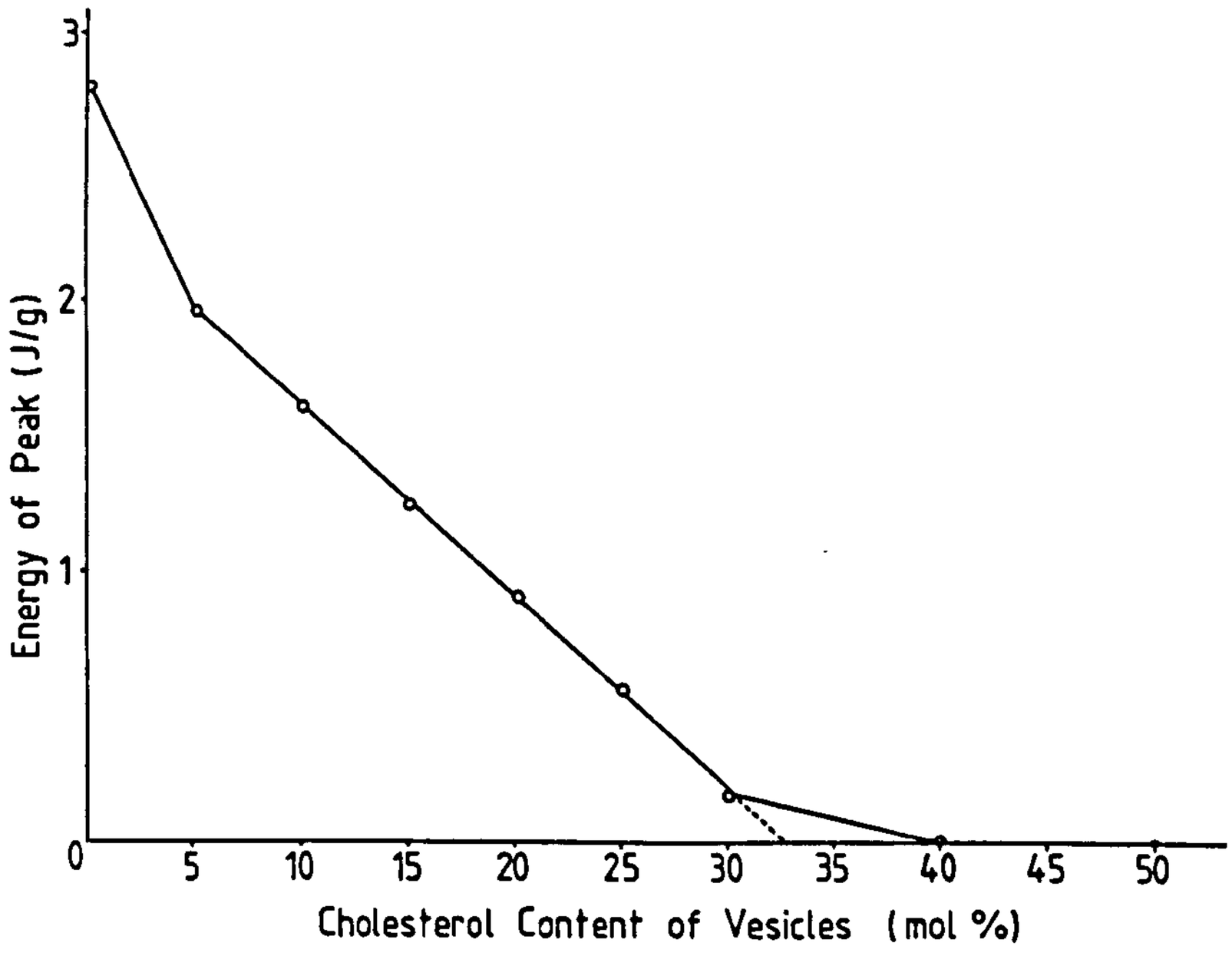


Fig. 3.21 The energy under the endothermic peak, produced by DSC, is plotted against the cholesterol content of vesicles containing Surfactant IV.

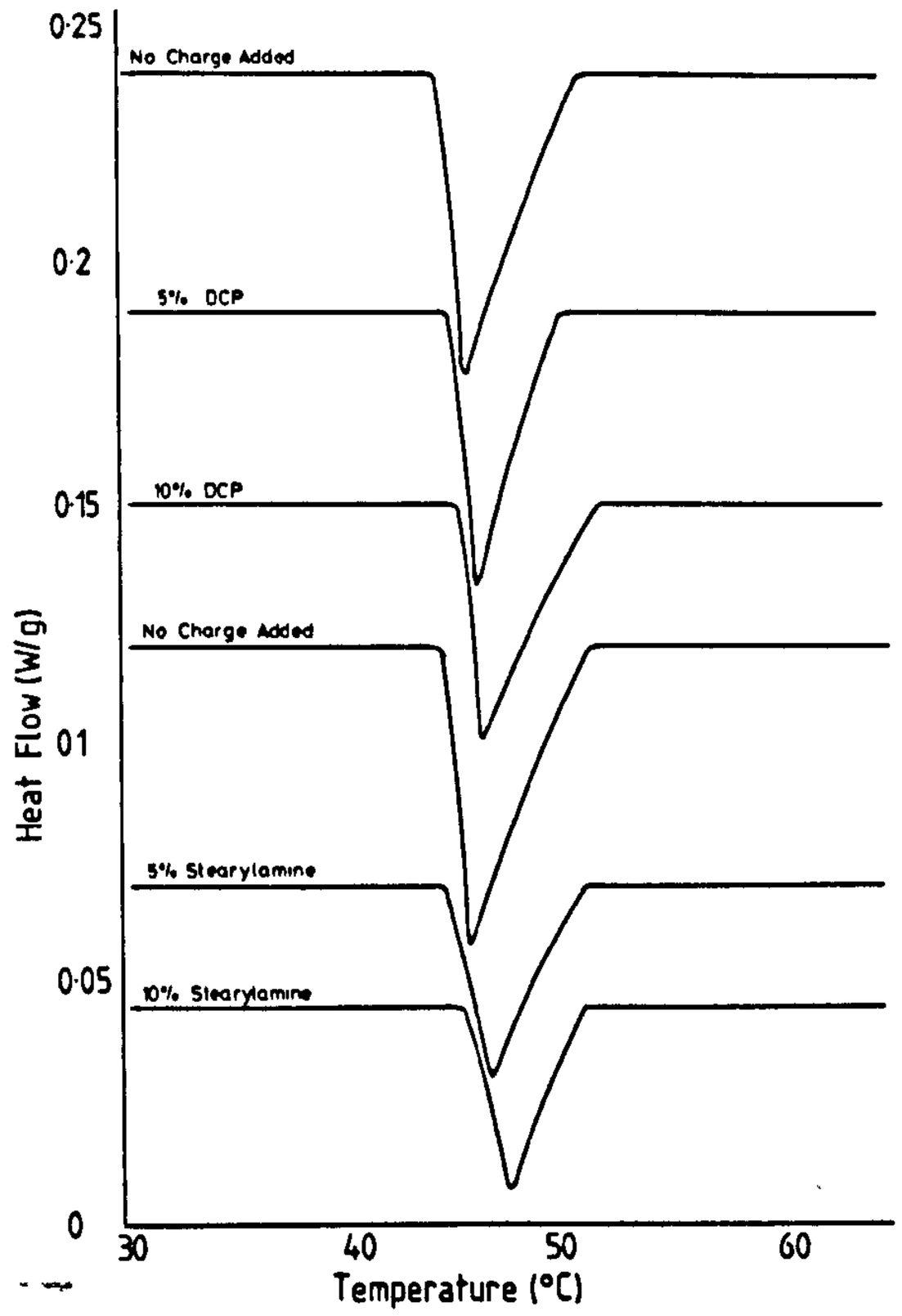


Fig. 3.22 DSC scans produced over the temperature range 30–60°C for vesicles composed of IV 50:CHOL 50 (No added charge), with an added 5 or 10mol% stearylamine or an added 5 or 10 mol% DCP.

When stearylamine was added, again a slight increase in the onset temperature was detected along with a very slight fall in the area under the endotherm peak. A change in the shape of the peak was also noted, it having a reduced height, but being broader than the corresponding peak with uncharged vesicles.

The addition of 5mol% DCP or stearylamine to vesicles with equimolar concentrations of surfactant and cholesterol resulted in no peaks being detected i.e. the same as when the charge was absent.

3.2.6.3 Discussion

The formation of stable liposomes from phospholipids was only possible at temperatures above the gel-to-liquid crystal phase transition (Tc) of the phospholipids, and represented the melting point of the acyl chains. At the Tc, a reduced cross sectional area of the molecule was observed in conjunction with a reduced bilayer thickness. The temperature at which the lipid underwent its transition was dependant on 1) the nature of the fatty acid side chain, with a short chain saturated lipids melting at a lower temperature than long chain saturated lipids, 2) the amount of unsaturation in the acyl chain, since unsaturated lipids melted at a lower temperature than the corresponding saturated lipids, and 3) the nature of the polar head group, since phosphatidylcholines melted 20° lower than the comparable phosphatidylethanolamines (Juliano and Layton, 1980).

When the temperature was raised from below the Tc, through the Tc to above it, the fatty acid side chains went from a closely packed, ordered gel-phase where the acyl chains have restrained mobility, to a more loosely packed, less ordered liquid-crystalline

state where the chains were capable of more rotational motion (Juliano and Layton, 1980).

The non-ionic C_{16} surfactant used in this study (Surfactant IV) produced a Tc of about 47°C, which was similar to the Tc recorded for unsonicated vesicles of a C_{16} dipalmitoyl phosphatidylcholine (Jacobson and Papahadjopoulos, 1975). This relatively high value may have been expected, given the long, saturated hydrocarbon chain of the surfactant. The extrapolated value of 33mol% cholesterol required to abolish the Tc of the NSV's was the same as that quoted for liposomes (Kimelberg and Mayhew, 1978), although the more common figure quoted for abolition of the endothermic peak was 50mol% (Ladbrooke et al, 1968)

When reporting Tc values, it was important to signify the method of manufacture, or the type of vesicles used in the determination. This was because sonicated vesicles produced a Tc which was broader and occurred at a temperature 5° lower than that produced by the corresponding unsonicated vesicles (Kimelberg and Papahadjopoulos, 1974). The effect of gradually increasing the mol% content of cholesterol in NSV's was similar to that seen using phospholipids. When the cholesterol content of 1,2-dipalmitoyl-L-lecithin : cholesterol mixtures was increased, no effect was seen initially on the sharp main endothermic peak. However, further increases resulted in a broadening of the main transition with a reduction in area, and 50mol% cholesterol produced no endothermic peak (Ladbrooke et al, 1968). In general, cholesterol could only be incorporated into vesicles up to a maximum molar ratio of 1:1 after which it separated out (Lecuyer and Dervichian, 1969).

The addition of cholesterol appeared to disrupt the ordered

array of the hydrocarbon chains in the gel-phase (Ladbrooke et al, 1968). Cholesterol had been called a "fluidity buffer", since below the Tc it made the membrane less ordered, while above the Tc it made the membrane more ordered, thus suppressing the dramatic shift in order normally seen at the Tc (Papahadjopoulos et al, 1973b). In liposomes prepared from lecithin and containing 50mol% cholesterol, all the chains were found to be in the fluid state (Ladbrooke et al, 1968) caused by an increase in fluidity below the Tc and a reduced fluidity above the Tc (Demel and de Kruyff, 1976).

Since the addition of SOL24 to NSV's produced no endothermic peak when included in vesicles in place of a portion of the cholesterol, it appeared that it acted in a similar manner to cholesterol in the way it intercalated in the bilayer and the effect it had on bilayer fluidity.

From mixed monolayer studies with lecithin and cholesterol, it had been suggested that cholesterol occupied a molecular cavity and therefore did not cause a proportional increase in the area of the mixed monolayer (Shah and Schulman, 1967). Other sterols were capable of replacing cholesterol in the bilayer but there appeared to be three requirements for a sterol-lipid interaction 1) a planar nuclear ring, 2) a cholestane type side chain, and 3) a 3β hydroxyl group (Demel and de Kruyff, 1976).

When two phospholipids were included in a bilayer, if the two lipids had fairly close Tc, the resulting Tc showed a broad peak, while for two lipids of very different Tc, two independent Tc traces were detected (Phillips et al, 1972).

The Tc of lipids and non-ionic surfactants had important ramifications for the release of ions, sugars and drugs from the

vesicles produced. At the Tc the vesicles showed a marked increase in permeability (Juliano and Stamp, 1979) and it has been suggested that this increase in permeability at the Tc was due to the coexistence of fluid and gel regions at the Tc. The interstices between the fluid and gel regions were areas for rapid efflux, with efflux being much lower at temperatures both above and below the Tc (Papahadjopoulos et al, 1973a). A decrease in permeability could also be produced by the addition of cholesterol (Scarpa and de Gier, 1971), which could again be explained by a change in the fluidity of the bilayers (Chapman and Penkett, 1966).

The addition of a charge to the vesicular bilayer resulted in the consideration of two additional factors, interactions between lipid bilayer surfaces and interactions between the charged species and other charged species in the aqueous phase (Lee, 1977a). Changes in the multilamellar bilayer packing were likely due to the charge-charge repulsion between the individual sheets (Hauser and Phillips, 1973). Ca^{2+} and Mg^{2+} ions have been shown to produce structural changes in vesicular shape (Verkleij et al, 1974; Papahadjopoulos et al, 1975) and the addition of Ca^{2+} caused lamellar phases of cardiolipin to convert to hexagonal phases (Rand and Sengupta, 1972). Ca^{2+} and Mg^{2+} were thought to form ion bridges between charged phospholipid molecules (Papahadjopoulos, 1968), while monovalent ions had no effect on the Tc (MacDonald et al, 1976).

Charged lipids have also been shown to be sensitive to pH, with changes in hydrogen ion concentration altering the ionization of the polar head groups resulting in a change in the Tc. Alternatively, by changing the H^+ concentration at a constant temperature it was possible to cause a change from the gel to the liquid crystalline

state (Trauble and Eibl, 1974).

In the case of the charged, multilamellar NSV's examined in this instance, the effect of interactions between the bilayers with the addition of charged molecules was likely to exist. The effects due to the challenge by Ca^{2+} and Mg^{2+} were less likely since all vesicles were prepared in deionized, distilled water at pH 6.9. Any changes in H^{+} concentration may have affected the ionization of the charged molecule but were unlikely to have had a significant effect on the headgroup of the non-ionic surfactant, and hence the Tc of the system.

Evidence of the ability of stearylamine (SA) to cause changes in the Tc of liposomes composed of dipalmitoyl phosphatidyl choline was given by Deleers et al (1982). When SA was ionized, a marked increase in the Tc was observed, while increasing the pH caused the Tc to fall. Although the most marked change in the Tc and fluidity of the vesicles were noted when SA was ionized, differences were seen when it was in its neutral form, although they were less marked. It has been proposed that this change in the Tc caused by the neutral form of the molecule was due to the long stearyl chain being inserted into the bilayer (Deleers et al, 1980).

The Tc of negatively charged vesicles, containing palmitic acid, was shown to be sensitive to pH in the physiological range, corresponding to the ionization of the carboxyl group of the palmitic acid (Fernandez et al, 1986).

It therefore appeared that the slight changes observed in the Tc of the NSV's with negative and positive charges could be explained by considering the ionization states of the charged molecules at the pH of the solution, and by considering the effects the insertion of

their hydrocarbon chains into the bilayer had on the overall fluidity of the bilayer.

3.2.7 **In Vitro** Dialysis of Doxorubicin Loaded NSV's

Measurement of the movement of drug across a dialysis membrane was useful for giving relative rates of release of drug from vesicles and has been used for examining release of small molecules from liposomes (Bangham et al. 1965). Absolute values of release cannot be obtained from these dialysis experiments as after drug was released from the vesicles it must then cross the dialysis membrane before being measured. It was also known that DOX could be adsorbed onto the dialysis membrane (Olson et al. 1982), and increasing the concentration of drug solution in contact with the dialysis membrane resulted in greater adsorption of DOX resulting in the membrane becoming more red.

3.2.7.1 Results

Samples were prepared by the sonication method, containing 75μmoles/ml material and dialysed to remove unentrapped drug. Vesicles containing I 50:CHOL 50 and I 50:CHOL 25:SOL24 25 were prepared using a hydrating solution containing 3mg/ml DOX (5ml), while those containing IV 47.5:CHOL 47.5:SA 5.0, IV 49.5:CHOL 49.5:SA 1.0 and IV 85.5:CHOL 9.5:SA 5.0 used a hydrating solution of 5mg/ml DOX (5ml). After dialysis, all drug loaded samples gave DOX entrapments of about 1mg/ml. A free drug solution containing 1mg/ml DOX (5ml) was used as a control. The total amount of DOX in the donor chamber of the dialysis cell at time zero was taken as 100%. The DOX concentration in the acceptor chamber was measured at defined time points and the total amount of DOX in the acceptor chamber expressed as a percentage of that in the donor chamber at time zero.

Fig. 3.23 shows the release rates from Surfactant I vesicles.

As expected, the rate of release of DOX across the dialysis membrane from vesicles was much slower than that of free drug. Release after 6h was approximately 3% from the I 50:CHOL 50 vesicles but only 2% from the SOL24 containing vesicles. The DOX release from the free drug solution and I 50:CHOL 50 vesicles was linear over the first 6h, while release from SOL24 vesicles was linear for the first 3h then began to slow down. The release rates must slow down substantially after the first 6h, since the 24h releases were not substantially greater than those measured at 6h (Table 3.21).

Table 3.21 Amount of DOX released across a dialysis membrane after 24h.

Sample	24h DOX release (%)
Free DOX in solution	39.33
I 50:CHOL 50	4.15
I 50:CHOL 25:SOL24 25	3.65

The DOX release rates of vesicles containing Surfactant IV and free drug in solution are shown in Fig. 3.24. Again the release rates for the vesicular forms were much slower than the free drug. DOX was released particularly slowly from vesicles containing IV 47.5:CHOL 47.5:SA 5.0 while reducing the amount of cholesterol or stearylamine in the vesicular composition increased the release rate by about 20 times. The release profiles for the vesicles were initially linear but began to deviate from linearity after 4-5h. The DOX release measured after 24h dialysis (Table 3.22) suggested

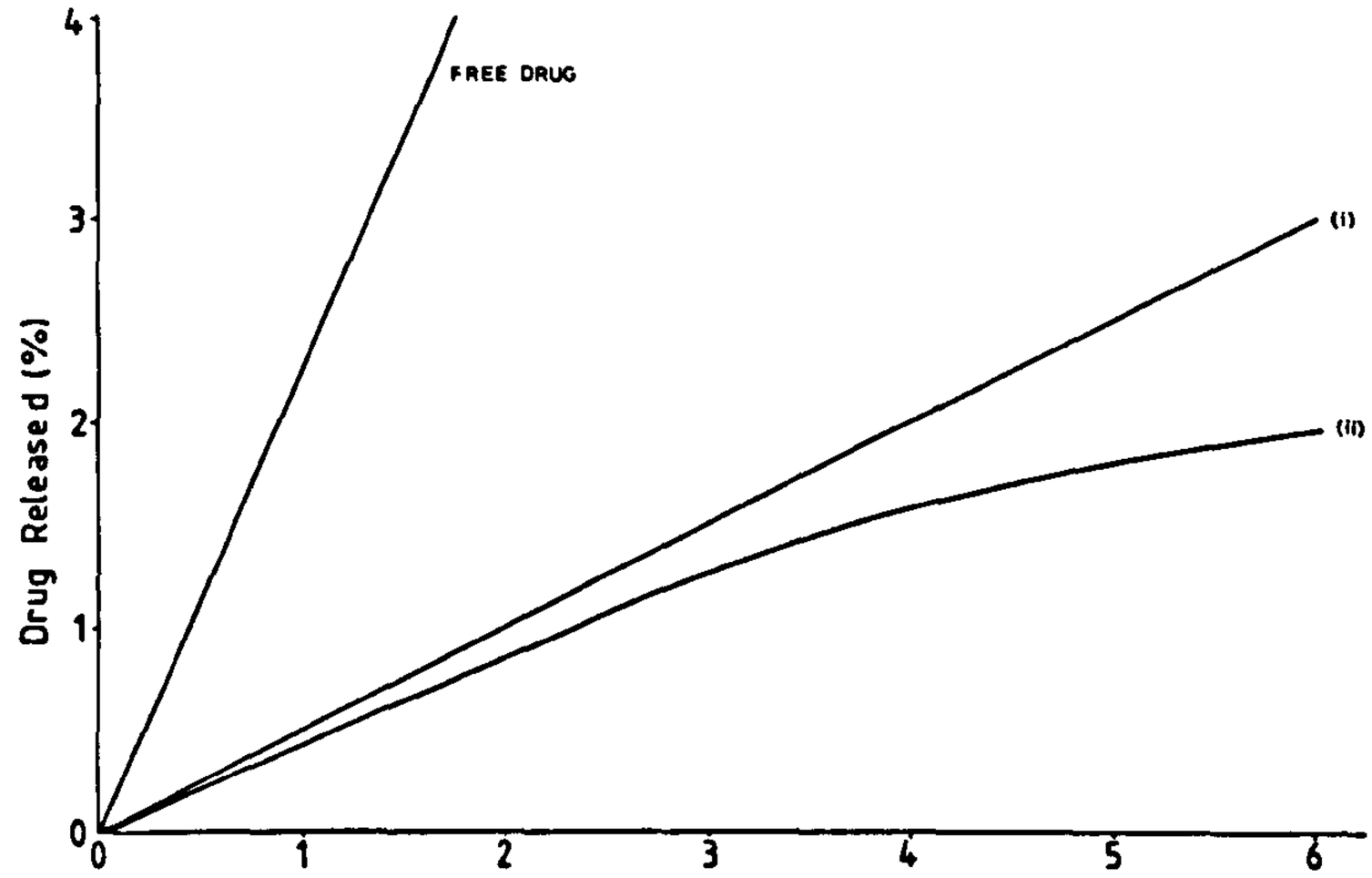


Fig. 3.23 Release of DOX across a dialysis membrane from a solution of free drug (1mg/ml, 5ml) and drug loaded vesicles composed of i) I 50:CHOL 50 and ii) I 50:CHOL 25:SOL24 25.

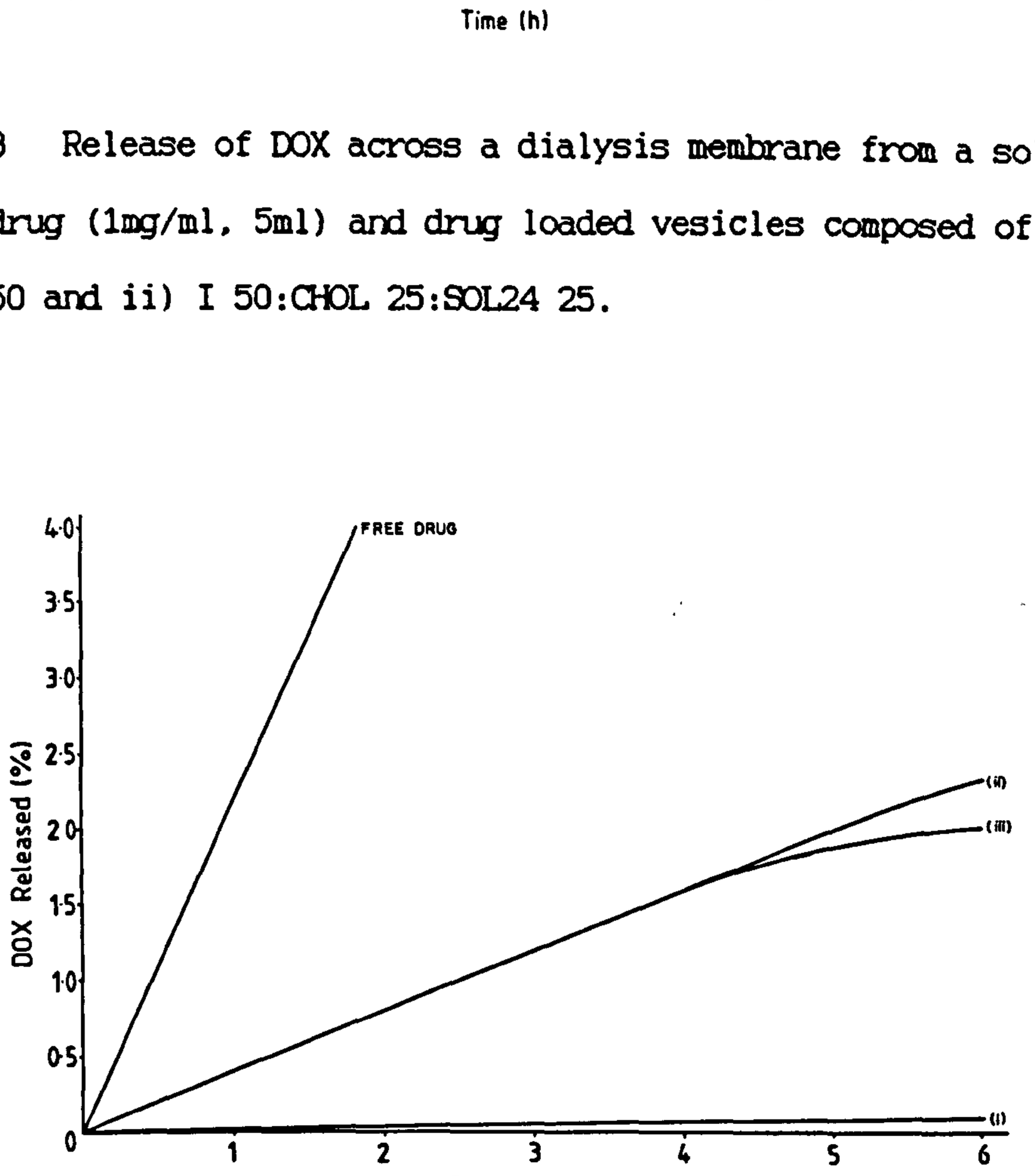


Fig. 3.24 Release of DOX across a dialysis membrane from a free drug solution (1mg/ml, 5ml) and drug loaded vesicles composed of i) IV 47.5:CHOL 47.5:SA 5.0, ii) IV 49.5:CHOL 49.5:SA 1.0 and iii) IV 85.5:CHOL 9.5:SA 5.0.

that DOX release slowed down considerably after the first few hours.

Table 3.22 Amount of DOX released across a dialysis membrane after 24h.

Sample	24h DOX release (%)
Free DOX in solution	40.35
IV 47.5:CHOL 47.5:SA 5.0	0.167
IV 49.5:CHOL 49.5:SA 1.0	3.50
IV 85.5:CHOL 9.5:SA 5.0	2.95

3.2.7.2 Discussion

The rates of DOX movement across the dialysis membrane, for all drug loaded vesicles, was slower than that for free drug. The release of both CF and DOX from non-ionic surfactant vesicles has been shown to be affected by the proportion of cholesterol present in the bilayer (Baillie et al, 1985; Rogerson et al, 1987). In general, vesicles without cholesterol were particularly leaky, so inclusion of cholesterol was essential to give reduced release rates, with a maximum reduction in DOX release with cholesterol contents of 25mol% or more (Khand et al, 1987). The drug release profile from vesicles generally had an initial fast release followed by a prolonged, slower release (Kimelberg and Atchison, 1978). This type of behaviour was seen with all the DOX loaded vesicles examined, although there were variations in the length of the initial fast release phase. The inclusion of SOL24 into vesicles in place of a proportion of cholesterol reduced the initial release of drug.

However, SOL24 containing vesicles produced a faster release at later times as shown by the 24h DOX releases.

Incorporation of SA into Surfactant IV vesicles markedly affected the release of DOX. The release from vesicles composed of IV 47.5:CHOL 47.5:SA 5.0 was very slow compared to other vesicular compositions examined. Positively charged liposomes, containing SA and encapsulating a variety of drugs, have been shown to produce particularly slow releases even in the presence of serum. Furthermore, encapsulation of amphipathic drugs, such as DOX, was likely to proceed by intercalation of the drug in the bilayer which may further modify its release (Juliano et al, 1978).

Reducing the cholesterol content of these vesicles increased the 24h release rate by a factor of 18, while reducing the SA content increased the release rate by a factor of about 21.

3.2.8 Effect of Empty Vesicles on Release of Free Doxorubicin

Empty vesicle samples were prepared by the sonication method using 300mM glucose(4ml) as the hydrating solution. Prior to commencing dialysis, 1ml of a DOX solution (5mg/ml in 300mM glucose) was mixed with the empty vesicle suspension.

The effect of increasing the concentration of vesicles, i.e. increasing the amount of starting material used to prepare the vesicles, on DOX release was examined for three vesicular compositions.

Empty vesicles composed of I 50:CHOL 25:SOL24 25 and I 50:CHOL 50, containing 150,300,600 and 1200μmoles total starting material in the 4ml dispersion, were prepared. Empty vesicle samples containing Surfactant IV (IV 47.5:CHOL 47.5:SA 5.0) were also prepared containing 150, 300, 450, 600 and 1200μmoles total material in 4ml. The release of free DOX in solution (1mg/ml in 300mM glucose, 5ml) was also examined and compared with the release of drug from empty vesicle/free drug mixtures.

3.2.8.1 Results

The release profiles for vesicles composed of I 50:CHOL 50 (Fig. 3.25) show that increasing the concentration of vesicles had no effect on the release of the drug across the membrane, with the possible exception of the most concentrated sample which showed a very slightly reduced release. All release rates were linear over 6h and were identical to the release measured for free drug in solution. The percentage of DOX released after 24h was identical for each sample, with only the most concentrated vesicle sample possibly showing a slightly reduced 24h release (Table 3.23).

Table 3.23 DOX released (%) after 24h from samples of empty vesicles with free drug as a function of concentration for vesicles composed of I 50:CHOL 50.

Concentration of Lipid in Sample (μmoles)	% DOX Released after 24h
0	39.57
150	39.71
300	38.87
600	40.40
1200	37.60

Incorporation of SOL24 into the vesicular bilayer had a marked effect on the drug release (Fig. 3.26). Over the first 6h all releases were linear, but as the concentration of vesicles was increased, the release rate was reduced. However, after 24h, the % DOX released with the 150 and 300μmole samples were the same as that of free drug, the 600μmole sample was slightly less and the 1200μmole sample less than half that of free drug (Table 3.24).

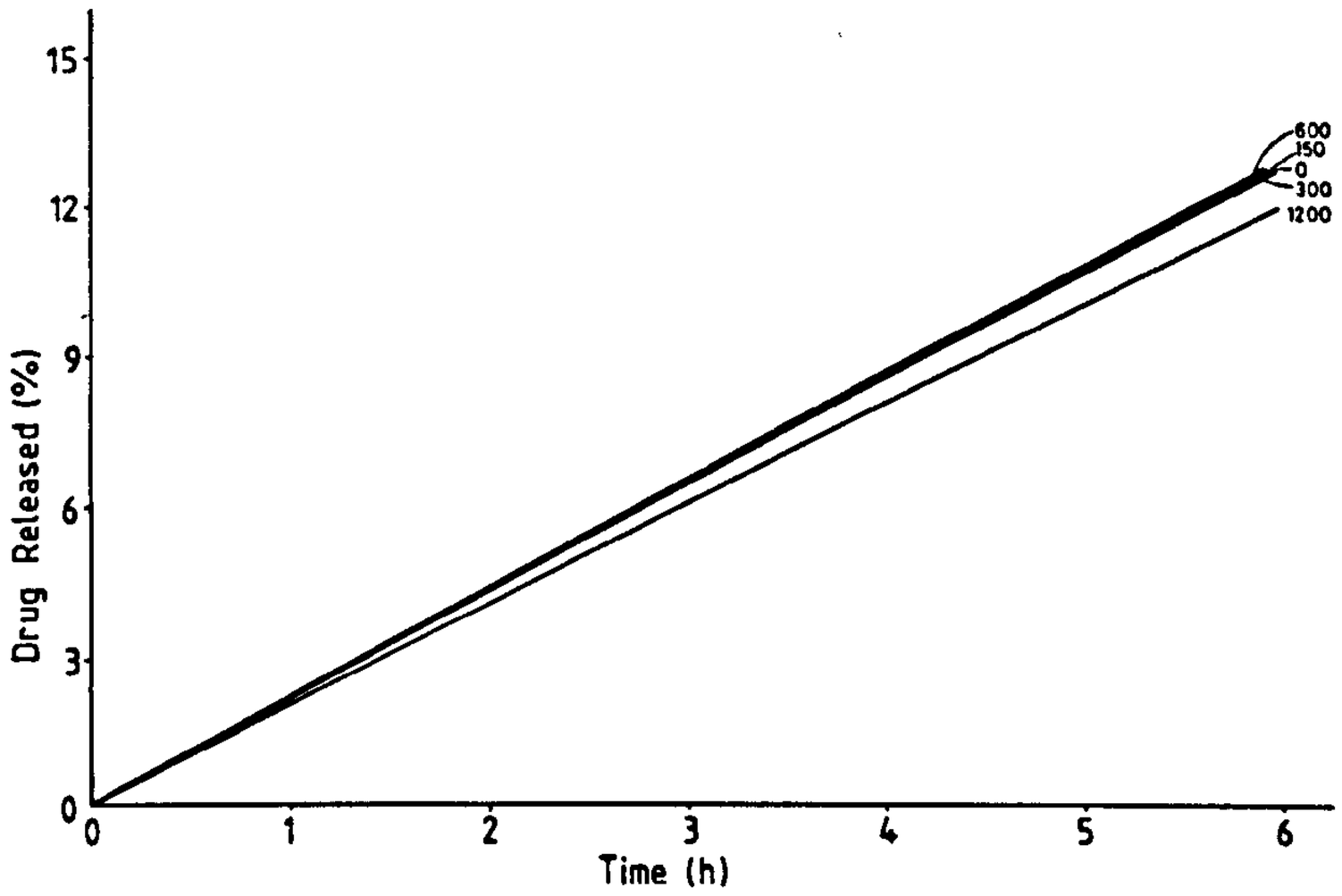


Fig. 3.25 Release of DOX across a dialysis membrane from a free drug solution and from samples where free drug is mixed with empty vesicle samples containing 150, 300, 600 or 1200μmoles total starting material. Vesicular composition - I 50:CHOL 50.

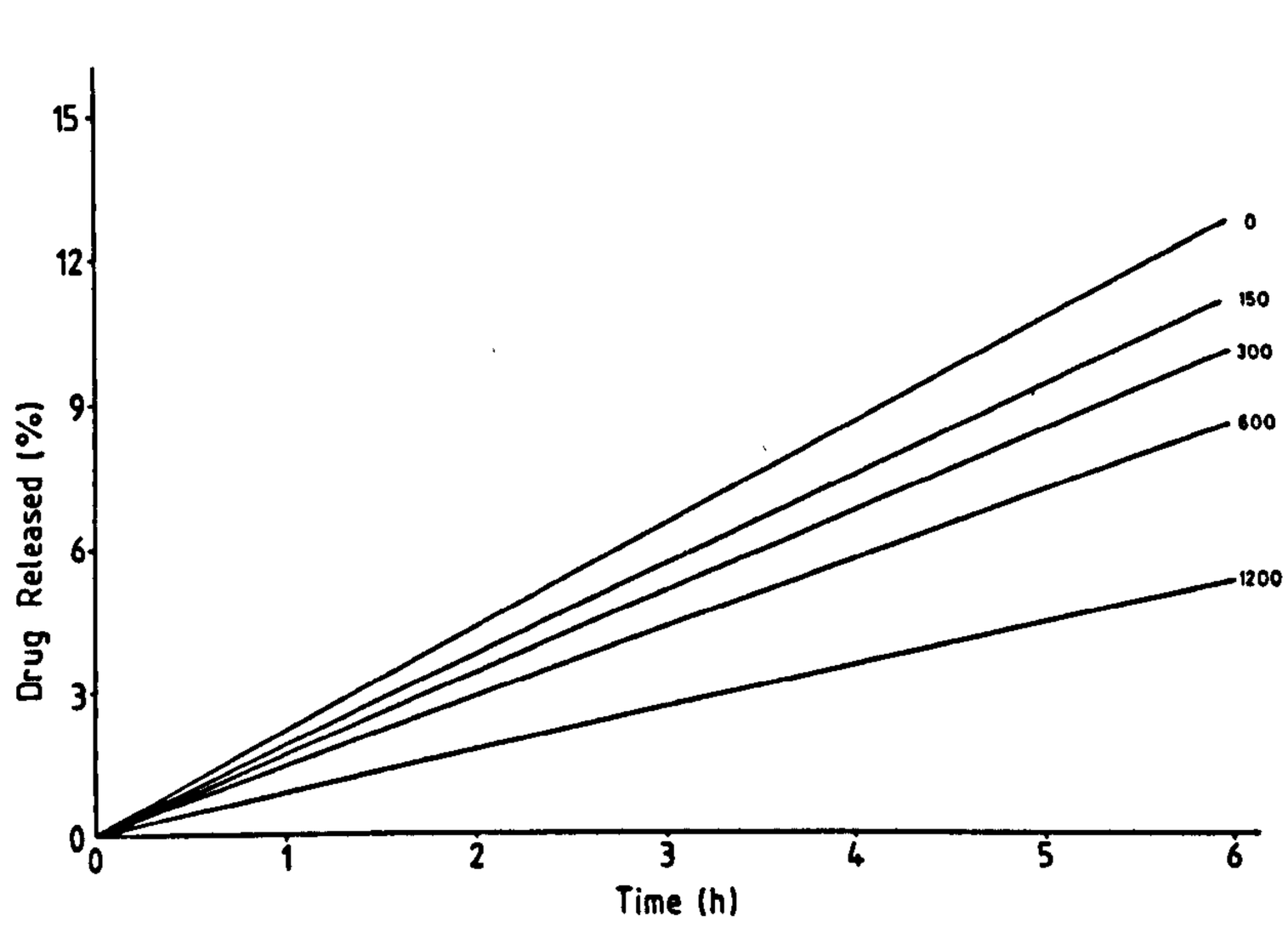


Fig. 3.26 Release of DOX across a dialysis membrane from a free drug solution or from samples where free drug is mixed with empty vesicle samples containing 150, 300, 600 or 1200μmoles total starting material. Vesicular composition - I 50:CHOL 25:SOL24 25.

Table 3.24 DOX released (%) after 24h from samples of empty vesicles with free drug as a function of concentration for vesicles composed of I 50:CHOL 25:SOL24 25.

Concentration of Lipid in Sample (μmoles)	% DOX Released after 24h
0	39.69
150	40.33
300	39.65
600	36.55
1200	17.85

The picture with the positively charged Surfactant IV vesicles was somewhat different. Free drug and all empty vesicle samples produced a linear release over the first 3.5h, with release rates equal to that of free drug in solution (Fig. 3.27). However, all the vesicle-containing samples then began to deviate from a linear release, with the more concentrated samples showing slower DOX release at earlier times. After 24h, the DOX release from all the empty vesicle samples was much less than that of free drug (Table 3.25), and in most samples the 24h release was actually less than that at 6h.

Table 3.25 DOX release (%) at 6h and 24h for the given concentrations of empty vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.

μmoles material	DOX Released (%)	
	6 hour	24 hour
0	12.90	38.50
150	12.81	15.35
300	11.80	10.55
450	11.40	9.27
600	11.06	7.12
1200	9.62	3.44

3.2.8.2 Discussion

The combination of free drug with empty vesicles produced results with completely different characteristics depending on the vesicular composition used. It appeared that I 50:CHOL 50 vesicles did not interact with DOX since for the most part they did not affect the release characteristics of the DOX molecules.

The inclusion of SOL24 produced a marked reduction in the in vitro release as the concentration of vesicles was increased. There appeared to be an immediate interaction between the vesicles and the drug molecules since release was affected from time zero. The interaction must also be reversible, since the 24h data for release for the least concentrated samples approached that of the free drug sample. Bearing this in mind it seemed likely that the drug molecules became trapped in and/or between the POE chains forming a

layer around the outside of the vesicle. This would initially reduce the number of DOX molecules available to set up an equilibrium on either side of the dialysis membrane, thus reducing the measured release. The more concentrated vesicle samples would be capable of trapping more DOX than the less concentrated samples and as the same number of DOX molecules was presented to each sample the net effect would be to reduce the measured drug release.

Estimating the "apparent binding" of DOX molecules to the empty vesicles from the slope of the drug release profiles over 6h of dialysis produced the trace shown in Fig. 3.28. From this, it appeared that the amount of DOX bound by the empty vesicles was related to the number of vesicles and hence the number of SOL24 molecules present. However, the amount of drug bound was not linear over the empty vesicle concentration range, with more drug bound per vesicle at lower vesicle concentrations.

DOX has been shown capable of binding to a wide variety of substances including inert materials such as glass containers and PTFE filters (Bosanquet, 1986) as well as forming complexes with proteins, nucleic acids, phospholipids, amino acids, biogenic amines and itself (Cummings and Smyth, 1988). This may help to explain the results obtained with the positively charged, stearylamine containing vesicles. It appeared that, initially, when the drug solution was mixed with these vesicles, little effect was seen since all samples produced the same initial release rate as the free drug alone (Fig. 3.27). However, with time, it appeared that the drug became bound to the vesicle, with the more concentrated empty vesicle samples capable of binding more DOX. Moreover, this binding did not appear to be reversible, since the 24h release of drug was less than that

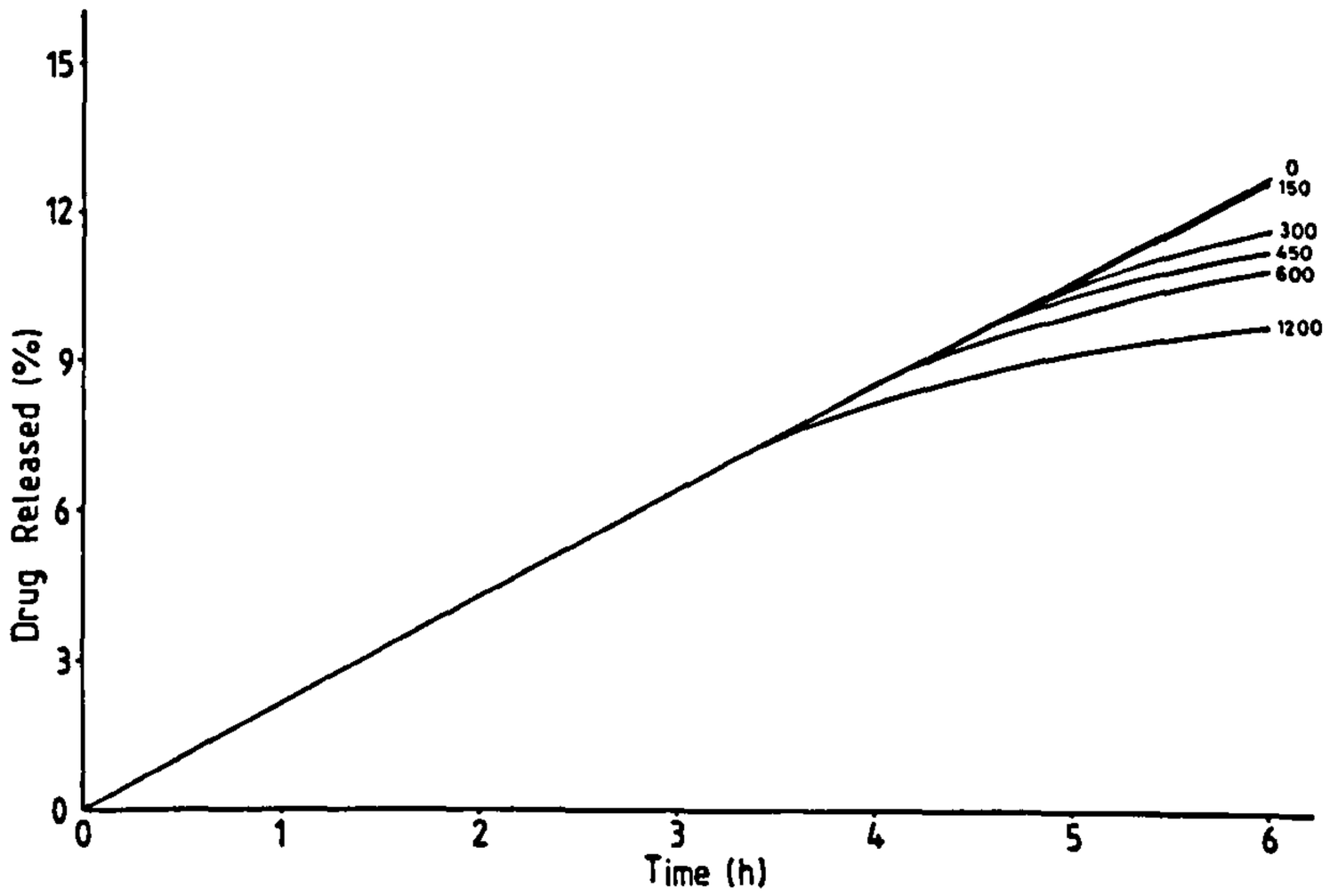


Fig. 3.27 Release of DOX across a dialysis membrane from a free drug solution and from samples where free drug is mixed with empty vesicle samples containing 150, 300, 450, 600 or 1200μmoles total starting material. Vesicular composition - IV 47.5:CHOL 47.5:SA 5.0.

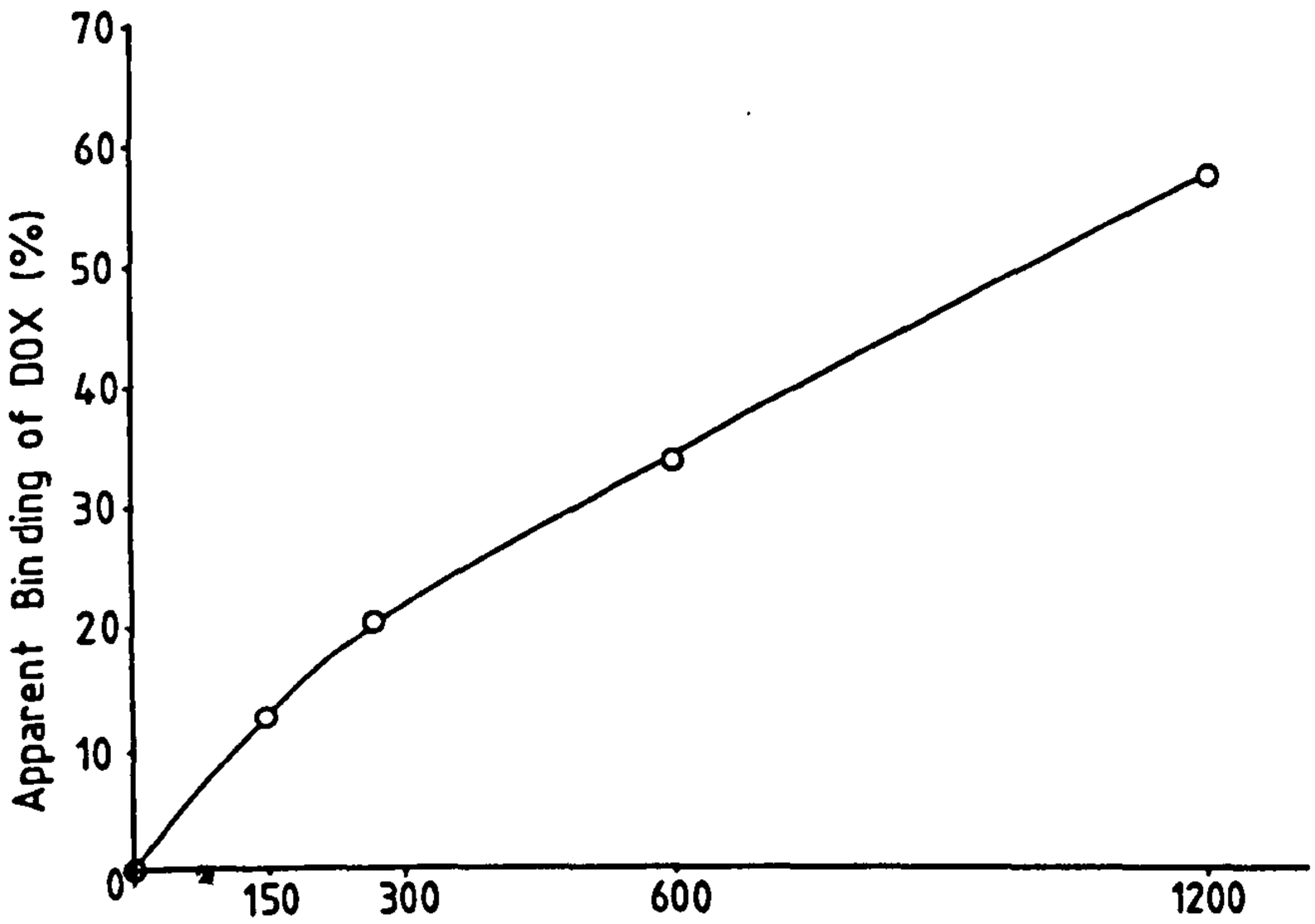


Fig. 3.28 The apparent binding of doxorubicin (DOX) to various concentrations of empty vesicles composed of I 50:CHOL 25:SOL24 25 after 6h dialysis.

at 6h for some samples.

Positively charged liposomes containing DOX were shown to have very slow releases over 10 days due to DOX being firmly associated with the bilayer (Crommelin et al, 1983). DOX was also known to form complexes with negatively charged phospholipids by electrostatic interaction (Goormaghtigh et al, 1980; Duarte-Karim et al, 1976). This was thought to take place between the amino group of the DOX and negatively charged phosphate groups, rather than carboxylic groups, on the phospholipid molecule (Nicolay et al, 1988; Burke et al, 1988; Garnier-Suillerot and Gattengo, 1988). It may be possible that the conditions around the outside of the positively charged vesicles were such that the DOX molecule reacted with the amine of the stearylamine molecule to form a bond which was not be reversible in vitro.

4 *In Vivo* Results and Discussion

4.1 Introduction

Doxorubicin (DOX) is an important member of the anthracycline class of anticancer drugs. It has been used to treat a wide range of human cancers such as lymphomas (Bonadonna *et al*, 1970; Wang *et al*, 1971), leukaemias (Bonadonna *et al*, 1970; Oldham and Pomeroy, 1972; Wang *et al*, 1971) and solid tumours including gastric and pancreatic tumours (Bonadonna *et al*, 1970; Middleman *et al*, 1971; Philips *et al*, 1975; Wang *et al*, 1971). However, the clinical usefulness of DOX is limited by a cumulative, irreversible and potentially lethal cardiotoxicity which manifests itself as a refractory congestive heart failure. The incidence of this major side effect increases steeply when the cumulative DOX dose exceeds 550mg/m^2, or less if the patient has received previous mediastinal irradiation. In animals, this cardiotoxicity has been shown to be reduced by encapsulation of DOX in liposomes (Rahman *et al*, 1980; Rahman *et al*, 1982) and many examples of reduced cardiac uptake of the drug after liposomal encapsulation have been reported (Rahman *et al*, 1980; Gabizon *et al*, 1982; van Hoesel *et al*, 1984; Forssen and Tokes, 1979). Nephrotoxicity of the drug may also be reduced by encapsulation in liposomes (van Hoesel *et al*, 1984).

In many cases the antitumour activity of the drug was maintained after encapsulation (van Hoesel *et al*, 1984; Gabizon *et al*, 1982; Forssen and Tokes, 1979). However, it should not be assumed that encapsulation of DOX will result in maintenance of the cytotoxic activity of the drug since it has been shown *in vitro* that encapsulation of the drug can result in less damage to cancer cells (Wolff and Rohdewald, 1984). Recently, a Phase I clinical trial, using a preparation called Lip-Dox, has been reported. Patients

tolerated the preparation well with none of the common side effects, such as nausea and vomiting, being evident (Sells et al, 1987). Other clinical trials being undertaken with liposomally entrapped DOX have also been reported (Treat et al, 1989; Cowens et al, 1989; Delgado et al, 1989).

Encapsulation of DOX (Rogerson et al, 1988; Kerr et al, 1988a), methotrexate (Azmin et al, 1985; Azmin et al, 1986) and sodium stibogluconate (Carter et al, 1989; Hunter et al, 1988) in non-ionic surfactant vesicles has shown that the in vivo distribution of these drugs can be modified by their encapsulation in NSV's. Rogerson et al (1988) found that injection of DOX encapsulated in vesicles resulted in a tumour growth delay in mice that was as good as, if not slightly better than, free drug and showed in addition a slight reduction in cardiac drug level. It thus appears that these non-ionic surfactant vesicles generally act in a similar manner to the more widely studied liposomes. This thesis extends the study of non-ionic surfactant vesicles as carriers of doxorubicin.

4.1.1 Analysis of Plasma and Tissue Samples

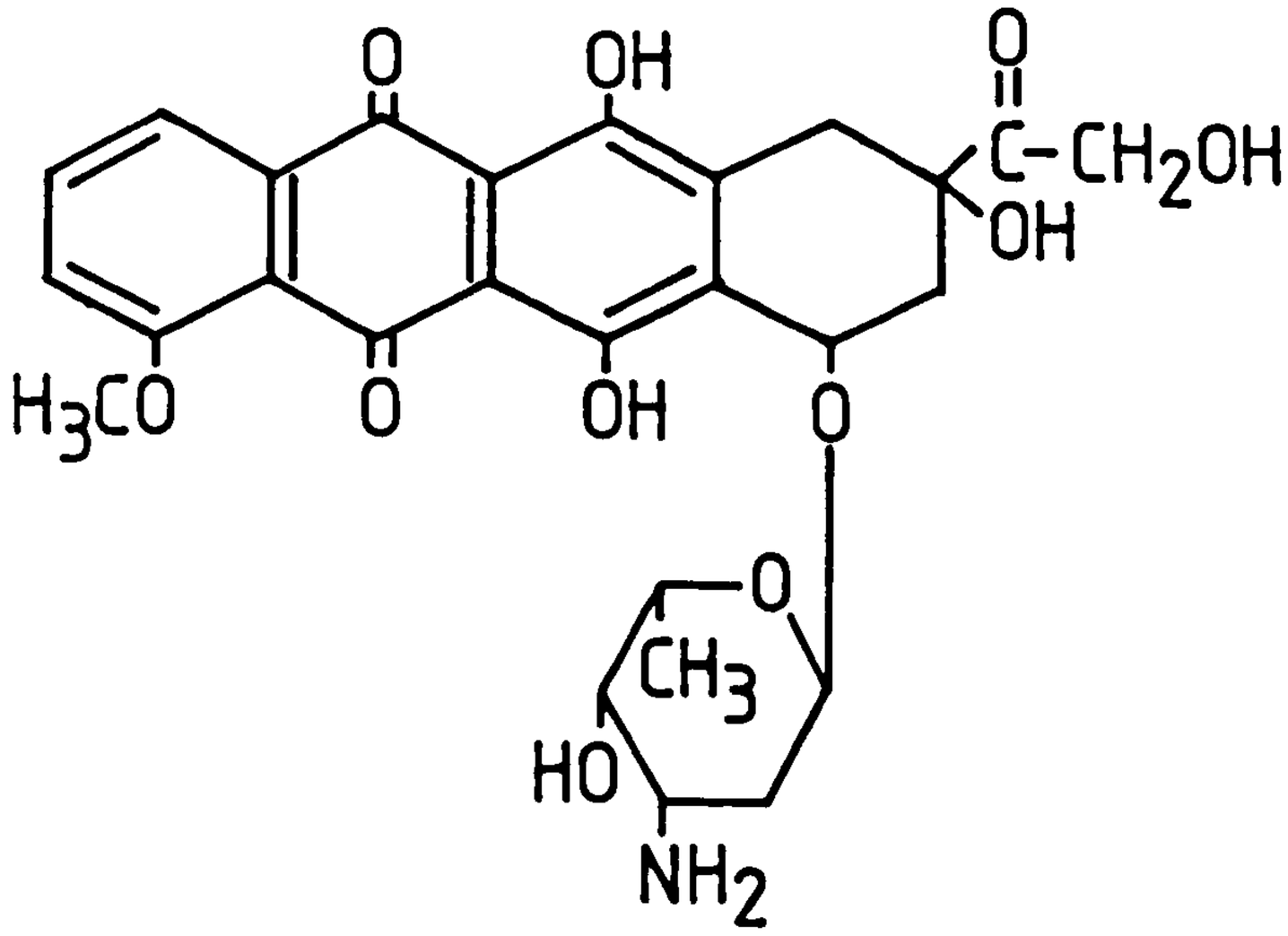
Many methods have been used to analyse DOX and other anthracyclines, with HPLC having been used extensively in conjunction with various detection methods, such as radioimmunoassay, fixed-wavelength UV absorbance, fluorescence and electrochemical detection. All these methods have disadvantages, with radioimmunoassay being unable to distinguish between parent drug molecules and metabolites, and underestimation of drug concentrations with other methods due to complexation with biomolecules or co-elution with impurities. However, of all these detection methods, fluorescence detection has been the one most widely used because of its high sensitivity and selectivity (Cummings and Morrison, 1986).

After intravenous injection of DOX solutions, the drug may undergo metabolic transformation. DOX (Fig. 4.1) may undergo C-13 carbonyl reduction to form doxorubicinol (DOL). Both the parent DOX and DOL may form 7-deoxyaglycone metabolites by reductive removal of of the C-7 linked daunosamine sugar group to form doxorubicin-7-deoxyaglycone (7-DOX) and doxorubicinol-7-deoxyaglycone (7-DOL). Doxorubicin-7-hydroxyaglycone (DOXONE) and doxorubicinol-7-hydroxyaglycone (DOLONE) may also be formed (Cummings et al, 1986). The structures of the metabolites described above are shown in Fig. 4.3.

All parent drugs and metabolites were kindly supplied by the CRC Department of Medical Oncology, University of Glasgow. Pure DOX.HCl and DOL had been received from Dr. S. Penco (Farmitalia, Milan, Italy) and the internal standard, daunorubicin HCl (DNR; Fig. 4.2) obtained from May and Baker Ltd. (Dagenham, U.K.). The 7-hydroxyaglycones (DOXONE, DOLONE) and 7-deoxyaglycones (7-DOX, 7-DOL) were synthesised in the Department of Medical Oncology, University of Glasgow (Cummings et al, 1984). A sample trace, obtained after HPLC separation of a solution containing DOX, DNR and the metabolites is shown in Fig. 4.4, and from this the relative fluorescence of each of the compounds is calculated (Table 4.1). Essentially, parent DOX, DNR and DOL are about half as fluorescent as the other metabolites. The limit of detection for the parent drugs and metabolites, as an amount injected onto the HPLC column, was 5ng for DOX, DNR and DOL and 2.5ng for the other metabolites.

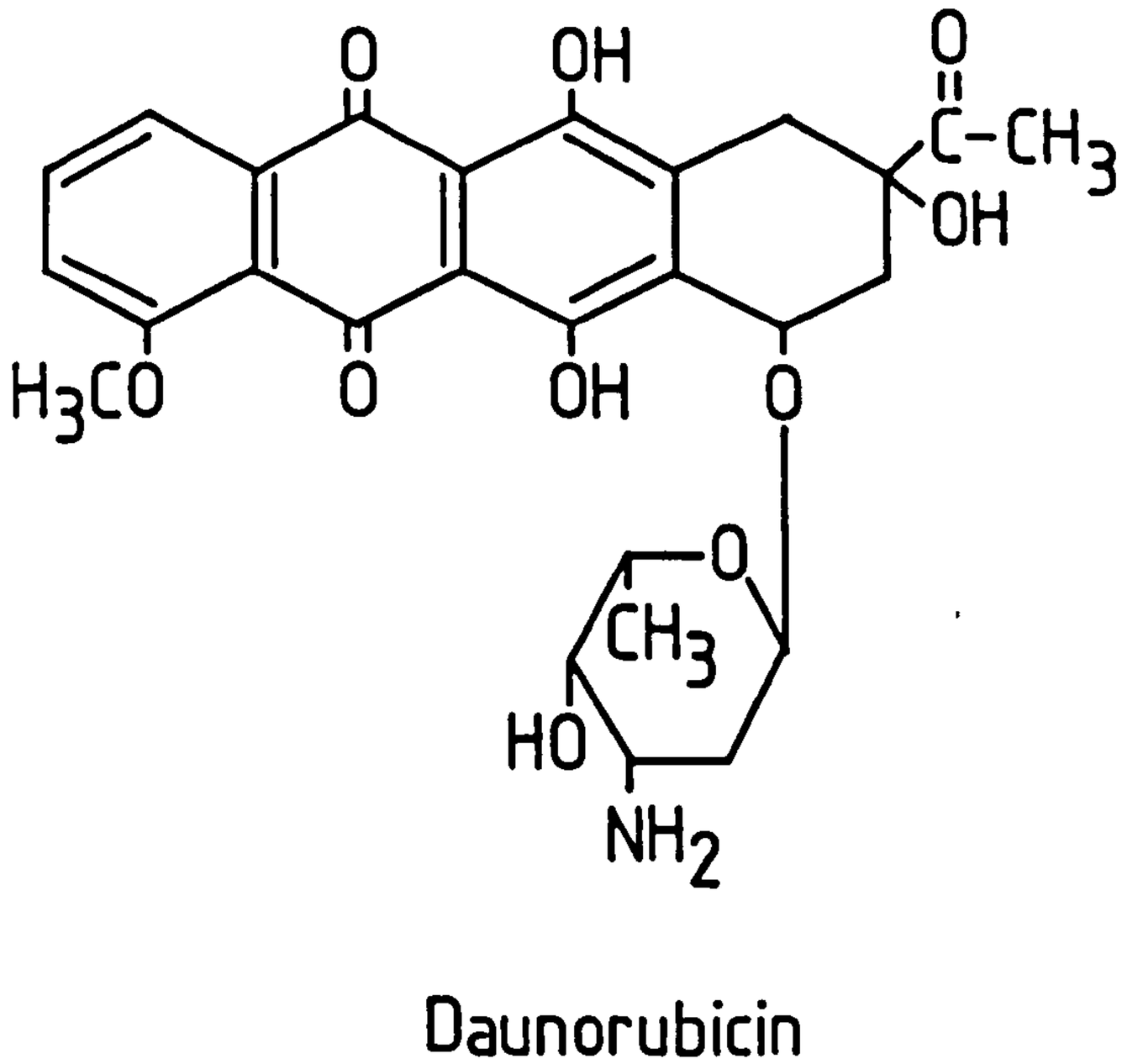
A standard calibration curve was prepared for DOX over the range 2-2000ng on column and the detector response was found to be linear. All samples for analysis were kept within this concentration range.

The addition of silver nitrate to the tissue homogenates has



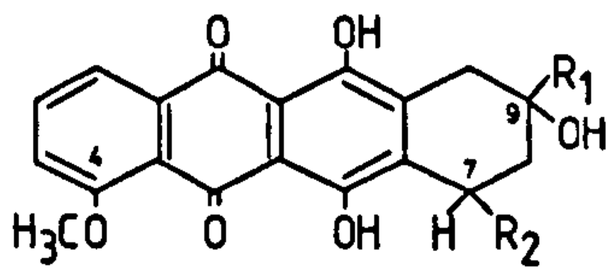
Doxorubicin

Fig. 4.1 The chemical structure of doxorubicin (DOX).



Daunorubicin

Fig. 4.2 The chemical structure of daunorubicin (DNR), the internal standard used in the HPLC analysis of the murine tissues.



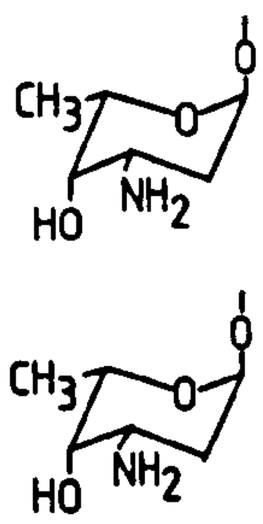
Compound	R_1	R_2
DOX	$-\overset{\overset{O}{\|}}{\underset{13}{C}}-CH_2OH$	
DOL	$-\overset{\overset{OH}{\|}}{\underset{H}{C}}-CH_2OH$	
DOXONE	$-\overset{\overset{O}{\|}}{C}-CH_2OH$	OH
DOLONE	$-\overset{\overset{OH}{\|}}{\underset{H}{C}}-CH_2OH$	OH
7-DOX	$-\overset{\overset{O}{\|}}{C}-CH_2OH$	H
7-DOL	$-\overset{\overset{OH}{\|}}{\underset{H}{C}}-CH_2OH$	H

Fig. 4.3 The structures of DOX and five of its possible metabolites capable of being detected by the HPLC method used to analyse the murine samples. The abbreviations used to describe the compounds are doxorubicin (DOX), doxorubicinol (DOL), doxorubicin-7-hydroxyaglycone (DOXONE), doxorubicinol-7-hydroxyaglycone (DOLONE), doxorubicin-7-deoxyaglycone (7-DOX) and doxorubicinol-7-deoxyaglycone (7-DOL).

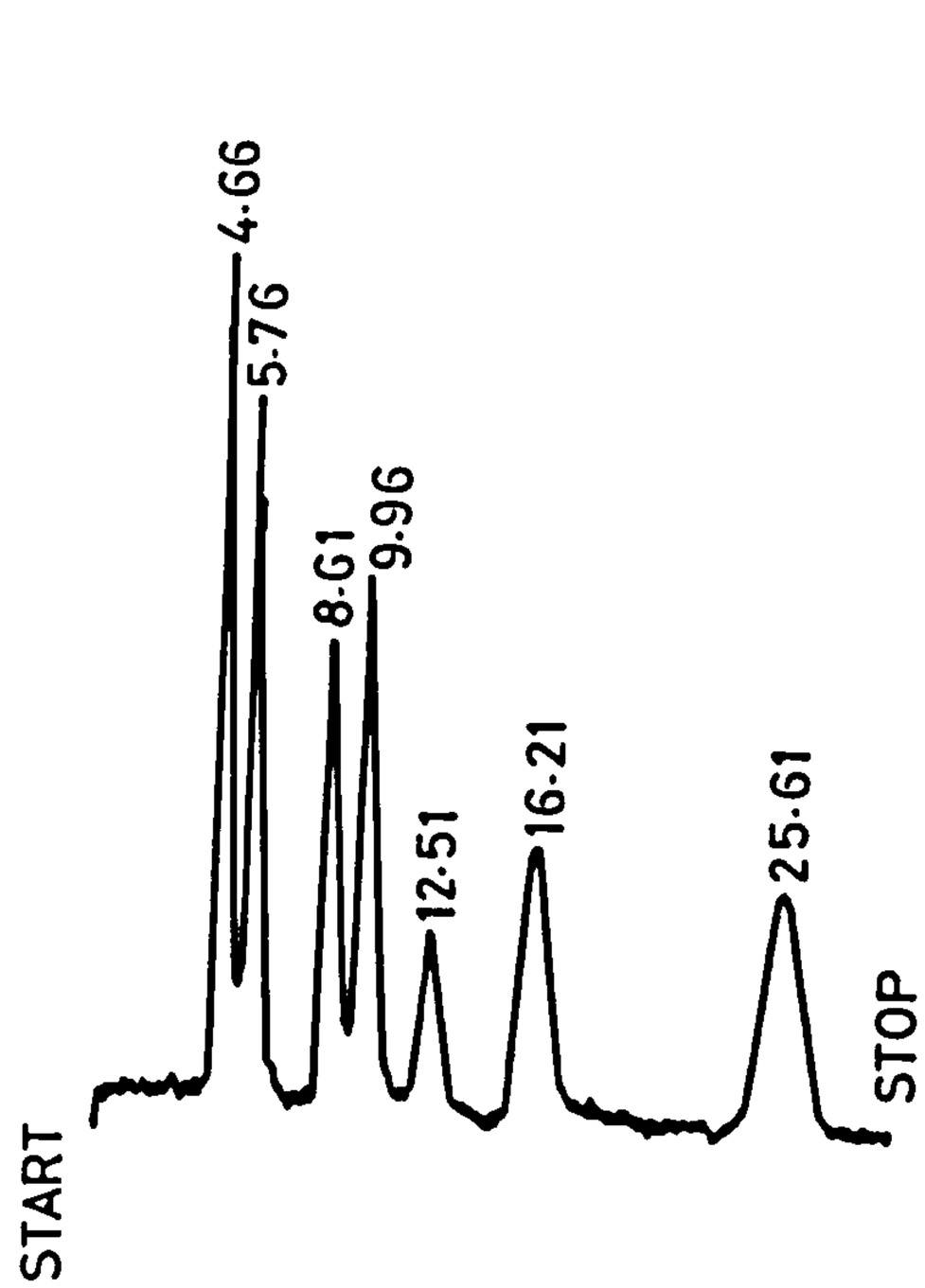


Fig. 4.4 A sample chromatogram showing the separation of DNR and DOX and its metabolites. The retention time (min) for each of the peaks is shown and the calibration factor calculated for each of the compounds is shown below in Table 4.1.

Table 4.1 The retention times and calibration factors are shown for each of the compounds separated in the chromatogram above. The compound abbreviations are the same as those used in Fig. 4.3.

Compound Abbreviation	Retention Time (min)	Calibration Factor
DOL	4.66	0.0231561
DOX	5.76	0.0257108
DOLONE	8.61	0.0100563
DNR	9.96	0.0217452
DOXONE	12.51	0.0100591
7-DOL	16.21	0.0100513
7-DOX	25.61	0.0100582

been shown to increase the amount of drug and metabolites recovered from the tissues (Cummings et al, 1986). The recovery of the internal standard (DNR) from the tissues was 60-95% and the recovery from plasma 85-98%. DNR makes a particularly good internal standard since it is structurally very similar to DOX and would be expected to behave in a similar manner to DOX throughout the extraction and analysis procedures.

The only endogenous fluorescent peak detected was seen in the liver. This peak was eluted before any of the known compounds and was close to the solvent front, being detected at about 2min.

4.1.2 Symbols Used in In Vivo Studies

In all the figures relating to the in vivo experiments, the following symbols will be used to define the sample types injected:- 1) injection of free doxorubicin in solution is shown in all figures as open circles, 2) DOX encapsulated within vesicles is shown in the figures as closed circles, and 3) injection of free DOX in conjunction with empty vesicles is shown in the figures as open squares. Empty vesicles were injected in conjunction with free DOX to observe if the empty vesicles in any way affected the distribution of the drug. All points in the figures are the mean of 5 values and the bar represents the standard deviation of these values.

4.1.3 Plasma and Tissue Doxorubicin and Metabolite Concentrations

All plasma and tissue levels were plotted as the amount of DOX, or metabolite, present per gram of that particular tissue, or per ml of plasma. The average weights of the murine tissues examined were heart (0.14g), liver (1.1g), kidney (0.3g), spleen (0.09g), tumour (0.4g) and lung (0.17g) and about 0.2ml plasma was recovered from each mouse.

4.2 *In Vivo* Distribution Study Using Modified NSV's Containing SOL24

The intravenous administration of colloidal carriers generally results in rapid removal of the carriers from the circulation by the organs of the mononuclear phagocytic system (MPS), particularly by the liver and spleen. However, several reports have indicated that by coating particles with hydrophilic block copolymers their *in vivo* fate can be modified after intravenous injection (Illum and Davis, 1987; Illum *et al*, 1986; Illum *et al*, 1987). In this part of the work, NSV's were prepared containing SOL24 (see section 2.2.3.1.1) in an attempt to make the surface of the vesicle slightly more hydrophilic. It was appreciated that the size of this POE chain and the degree of hydrophilicity it was likely to impart on the vesicle surface was very much less than that used by Illum *et al* (1986; 1987) but since the POE chain was attached to a cholesterol molecule it gave a convenient method of incorporating the chain into the bilayer.

4.2.1 Sample Details

DOX loaded vesicles and empty vesicles were prepared by the sonication method, as described previously with the composition I 50:CHOL 25:SOL24 25. The empty vesicles had a mean diameter of 484.9nm ± 24.46nm and a mean polydispersity factor of 0.2186. Drug loaded vesicles had a mean diameter of 421.0nm ± 17.4nm and a mean polydispersity factor of 0.2478. DOX entrapment was 0.8092mg/ml ± 0.1754 and represented an average of 20% DOX entrapment (range 17-35%) from a hydrating solution containing 3mg/ml DOX and 75μmoles/ml starting material.

4.2.2 Distribution Study Results

Figs. 4.5–4.8 show the DOX concentrations in plasma, heart, kidney and liver after bolus intravenous injection of the three sample types.

Plasma (Fig. 4.5) – Administration of a free drug solution resulted in a bi-, or possibly tri- phasic removal of the drug from the plasma, with an initial fast phase followed by a much slower phase. Entrapment of DOX in NSV's produced a higher initial peak which fell slowly and produced a concentration at 48h that is some 10 times higher than that obtained after injection of a free drug solution. Injection of empty vesicles with free drug produced a curve similar to that of free drug alone but was slightly higher at later time points.

Heart (Fig. 4.6) – The peak DOX concentration was reduced by encapsulation of the drug in NSV's (Table 4.2). However, the loss of DOX from the tissue was much slower than for the other two sample types. Administration of empty vesicles with free drug produced a profile similar to free drug in solution but gave a much higher peak.

Kidney (Fig. 4.7) – All three sample types produced similar drug concentration-time profiles i.e. an early peak followed by a continual fall in tissue DOX concentration. The drug loaded vesicles produced the smallest peak (Table 4.2) while the empty vesicles with free DOX produced the highest peak concentration.

Liver (Fig. 4.8) – The peak concentration for all three sample types was essentially the same in the liver (Table 4.2). All samples exhibited similar falls in concentration over the first 4h, with the free drug and empty vesicle with free drug samples continuing to fall at the same rate thereafter. From 4h until 48h the drug loaded vesicle sample concentration remained almost the

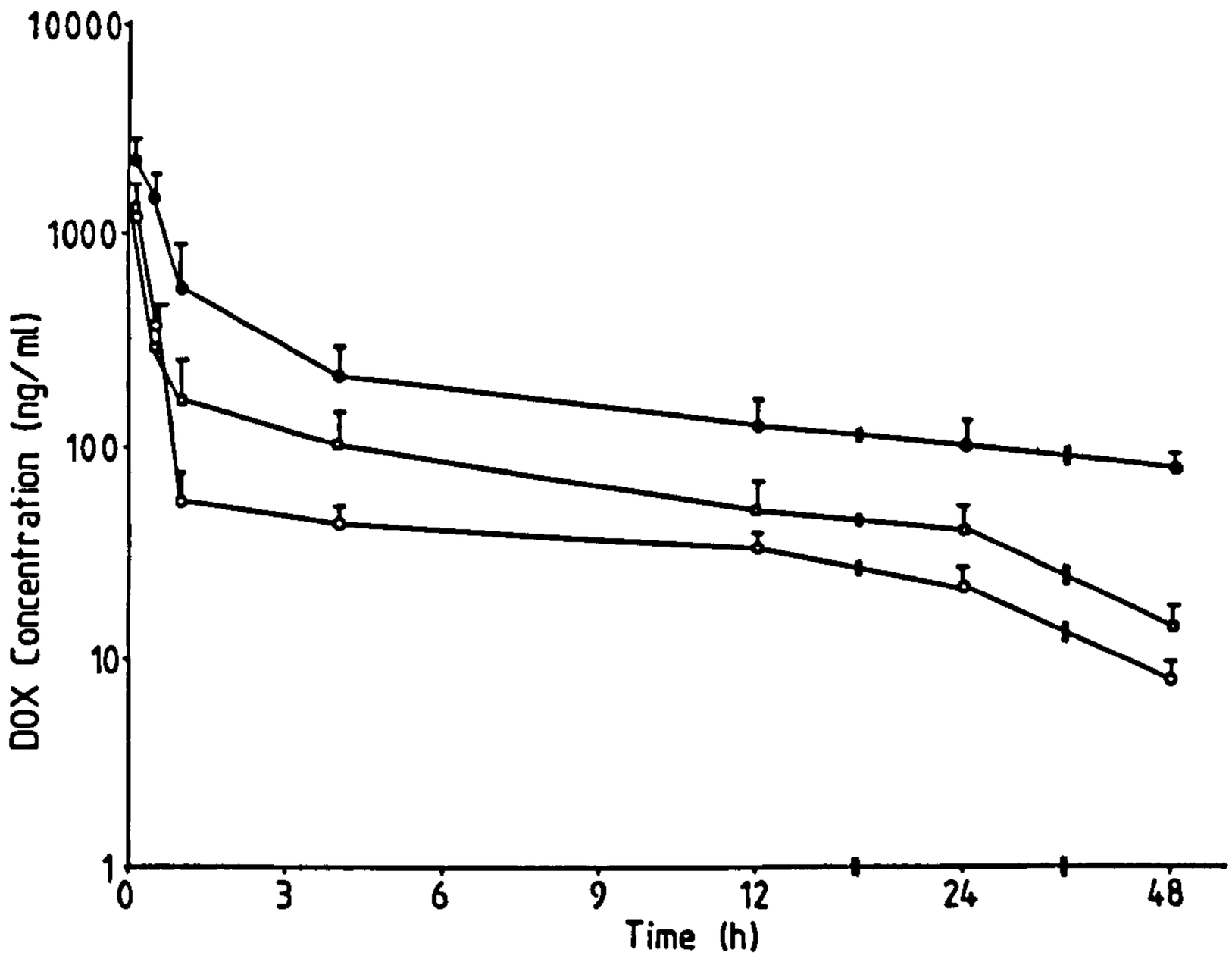


Fig. 4.5 Plasma concentrations of DOX as a function of time after bolus injection of 5mg kg^{-1} into the tail vein of AKR mice of free drug in solution (O), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of I 50:CHOL 25:SOL24 25.

The vertical bar denotes the standard deviation of the drug concentration.

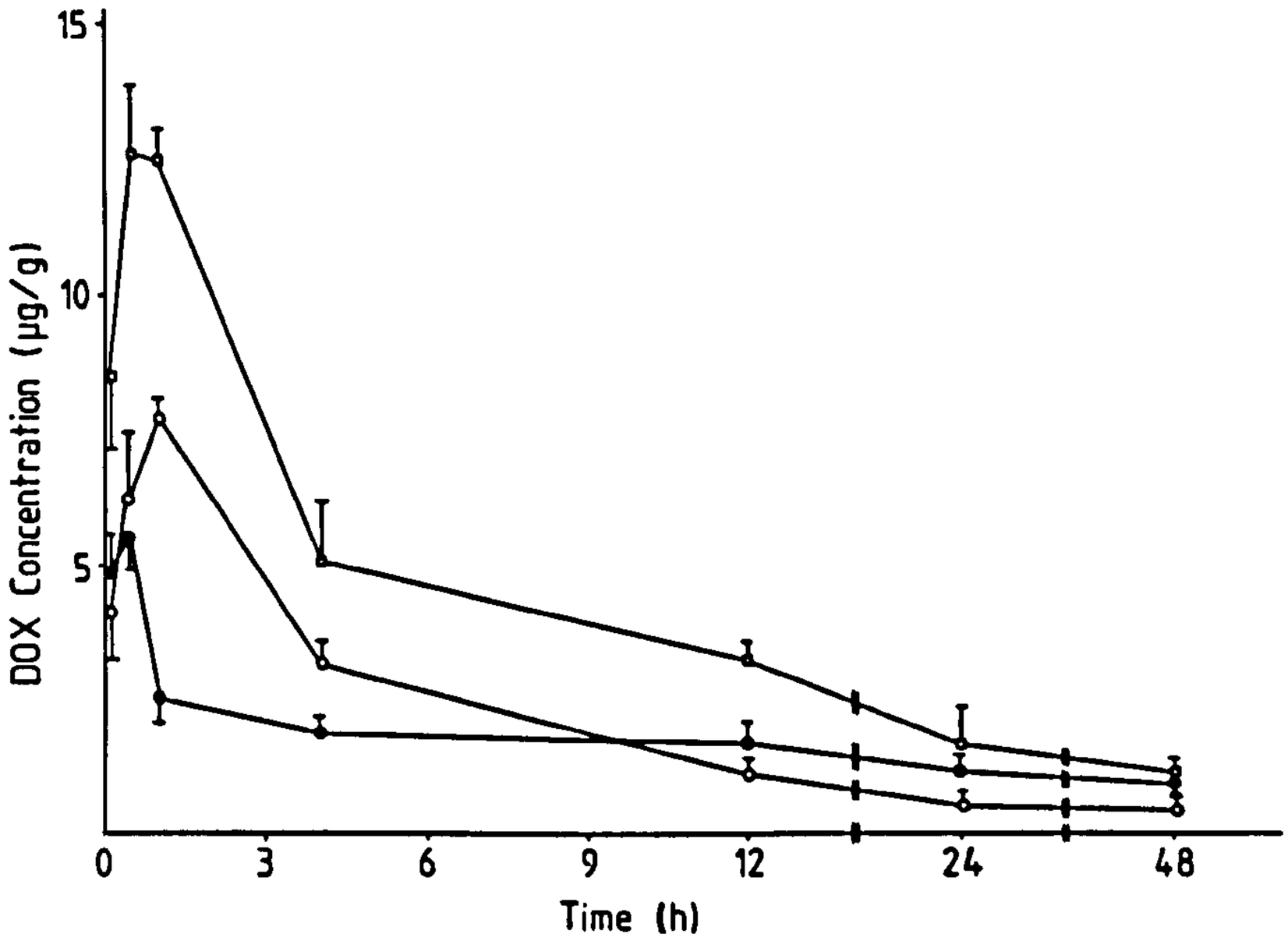


Fig. 4.6 Heart concentrations of DOX as a function of time after bolus injection of 5mg kg^{-1} into the tail vein of AKR mice of free drug in solution (O), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of I 50:CHOL 25:SOL24 25.
The vertical bar denotes the standard deviation of the drug concentration.

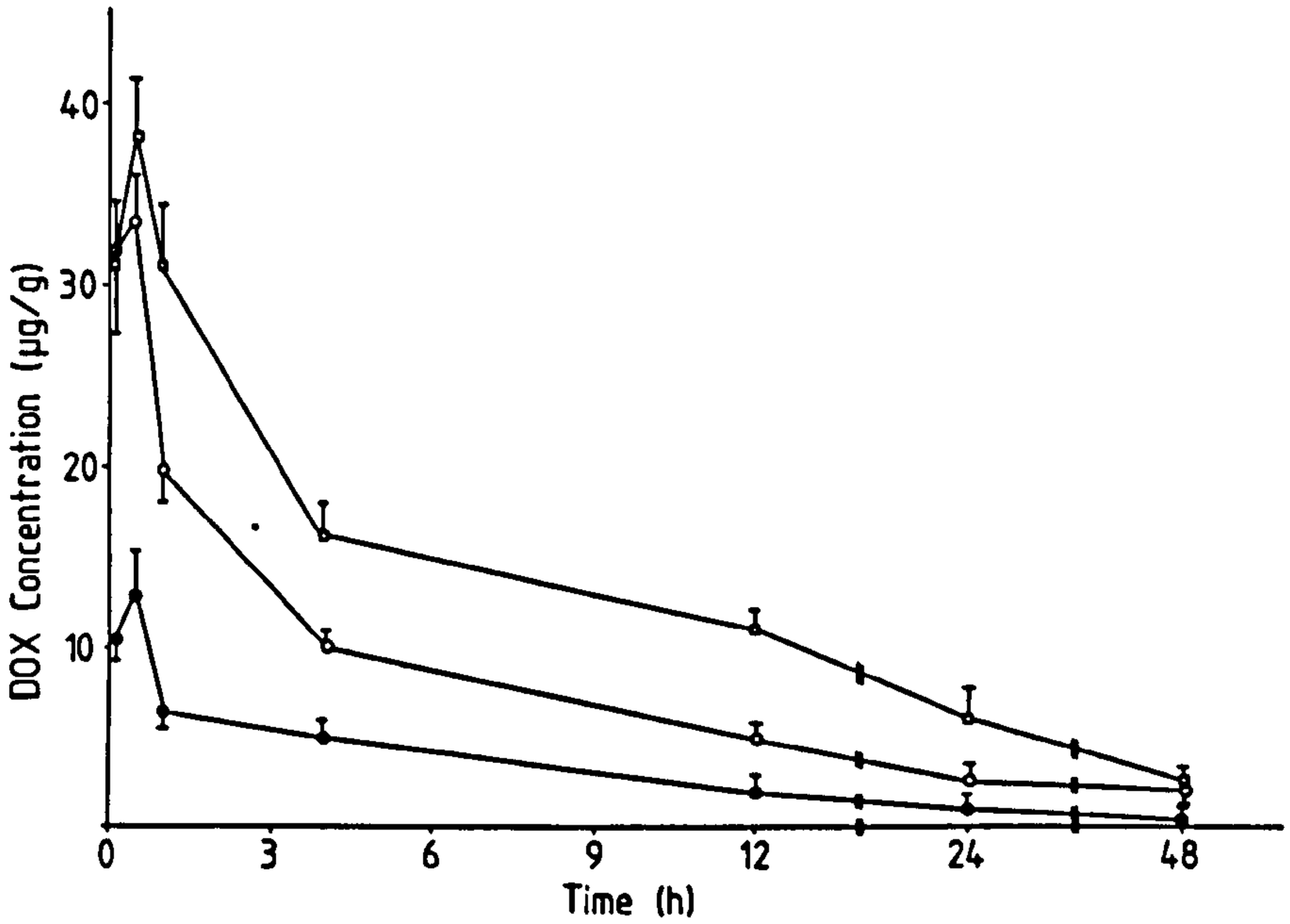


Fig. 4.7 Kidney concentrations of DOX as a function of time after bolus injection of 5mg kg^{-1} into the tail vein of AKR mice of free drug in solution (O), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of I 50:CHOL 25:SOL24 25.
The vertical bar denotes the standard deviation of the drug concentration.

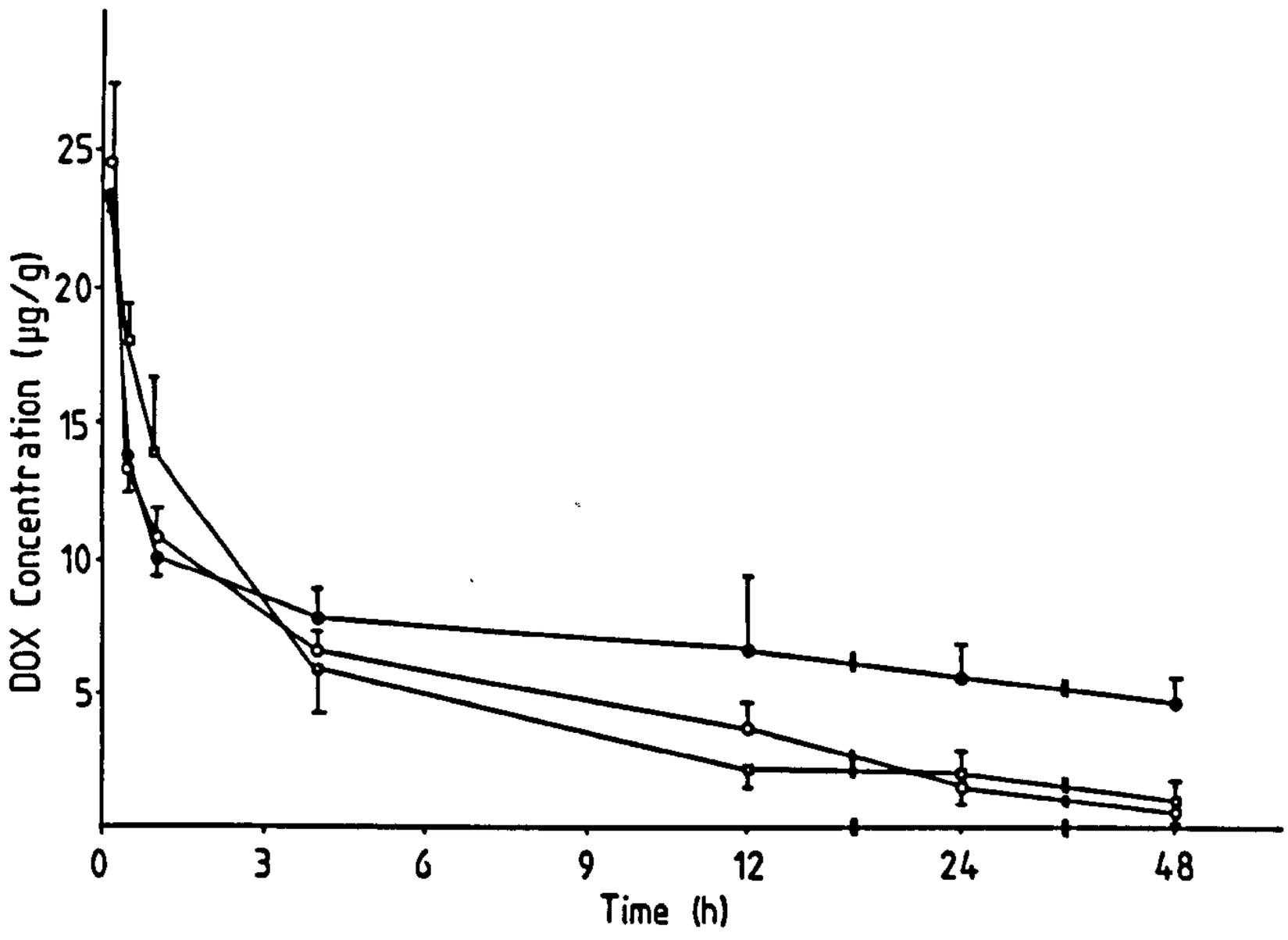


Fig. 4.8 Liver concentrations of DOX as a function of time after bolus injection of 5mg kg^{-1} into the tail vein of AKR mice of free drug in solution (o). empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of I 50:CHOL 25:SOL24 25.

The vertical bar denotes the standard deviation of the drug concentration.

same.

Metabolites - Many metabolites were detected in the tissues examined. However, in most tissues and for most metabolites detected, they were not produced in sufficient quantities to allow meaningful conclusions to be drawn. Only liver produced sufficient quantities of 7-DOX (Fig. 4.9) and 7-DOL (Fig. 4.10) in most of the mice at most time points to allow mean values to be calculated. For both metabolites, injection of free drug in solution or empty vesicles with free DOX produced peaks at 4h with subsequent rapid falls. The DOX loaded vesicles produced no peak, but gave a low, sustained concentration over the 48h.

Table 4.2 Peak DOX concentrations (μg/g ± standard deviation) obtained in heart, kidney, liver and plasma (μg/ml). (F.DOX represents free drug in solution, L.DOX represents drug loaded vesicles and E.DOX represents empty vesicles given in conjunction with free drug in solution.)

Tissue	F.DOX	L.DOX	E.DOX
Heart	7.75 ± 0.198	5.6 ± 0.62	12.6 ± 0.159
Kidney	33.6 ± 2.1	13.11 ± 1.98	38.0 ± 3.4
Liver	24.6 ± 1.36	23.2 ± 2.6	23.1 ± 1.82
Plasma	1.307 ± 0.018	2.215 ± 0.175	1.36 ± 0.159

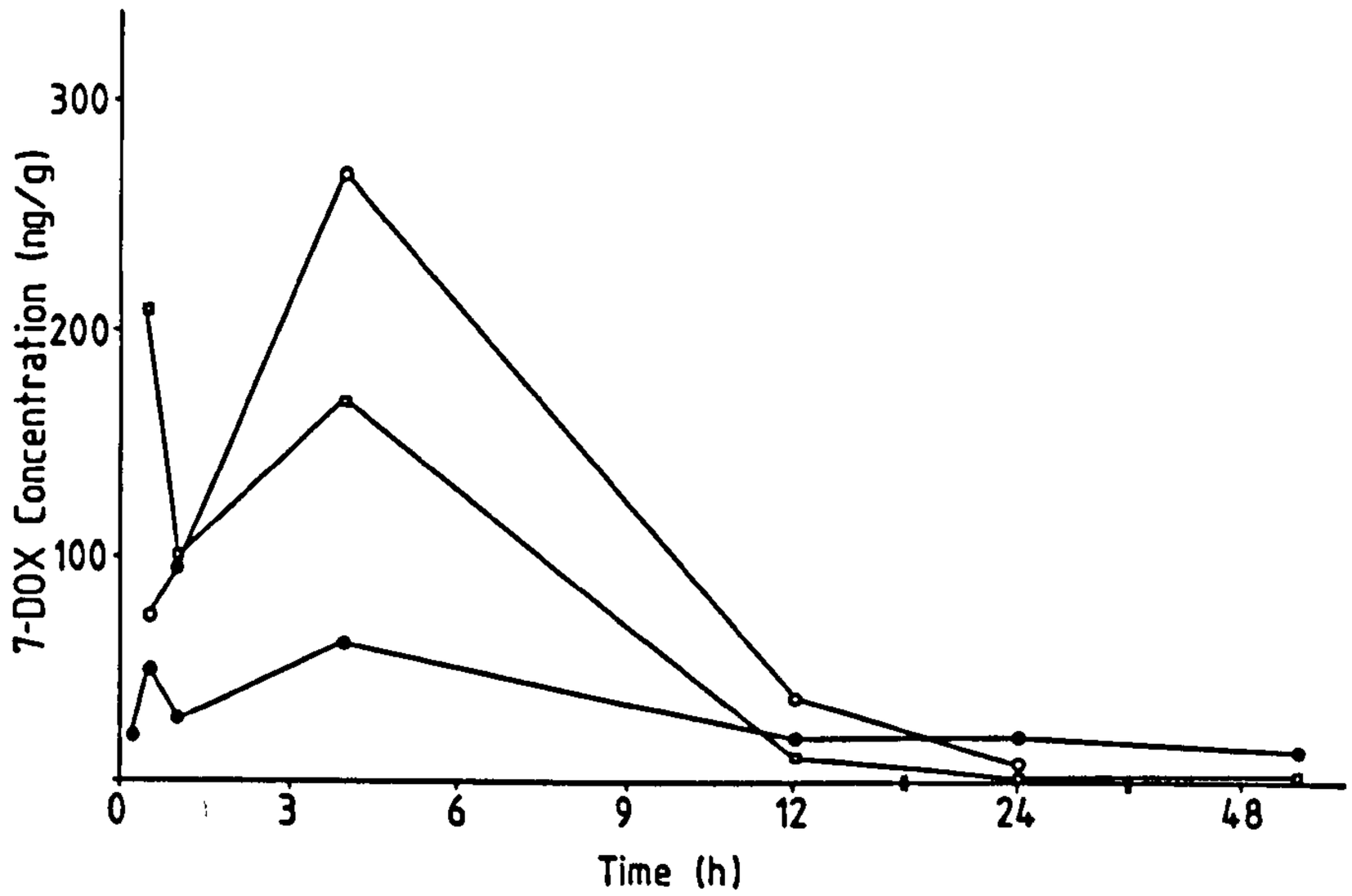


Fig. 4.9 Liver doxorubicin-7-deoxyaglycone (7-DOX) metabolite concentrations as a function of time after bolus injection of 5mg kg^{-1} into the tail vein of AKR mice of free drug in solution (O), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of I 50:CHOL 25:SOL24 25.

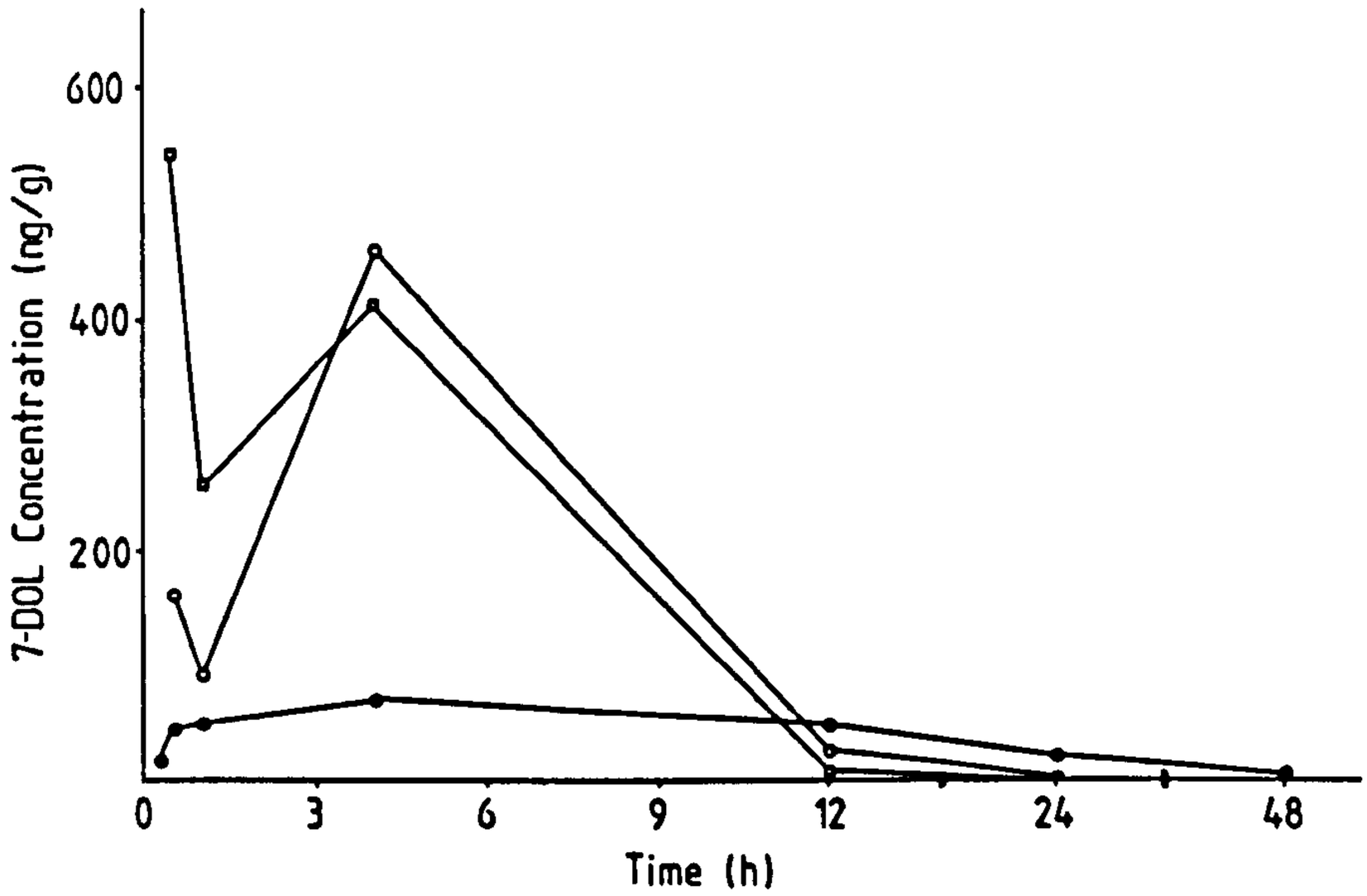


Fig. 4.10 Liver doxorubicinol-7-deoxyaglycone (7-DOL) metabolite concentrations as a function of time after bolus injection of 5mg kg^{-1} into the tail vein of AKR mice of free drug in solution (o), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of I 50:CHOL 25:SOL24 25.

4.2.3 Discussion

In plasma and all the tissues examined in this study, the DOX concentration measured was assumed to be the total DOX present in the sample i.e. the DOX which was measured represented free drug as well as any drug which was still entrapped or associated with vesicles. Recently, methods have been suggested where the amount of DOX circulating in the plasma can be separated into a vesicularly associated fraction and a non-vesicularly associated fraction, with the non-vesicularly associated fraction containing both the drug bound to plasma proteins as well as the free drug (Druckmann et al, 1989). DOX is known to be about 80% protein bound in plasma (Greene et al, 1983) with most of this bound to low molecular weight proteins such as albumin (Eksborg et al, 1982).

It has been shown that when DOX was injected intravenously as a free drug solution to mice, the drug was removed from the plasma in a triphasic manner. The initial distribution to the tissues was very rapid, with an α half life of 1.3min (Cummings et al, 1986). Distribution to the tissues was also rapid with equilibration in heart and liver occurring after 10min (Cummings et al, 1986). When the drug became internalized within cells, it was almost entirely associated with the nucleus of the cell (Egorin et al, 1974; Egorin et al, 1980). The slow plasma terminal half life of DOX observed in man suggested that its elimination may be governed to some extent by the slow release of nuclear bound drug from the tissues (Cummings et al, 1986).

In this study, removal of DOX from plasma, after injection of a free drug solution, occurred in a similar manner to that described above.

Administration of drug loaded vesicles resulted in higher plasma

DOX concentrations, than those obtained after injection of free drug in solution, at all time points examined. This appears to be a feature of the kinetics of DOX when it is encapsulated in vesicles, since similar features were observed using NSV's of different composition (Rogerson et al, 1988) and with liposomes (Olson et al, 1982; Rahman et al, 1986a). The increased initial plasma concentration observed with the drug loaded vesicles is likely to be due to the slower removal of the vesicles with their encapsulated drug from the circulation, compared with removal of drug molecules from the circulation when administered as a free drug solution. The maintenance of the plasma concentration by the drug loaded vesicles is likely to be due to the "depot" effect created by the vesicles removed from the circulation by the tissues slowly releasing their contents.

The administration of empty vesicles in conjunction with free drug produced a plasma profile similar to that obtained by injection of free drug alone, except that a slightly higher drug concentration was measured at later time points. It has been shown in dialysis experiments (see section 3.2.8) that empty vesicles containing SOL24 appeared to reversibly bind DOX. The empty vesicles, with any associated DOX, would be removed from the circulation in a similar manner to the drug loaded vesicles and act as a "depot" of DOX, albeit a far smaller reservoir than the drug loaded vesicles.

Much of the promise shown by preparations encapsulating DOX has been due to their ability to reduce the amount of drug being delivered to the heart (Olson et al, 1982), the major organ of toxicity of DOX. The question of whether vesicular systems will reduce cardiotoxicity and/or cardiac DOX levels appears to be dependant on the properties of the vesicles used. Several groups of

workers have found a reduced cardiac drug concentration when DOX was encapsulated (Rogerson et al, 1988; Rahman et al, 1982; Olson et al, 1982; Forssen and Tokes, 1979) and this reduced drug uptake by cardiac tissue correlated well with fewer cardiac lesions being detected (van Hoesel et al, 1984). Rahman et al (1980) reported that negatively charged liposomes produced higher cardiac DOX concentrations compared with a free drug solution, while positively charged vesicles reduced the cardiac uptake of drug. Gabizon et al (1982) noted that the composition and resulting permeability of the vesicles when in contact with serum was important in determining whether a reduced cardiac uptake of DOX was observed in vivo. Cardiolipin containing liposomes were found to be particularly unstable in the presence of serum. Thus, when these liposomes were injected intravenously, any encapsulated DOX was rapidly released and a minimal reduction in the cardiac drug concentration was observed. Administration of liposomes with compositions which were less susceptible to the effects of serum all reduced the cardiac drug concentration to a much greater extent.

Cellular uptake of liposomes may take place by membrane fusion, but is more likely to occur by endocytosis (Poste and Papahadjopoulos, 1976), while free DOX entered cells by passive diffusion or carrier mediated transport (Skovsgaard, 1978). Tissues vary in their endocytotic activity, with liver, spleen and some tumours having high activity compared to heart and kidney (de Duve et al, 1974; Gregoriadis et al, 1974; Segal et al, 1974; Trouet et al, 1972).

After injection of the DOX loaded NSV's in this particular study, the cardiac peak was reduced compared with free DOX, but a plateau level was reached at later times with drug loaded vesicles,

while the free drug curve fell over the 48h. Since cardiac tissue has low endocytotic activity, low drug uptake would be expected when vesicular carriers are administered intravenously. However, to account for the plateau level obtained, either some uptake of vesicles must be occurring in the heart or vesicles may become trapped within the vasculature of the tissue.

Injection of empty vesicles in conjunction with free DOX produced much higher peak levels than either of the other two preparations. The peak concentration was about twice as high as that of free drug and it appeared that the presence of empty vesicles was increasing the uptake of the free DOX molecules into the tissues. Increased absorption, induced by polyoxyethylene-24-cholesteryl ether (SOL24) has been observed previously for water soluble antibiotics (Davis et al, 1970) and ergot peptide alkaloids (Franz and Vonderscher, 1981).

The kidney is another potential organ of toxicity of DOX and another area where vesicular encapsulation may reduce this toxicity (Forssen and Tokes, 1979; van Hoesel et al, 1984).

The results obtained were similar to those seen in the heart, with encapsulation reducing the peak drug concentration, compared to free drug, although in this case there appeared to be no plateau concentration at later time points with the drug loaded vesicle sample. Again the reduced peak concentration, seen with the drug loaded NSV's, was probably due to the poor endocytotic capacity of kidney tissue. The fact that no plateau was seen suggested very few vesicles were taken up into the tissue.

Injection of the empty vesicles in conjunction with free DOX produced a larger peak and again this was likely to be due to the increased uptake of DOX induced by the empty vesicles in a similar

manner to that seen in heart tissue.

The liver profile obtained by encapsulating DOX in liposomes appeared to be dependant on the vesicular composition used. Gabizon et al (1982) found increased liver DOX levels and decreased hepatic clearance for most compositions of liposomes encapsulating DOX, but found that incorporation of cardiolipin resulted in a smaller increase. This smaller increase in liver DOX level was thought to be due to the leakiness of this particular liposomal composition, which had been observed in the presence of serum in vitro. However, this reduction must be due to other factors as well as to the incorporation of cardiolipin, since Rahman et al (1986a) found increased liver uptake and a 15 fold reduction in the terminal half life when using cardiolipin containing liposomes.

Increased liver uptake of DOX loaded liposomes was also observed with negatively charged liposomes (Forssen and Tokes, 1979) although Rahman et al (1980) found DOX entrapped in positive liposomes gave an area under the curve which was almost twice that of negatively charged vesicles.

Non-ionic surfactant vesicles containing methotrexate were shown to accumulate in the liver to a greater degree than free methotrexate (Azmin et al, 1985), while the concentration of sodium stibogluconate in liver 24h after administration showed encapsulating the drug in either liposomes or NSV's produced tissue levels 6-7 times higher than the free drug solution (Hunter et al, 1988).

In this study, little liver accumulation of DOX loaded NSV's appeared to be taking place compared with the drug levels obtained for free DOX solutions and all three samples produced similar initial concentrations. This indicates that the liver is capable of removing both free drug and particulate matter from the plasma.

These results are similar to those obtained by Rogerson et al (1988) and Kerr et al (1988a) who noted that the peak DOX levels in murine liver were reduced after administration of DOX loaded NSV's. Kerr et al (1988a) found that the peak hepatic levels, areas under the curve and half-lives were statistically indistinguishable, irrespective of whether the DOX was administered as a free drug solution or in encapsulated form.

Apparent differences in the peak liver concentration were observed after administration of DOX loaded NSV's in different studies in this laboratory, which may be due to differences in the size of the vesicles injected. The NSV's prepared by Rogerson et al (1988) and Kerr et al (1988a) measured about 1μm in diameter, while those injected in this study were 421nm in diameter. The smaller peak liver DOX concentrations noted by Rogerson et al (1988) and Kerr et al (1988a) may be due, at least in part, to a greater physical trapping of the NSV's in the lungs. The lung removal of the smaller NSV's used in this study should be much less, resulting in more vesicles being available for uptake by the liver.

Greatly increased peak liver methotrexate levels were measured by Azmin et al (1985) after encapsulation of the drug in NSV's. Since the vesicles used by Azmin et al (1985) were about 120nm in diameter, few would be expected to be trapped in the lungs. If the peak liver concentration for encapsulated methotrexate is compared with the peak liver doxorubicin concentration measured in this study, the drug levels compare favourably when the amount of drug injected into the mice is taken into consideration. This suggests that the vesicles are removed by the liver to a similar degree in both cases.

It is well known that the potential usefulness of vesicular carriers after intravenous administration is hampered by their uptake

by the mononuclear phagocyte system (MPS). The removal of vesicles which are classed as being large i.e. have diameters greater than 100nm, was primarily by the phagocytic cells of the liver and spleen such as the Kupffer cells (Scherphof et al, 1987). The precise kinetics of vesicle removal, and the role played by each organ in this, will depend on their size and charge (Juliano and Stamp, 1975), hydrophilicity (Illum et al, 1987) and by the effect of the encapsulated drug on these parameters.

The metabolite concentrations measured at each time point, for a specific tissue, were particularly variable due to the erratic nature of metabolism in vivo. Thus, care must be taken not to infer too much from the data and only attempt to make general comments about the effect various parameters have on the metabolism of DOX.

Metabolism was only seen to any significant extent in the liver where the parent drug was converted to 7-DOX and 7-DOL. The free drug sample showed the greatest potential for metabolism with the empty vesicle with free drug sample producing slightly less metabolism. Both these sample types produced a peak at 4h followed by a rapid fall in the metabolite concentration. The slight reduction in metabolism seen with the empty vesicles with free drug may possibly be due to the association of free drug molecules with the vesicles resulting in some degree of protection of the DOX molecule to metabolism. However, much more data would be required before this hypothesis could be confirmed. The DOX loaded vesicle sample produced a prolonged, but low metabolite concentration, compared to the other two sample types, and again may be due to some degree of protection to metabolism being conferred by encapsulation in vesicles, with only DOX that is released from the vesicle capable of undergoing metabolism.

This compared favourably with the results obtained by Azmin et al (1985; 1986) who showed that encapsulation of methotrexate in NSV's resulted in a much lower level of metabolism in murine liver.

The injection of DOX loaded NSV's into mice resulted in reduced peak levels but higher plateau concentrations of metabolites in plasma (Rogerson et al, 1988) and liver (Kerr et al, 1988a), compared with free drug in solution.

The cardiotoxicity seen after the administration of DOX may be caused by some of the metabolites formed in vivo. It has been suggested that reactive intermediates were formed in the conversion of DOX to the 7-deoxyaglycones and that these intermediates may be responsible for DOX induced toxicity (Goodman and Hochstein, 1977; Myers et al, 1977). More recently, doxorubicinol, the primary circulating metabolite of DOX, has been shown to be more potent than the parent drug at compromising both systolic and diastolic cardiac function (Olson et al, 1988).

The exact mechanism of production of metabolites and their activities and toxicities from parent DOX is beginning to be understood. However, much more work has to be carried out to fully ascertain the effect of encapsulation on the metabolism of DOX.

Whether encapsulation of DOX increases tissue uptake of the drug, compared with free drug in solution, will depend on the uptake of free drug and carriers by the tissue. The uptake of free DOX will depend on the physicochemical properties of the drug and the transport mechanisms of the drug into the tissue, while the properties of the carrier, as discussed above, will affect its uptake. All these factors, along with differences in drug and carrier handling by different animal species, may explain some of the apparent contradictions reported with different compositions of

liposomes encapsulating the same drug, or using different drugs with the same liposomal composition.

4.3 *In Vivo* Distribution and Tumour Growth Delay Study for Vesicles containing Surfactant IV

In all the previous *in vivo* experiments carried out using vesicles composed of non-ionic surfactants of the mono/di alkyl polyglyceryl ether class, none of the surfactants used were totally homogenous products since they contained hydrophilic headgroups whose length was an average value. In this experiment the homogenous product, Surfactant IV was used in an attempt to ascertain if having a defined, and smaller headgroup length (see Fig. 2.1) made any difference to the way in which the vesicles behaved *in vivo*. The results of the *in vitro* studies showed that vesicles prepared from Surfactant IV were particularly susceptible to aggregation. It therefore came as no surprise to find that uncharged vesicles (IV 50:CHOL 50) aggregated when attempts were made to produce dispersions of DOX loaded vesicles. The addition of dicetyl phosphate (DCP) induced a negative charge (IV 47.5:CHOL 47.5:DCP 5.0) which allowed non-aggregated systems to be produced using dilute solutions of DOX as the hydrating solution. Increasing the DOX concentration of the hydrating solution again produced aggregation. The inclusion of a positive charge, via stearylamine (SA), (IV 47.5:CHOL 47.5:SA 5.0) allowed formation of vesicles in a concentrated DOX hydrating solution without aggregation. Thus, this vesicular composition was used for the *in vivo* studies.

The inclusion of a high concentration of cholesterol in the bilayer of the vesicles reduced the rate of release of DOX from the vesicles (Storm *et al*, 1989) as shown previously (see section 3.2.7). It may also affect the distribution of the vesicles since *in vitro* work has shown that Kupffer cells of the liver avidly take up cholesterol-poor but not cholesterol-rich liposomes whereas splenic

macrophages preferentially take up cholesterol-rich rather than cholesterol-poor liposomes (Moghimi and Patel, 1988).

Stearylamine has been implicated as being potentially toxic to cells. It has been shown to cause significant haemolysis of rabbit red blood cells, with the amount of haemolysis related to the amount of stearylamine in the liposomal membrane. This haemolysis was most marked with rabbit, horse and guinea pig erythrocytes while those from man, sheep, cow and chicken were less susceptible (Yoshihara and Nakae, 1986). Positively charged liposomes containing stearylamine were found to be more toxic to chick heart cells in vitro and produced a 5-fold reduction in the LD_{50} in mice in vivo when compared with negatively charged or neutral vesicles (Olson et al, 1982).

However, stearylamine containing liposomes with entrapped doxorubicin have been injected intravenously to mice (Rahman et al, 1986b) and rats (Rahman et al, 1986a; van Hoesel et al, 1984), and injected into the cerebrospinal fluid of cynomolgus monkeys (Kimelberg et al, 1978) with no evidence of overt toxicity.

4.3.1 Sample Details

DOX loaded vesicles and empty vesicles were prepared by the sonication method with the composition IV 47.5:CHOL 47.5:SA 5.0. The empty vesicles had a mean diameter of 179.6nm ± 15.96nm and a polydispersity factor of 0.1934. The drug loaded vesicles had a mean diameter of 194.3nm ± 8.7nm and a polydispersity factor of 0.2585. DOX entrapment was 1.0533mg/ml ± 0.1445mg/ml and represented an entrapment of about 21% (range 18-24%) from a hydrating solution containing DOX 5mg/ml and containing 75μmoles/ml starting material.

4.3.2 Distribution Study Results

Figs. 4.11–4.16 show the DOX concentrations, detected by the HPLC method described previously, in plasma and tissues and metabolite concentrations in lung (Fig. 4.17) and liver (Figs. 4.18 and 4.19).

Plasma (Fig. 4.11) - Administration of DOX as a free drug solution or empty vesicles in conjunction with free drug resulted in the bi/tri phasic removal of drug from plasma expected, with both sample types producing virtually identical traces. Injection of drug loaded vesicles resulted in a markedly different plasma profile, with a virtually constant drug concentration detected throughout the 48h of the study. Initially the drug concentration was less than the other two samples, but after 4h was much higher.

Heart (Fig. 4.12) - Once again the free drug solution and empty vesicles with free drug sample produced traces which were the same. The drug loaded NSV's produced a very small peak at 15min followed by a gradual fall then a slight rise at 48h.

Lung (Fig. 4.13) - Free drug in solution and empty vesicles with free drug again produced very similar curves. Very low levels of DOX were detected in the tissue after injection of the DOX loaded sample with a virtually constant concentration throughout, save a slight rise at 48h.

Liver (Fig. 4.14) - Curves for the free drug solution and empty vesicles in conjunction with free drug were very alike, falling from the initial peak level. When the drug loaded sample was injected, a small peak was measured at 1h which was maintained over 48h. At 48h, the DOX concentration measured for the drug loaded vesicle sample was greater than that measured for the other two sample types.

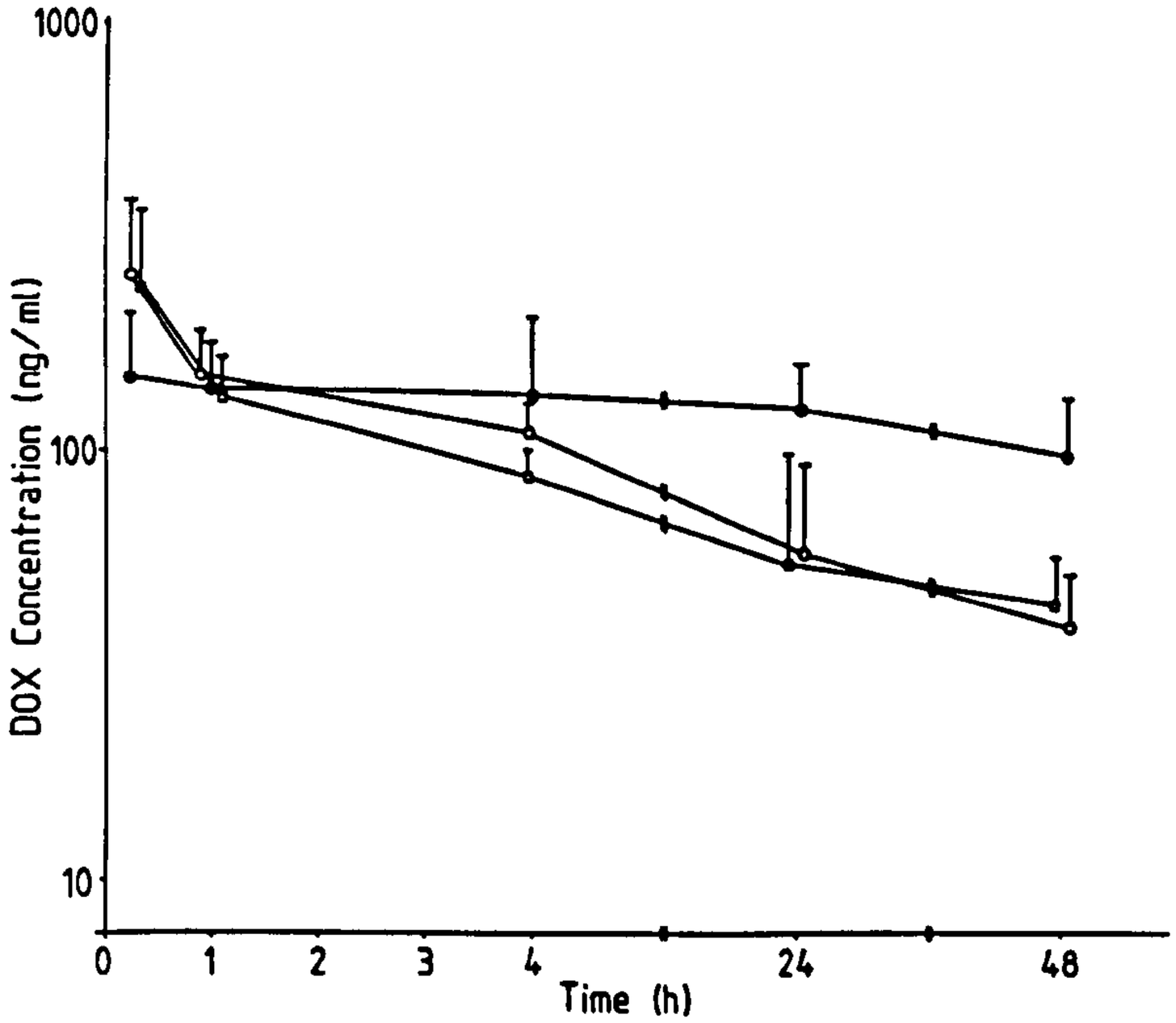


Fig. 4.11 Plasma concentrations of DOX as a function of time after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (O), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.
The vertical bar denotes the standard deviation of the drug concentration.

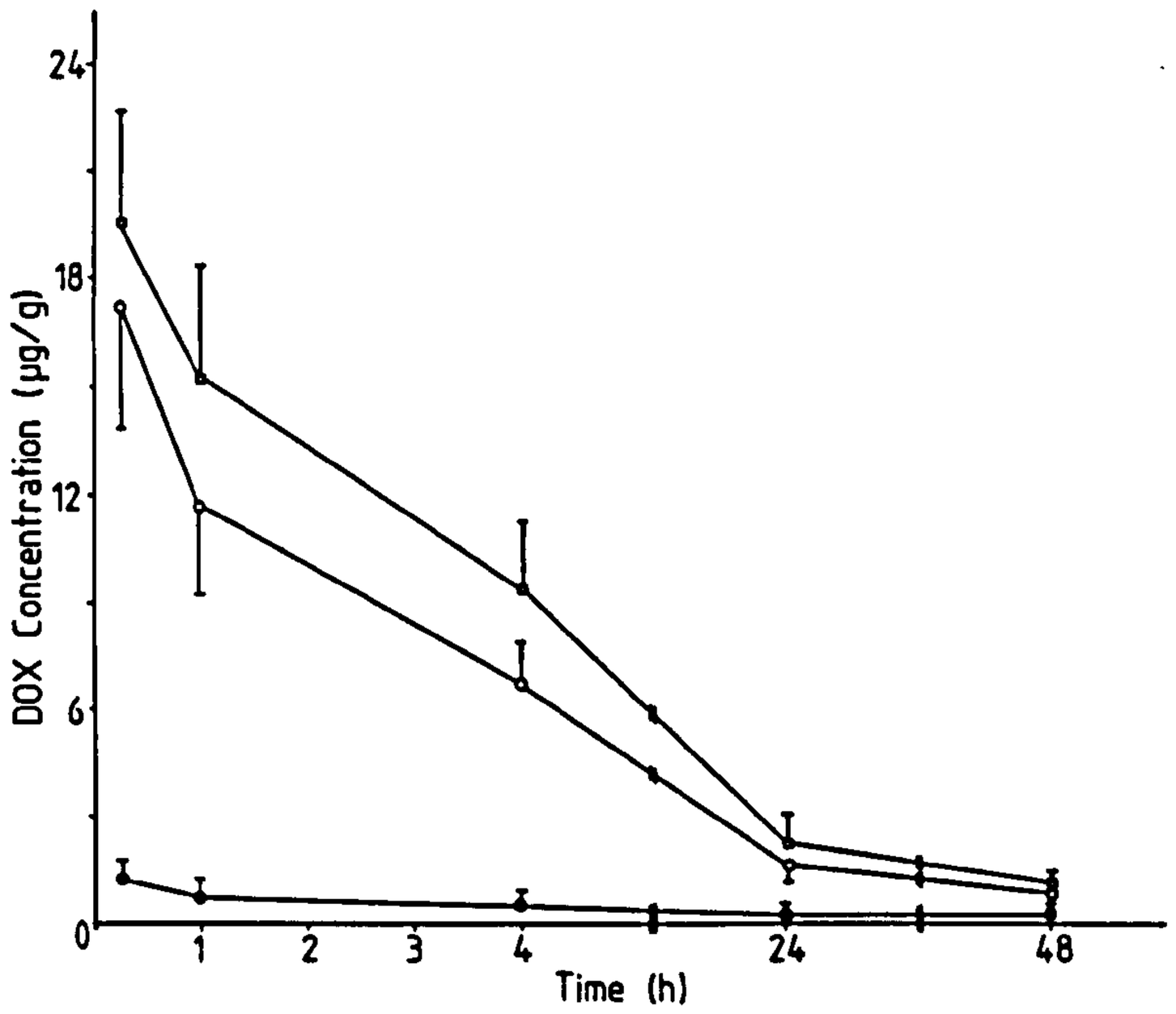


Fig. 4.12 Heart concentrations of DOX as a function of time after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (o), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.
The vertical bar denotes the standard deviation of the drug concentration.

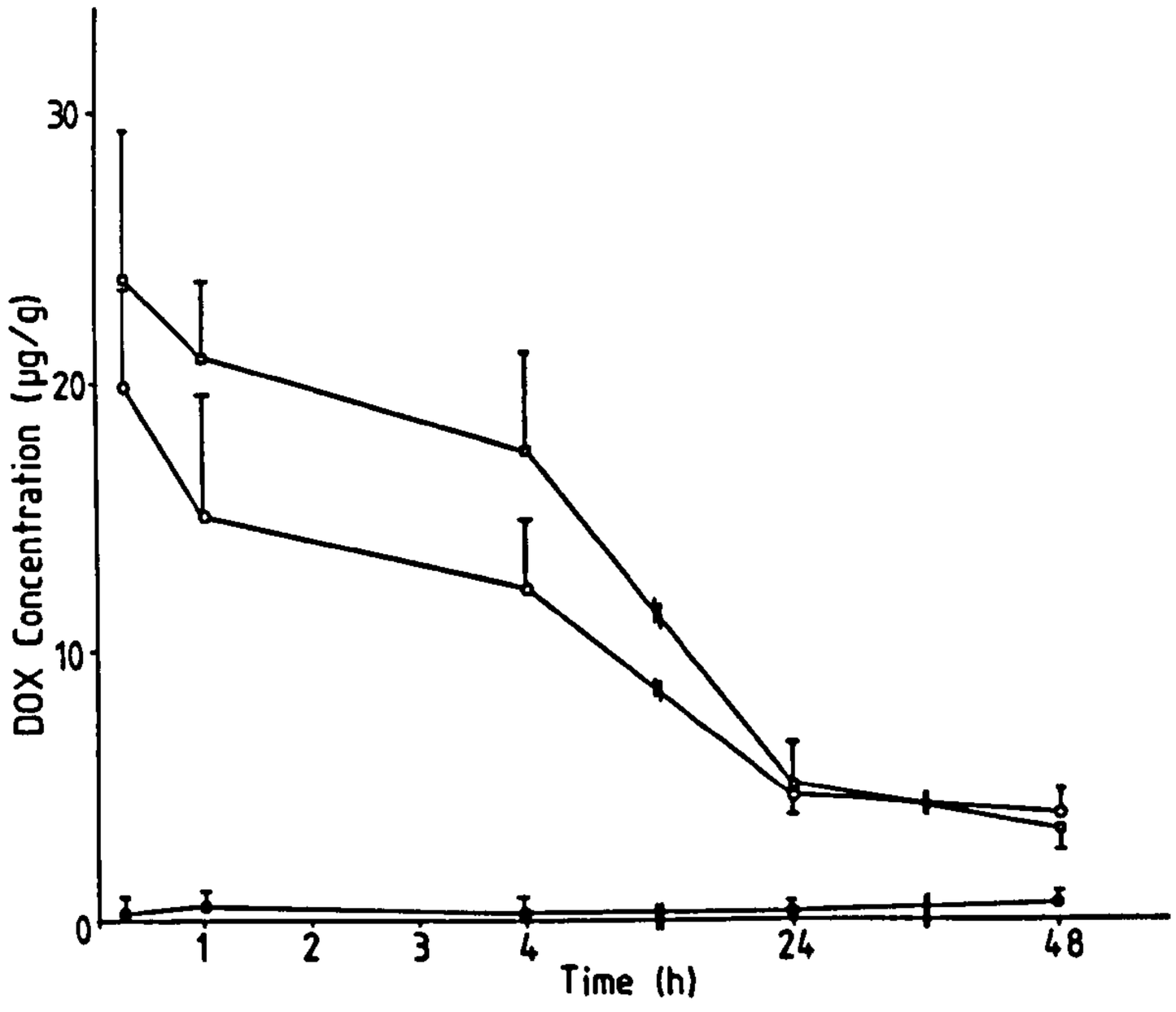


Fig. 4.13 Lung concentrations of DOX as a function of time after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (O), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.
The vertical bar denotes the standard deviation of the drug concentration.

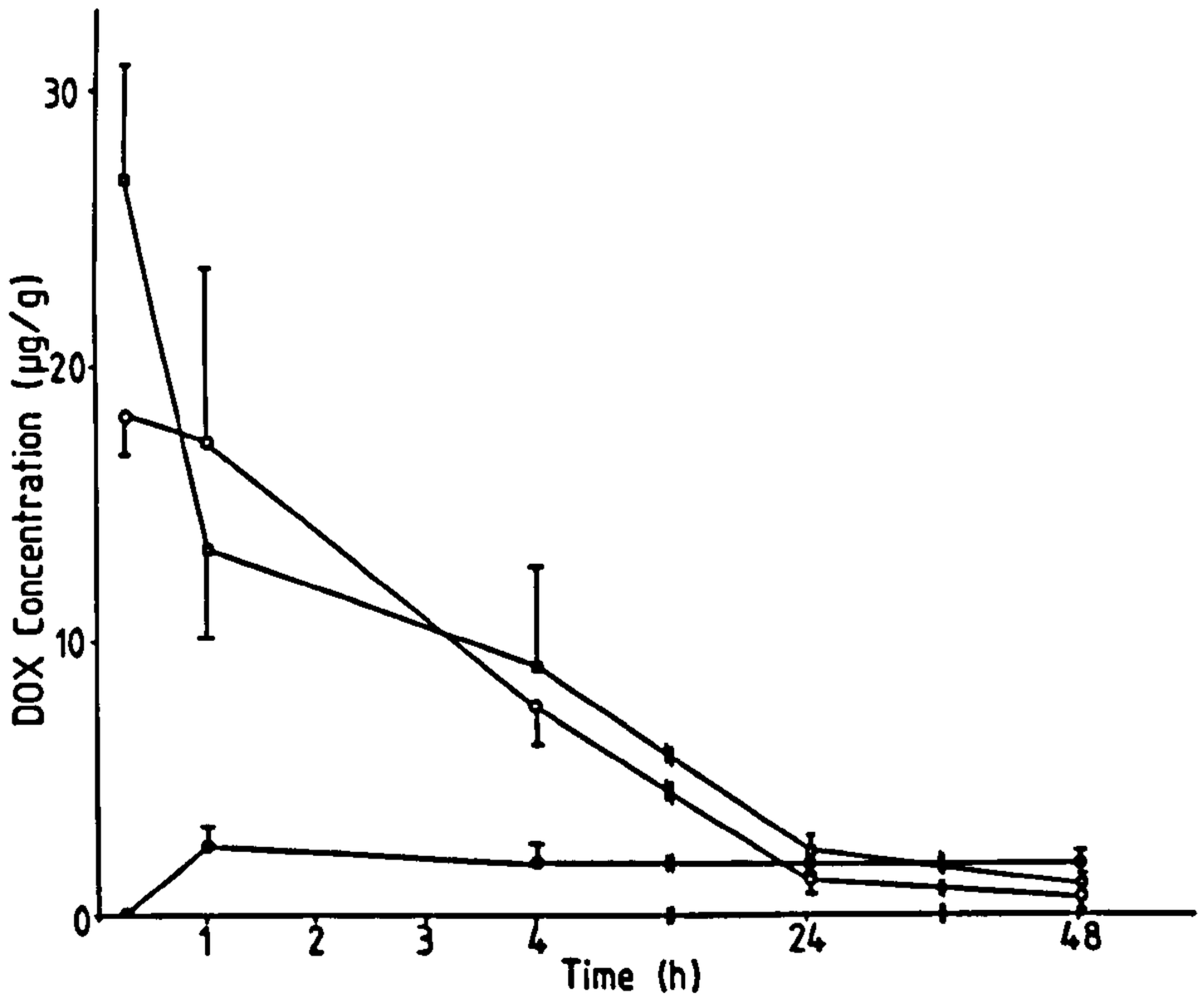


Fig. 4.14 Liver concentrations of DOX as a function of time after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (o), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.
The vertical bar denotes the standard deviation of the drug concentration.

Spleen (Fig. 4.15) - The DOX concentrations of all samples were very variable in this particular tissue. As with other tissues the free DOX solution and empty NSV's with free drug samples produced curves which were very similar while the drug loaded sample produced a small peak at 4h followed by a slight fall in DOX concentration over 48h.

Tumour (Fig. 4.16) - Free drug solution and empty vesicles with free drug produced curves which were essentially the same, giving a peak at 1h followed by a fall in the DOX concentration of the tissue. Injection of the DOX loaded vesicle sample produced a much smaller peak at 1h with a slight drop in DOX concentration thereafter. In this tissue, drug concentrations at given time points for each sample type were particularly variable.

Metabolites - The metabolites which were detected in sufficient quantities at enough time points to plot graphs of concentration against time were lung 7-DOL (Fig. 4.17), liver 7-DOL (Fig. 4.18) and liver 7-DOX (Fig. 4.19).

In all cases similar trends were seen, with high initial concentrations detected for the free DOX in solution and empty vesicles with free drug samples which fell rapidly. The drug loaded vesicle sample increased slowly to a plateau level which remained virtually constant throughout the 48h duration. In all cases the metabolite levels from the drug loaded vesicle samples were higher than either of the other sample types at 48h.

4.3.3 Tumour Growth Delay Study Results

The graph obtained for the tumour volume measured for each of the samples examined is shown in Fig. 4.20.

The tumours in the control group of mice became palpable 11 days after implantation of the tumour fragments and tumour volumes were

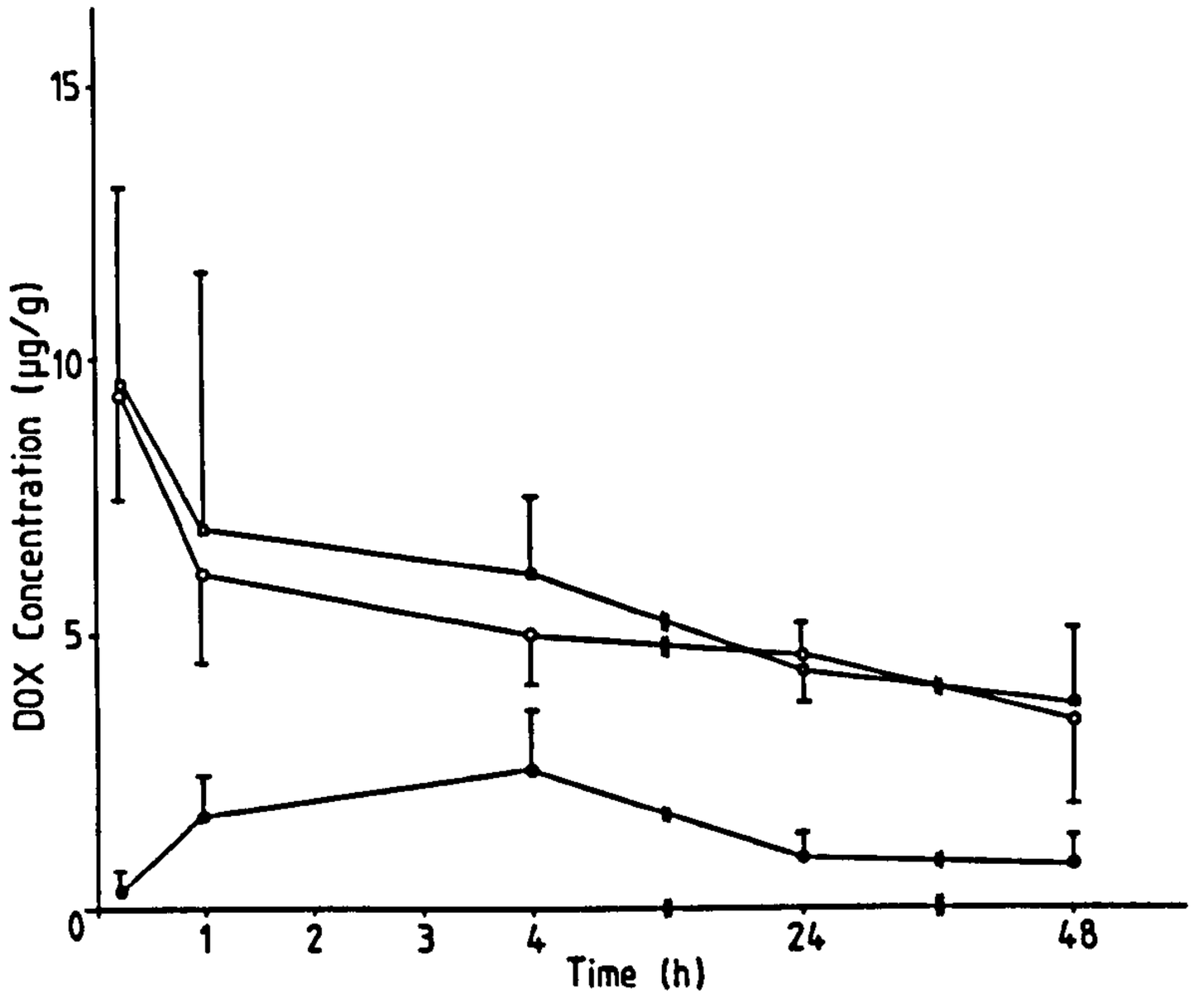


Fig. 4.15 Spleen concentrations of DOX as a function of time after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (o), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.
The vertical bar denotes the standard deviation of the drug concentration.

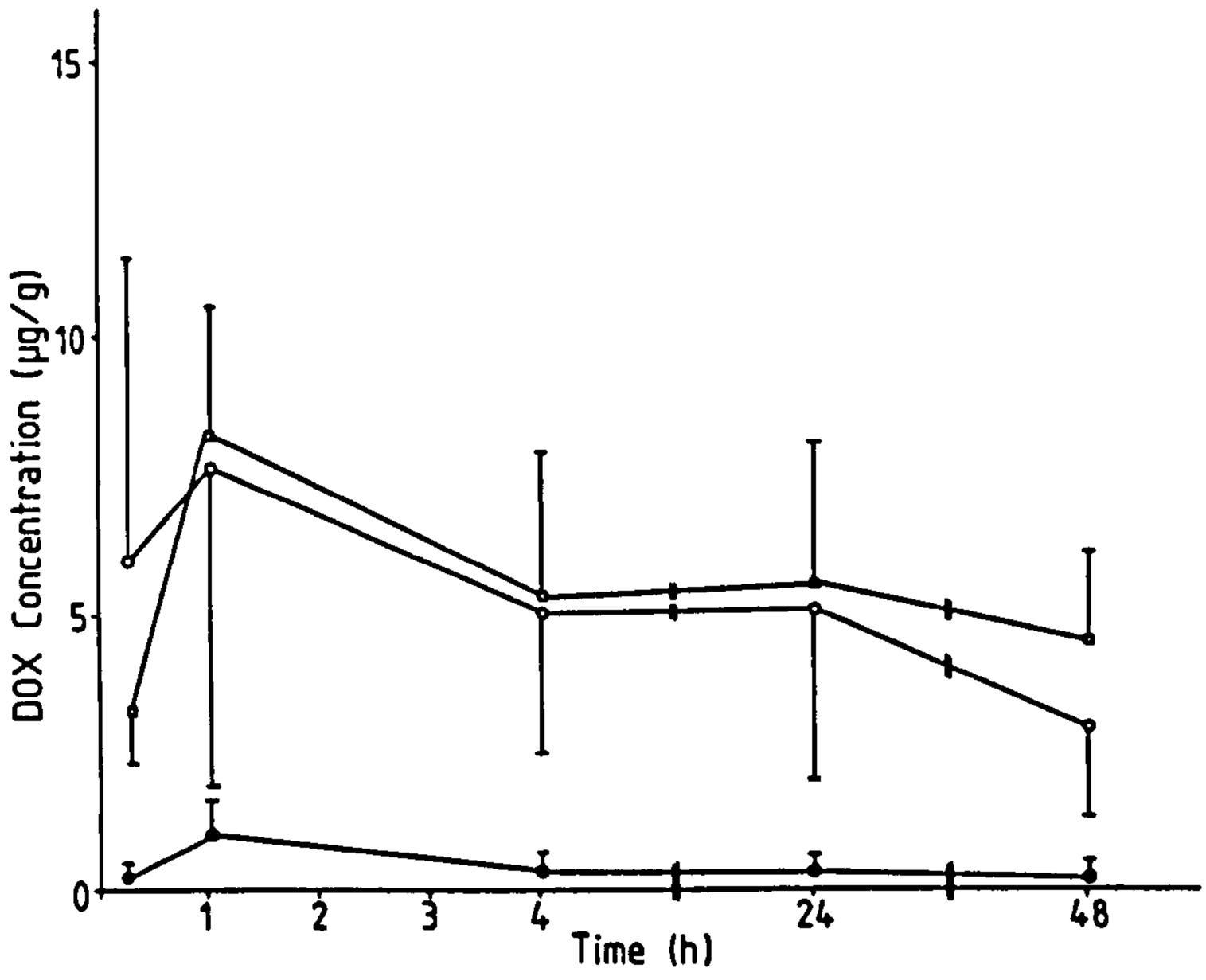


Fig. 4.16 ROS tumour concentrations of DOX as a function of time after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (O), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.

The vertical bar denotes the standard deviation of the drug concentration.

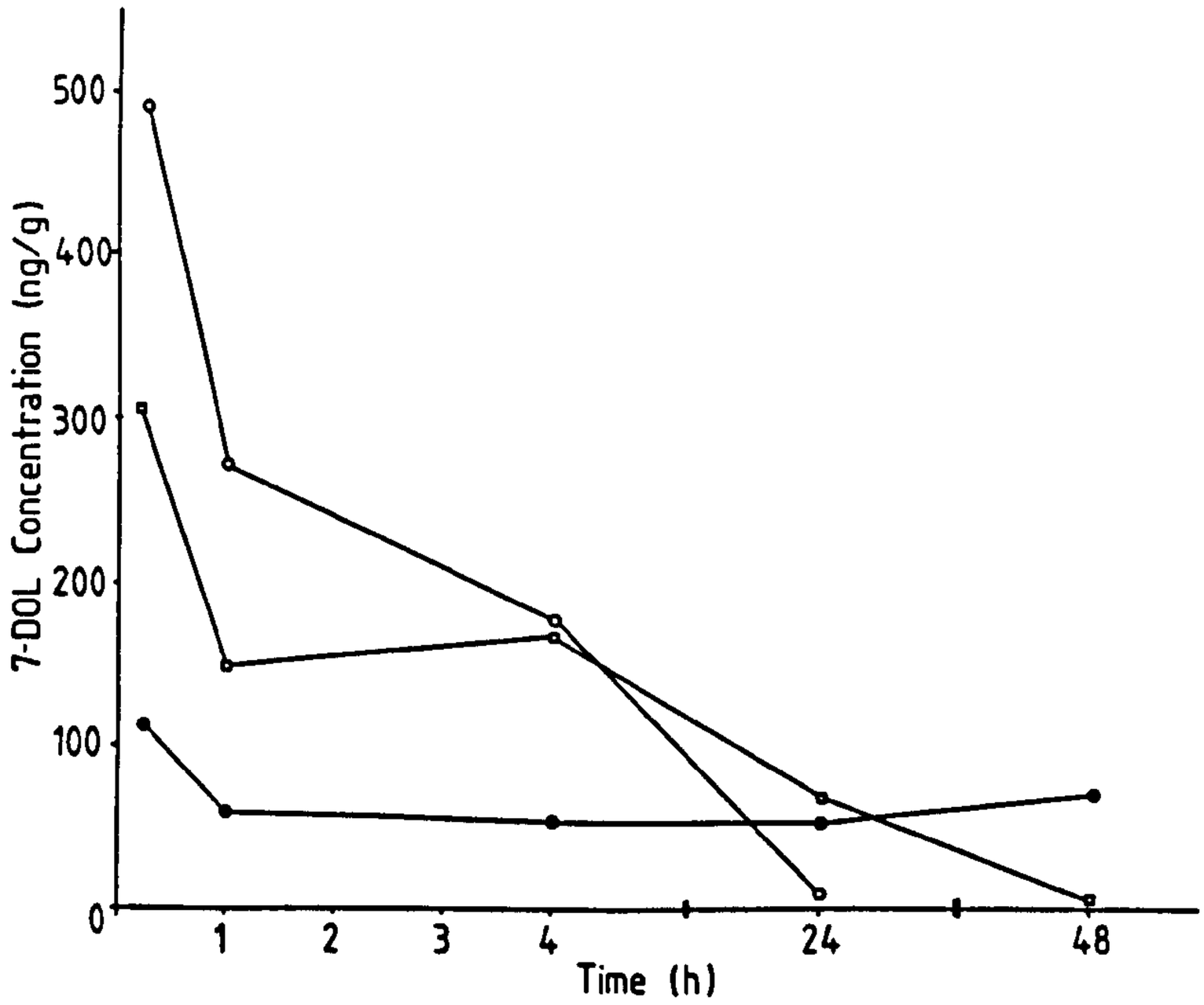


Fig. 4.17 Lung doxorubicinol-7-deoxyaglycone (7-DOL) metabolite concentrations as a function of time after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (o), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.

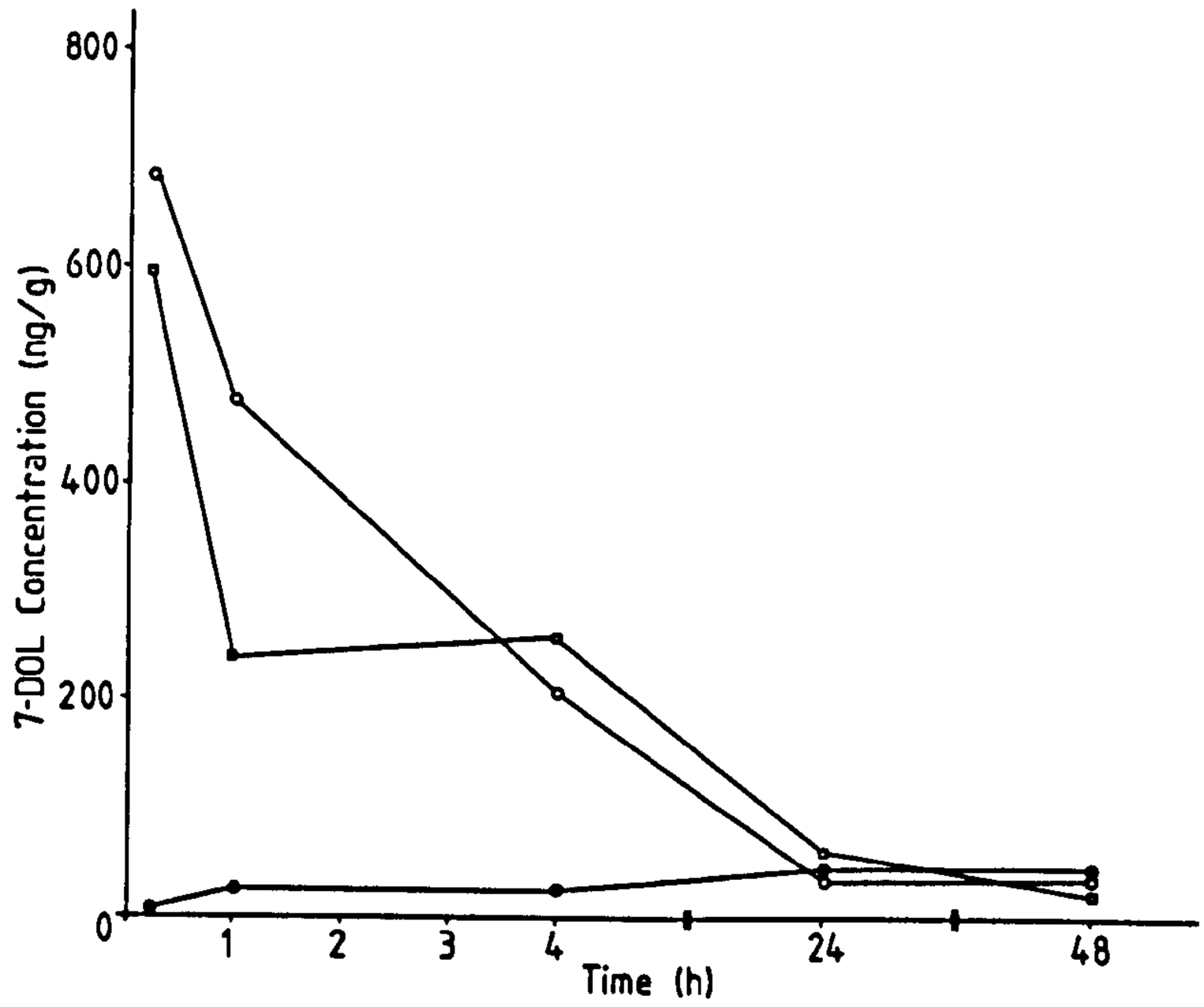


Fig. 4.18 Liver doxorubicinol-7-deoxyaglycone (7-DOL) metabolite concentrations as a function of time after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (O), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.

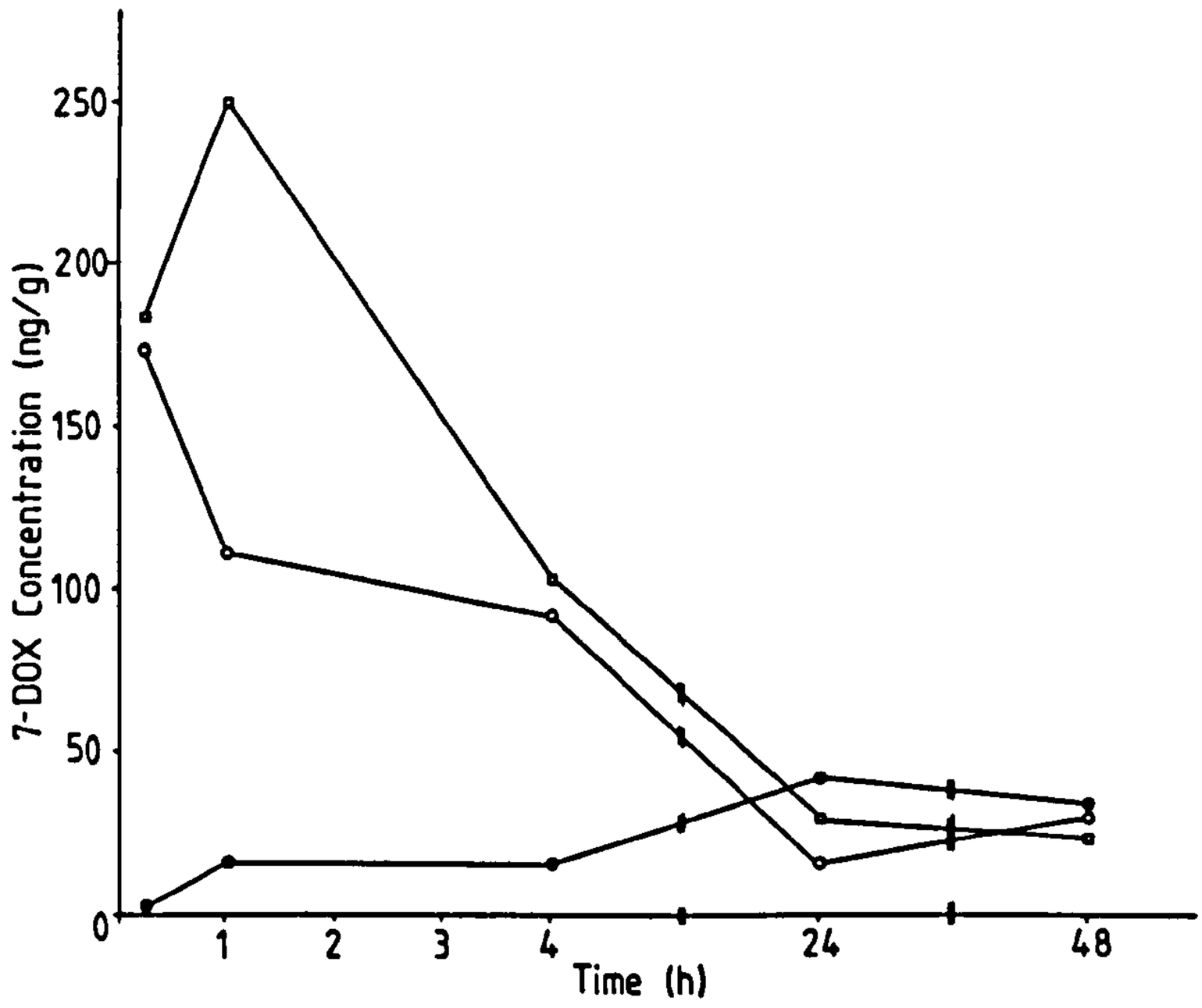


Fig. 4.19 Liver doxorubicin-7-deoxyaglycone (7-DOX) metabolite concentrations as a function of time after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (O), empty vesicles given in conjunction with free drug (□) or DOX entrapped in vesicles (●) for vesicles composed of IV 47.5:CHOL 47.5:SA 5.0.

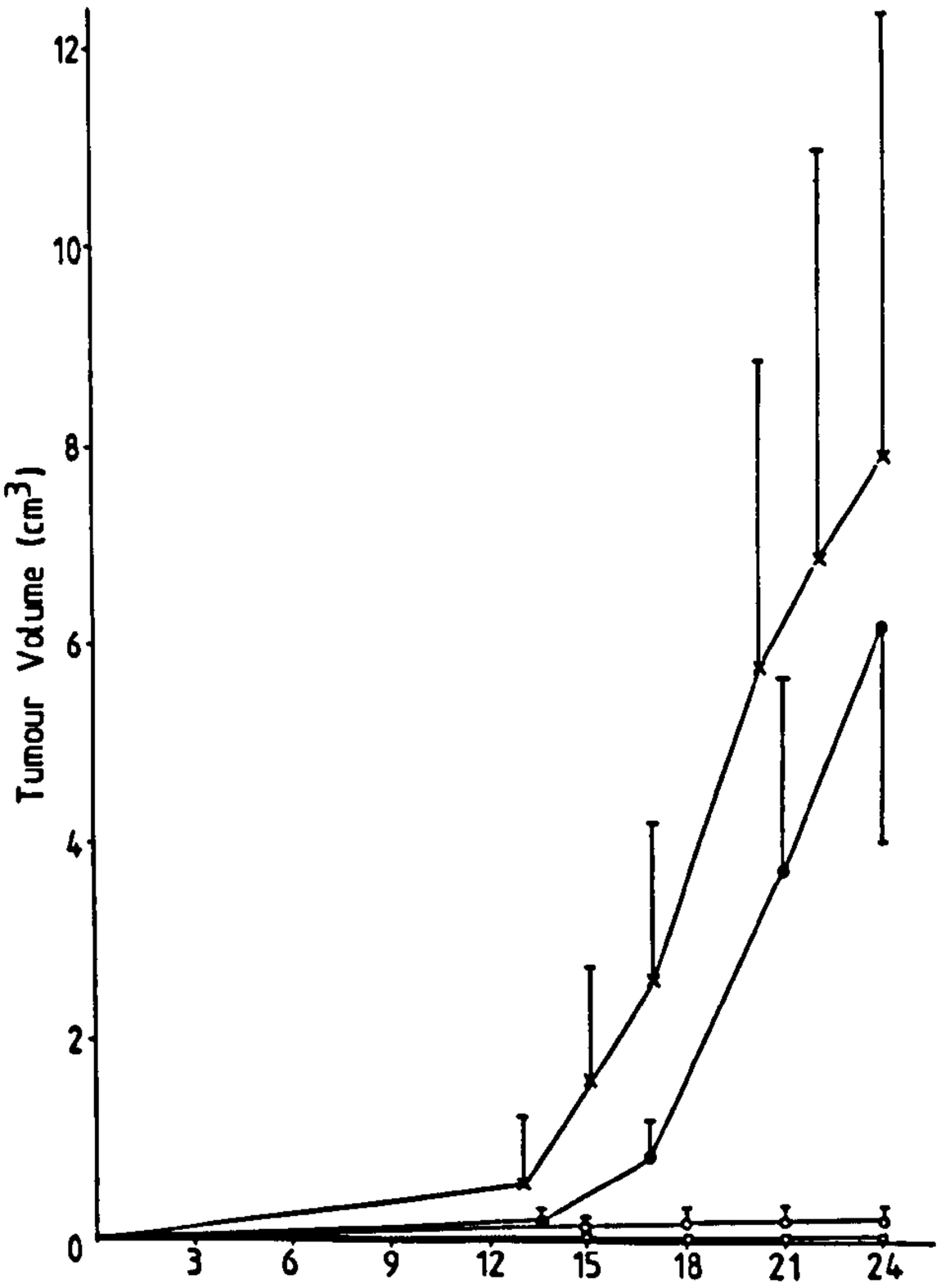


Fig. 4.20 ROS tumour volume against time after tumour fragment passage following after bolus injection of 10mg kg^{-1} into the tail vein of AKR mice of free drug in solution (o), empty vesicles given in conjunction with free drug (□), DOX entrapped in vesicles (●) or 300mM glucose solution (x). The vesicles were composed of IV 47.5:CHOL 47.5:SA 5.0. The vertical bar denotes the standard deviation of the tumour volume.

estimated every few days thereafter. After an initial period of slow growth, the tumour showed an exponential increase in size. In the group treated with free DOX in solution, only one mouse developed a tumour which became palpable, although the growth of this tumour was much slower than in any of the mice in the control group.

The group of mice injected with empty vesicles in conjunction with free DOX produced no tumours which were palpable over the 24 day duration of the experiment.

All of the mice injected with the DOX loaded vesicle sample developed tumours which grew in a similar manner to the control tumours. There appeared to be a slight tumour growth delay in these mice since the growth curve was slightly pushed to the right i.e.the curve was moved to the right of the control curve by about three days during the exponential growth phase.

4.3.4 Fluorescence Photomicrographs

The fluorescence photomicrographs produced some interesting results bearing in mind the apparent lack of effect the DOX loaded vesicles had on tumour growth. Fixation of the tissues by instant freezing in liquid nitrogen or in propan-2-ol:water (80:20, v/v) produced no difference in the photomicrographs obtained. Examination of the tissues in the absence of vesicles produced a dull, orange/green fluorescence, while after injection of DOX loaded vesicles, bright orange points of fluorescence were observed.

At 5min no vesicles were detected in any of the tissues examined. After 4h and 22h very few vesicles were observed in heart, kidney and lung. Vesicles were observed in liver and spleen at both 4h and 22h. At both these times, points of fluorescence could be seen in the phagocytic cells and sinusoids of the spleen

(Plate 4.1) and in the Kupffer cells and sinusoids of the liver (Plate 4.2). Most vesicles were observed in the tumour at 4h and 22h. The majority of the vesicles appeared to be attached to the inner surface of the blood vessels within the tumour although some did appear to have passed across the vessel wall and were residing in the tumour mass. At 4h, the vesicles and the surrounding area appear well defined (Plates 4.3; 4.4) with the vesicles appearing as pinpoints of orange and the tumour cells as larger, less intensely fluorescing areas. After 22h the orange colour at the wall of the blood vessel had become much more diffuse, with the majority of the vesicles still appearing to be located on the vessel wall (Plates 4.5; 4.6).

When free DOX in solution was injected, no obvious signs of fluorescence being localized were observed in any of the tissues examined after 5min, 4h or 22h.

4.3.5 Discussion

Analysis of the results produced by this *in vivo* study was not as straightforward or as easy to interpret as those obtained in the previous study. In other *in vivo* studies using DOX encapsulated in NSV's, no problems had been encountered in recovering the drug from the tissues since the extraction solvent of chloroform:propan-2-ol (2:1) disrupted any vesicles present. The DOX which was liberated, partitioned into the extraction solvent and was subsequently analysed (Rogerson *et al*, 1988; Kerr *et al*, 1988a). However, it soon became clear that with vesicles having this particular composition (IV 47.5:CHOL 47.5:SA 5.0), total vesicular disruption was not occurring and much of the DOX was not extracted, resulting in very low DOX concentrations being measured in the tissues.

The addition of a volume of extraction solvent to a volume of

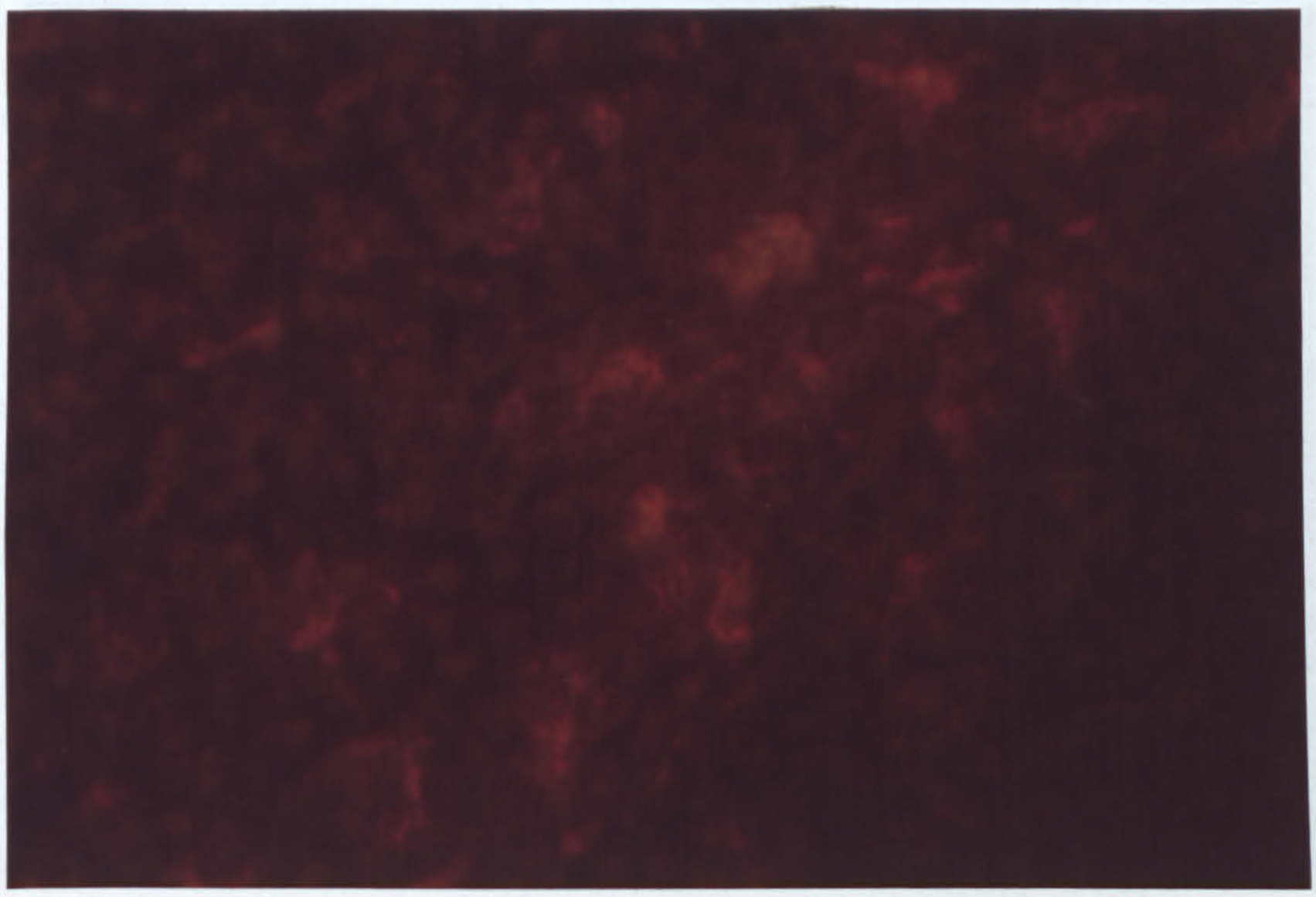


Plate 4.1 Fluorescent photomicrograph of murine spleen 4h after intravenous injection of doxorubicin loaded, non-ionic surfactant vesicles composed of IV 47.5:CHOL 47.5:SA 5.0. (Magnification x50)

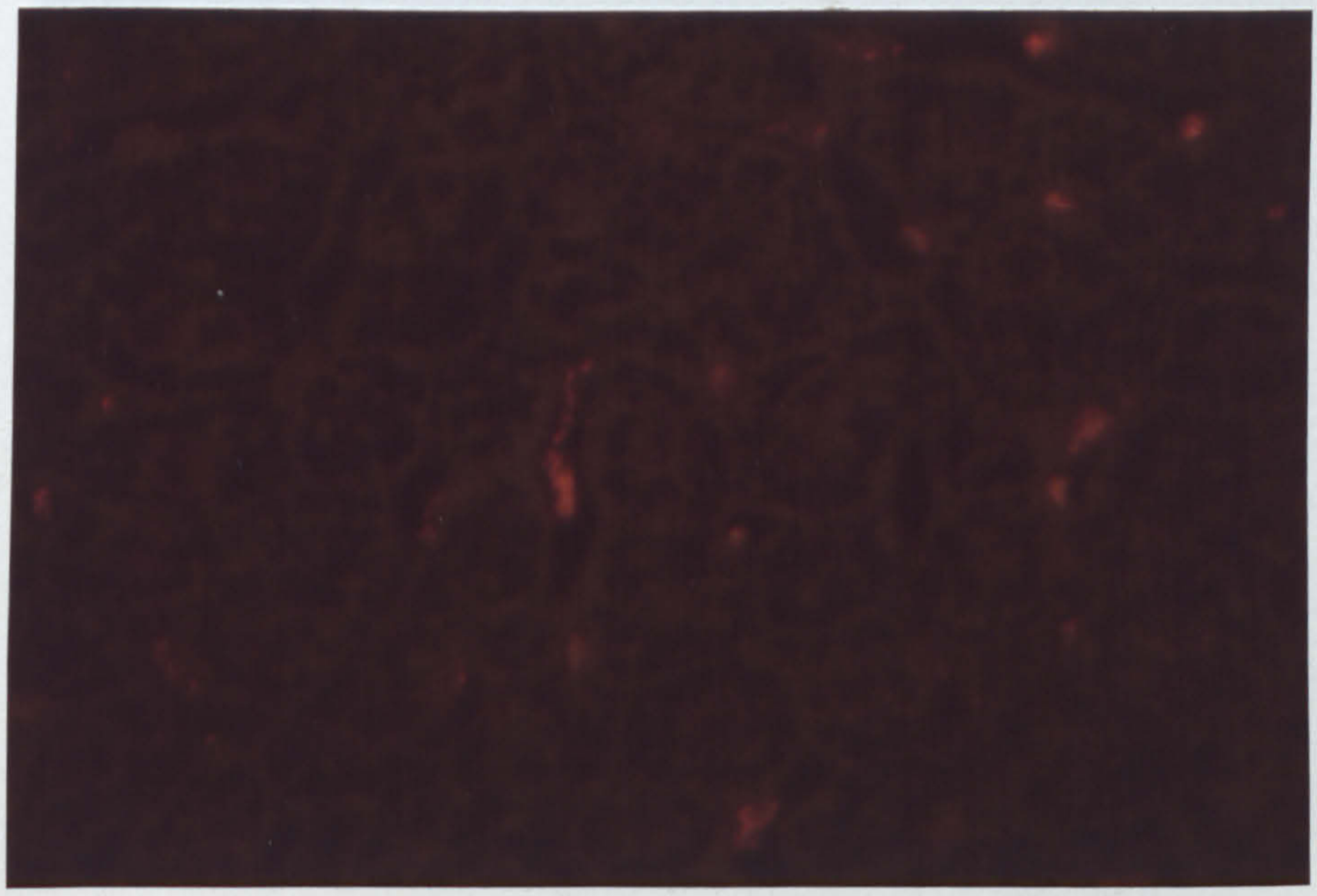


Plate 4.2 Fluorescent photomicrograph of murine liver 22h after intravenous injection of doxorubicin loaded, non-ionic surfactant vesicles composed of IV 47.5:CHOL 47.5:SA 5.0. (Magnification x50)

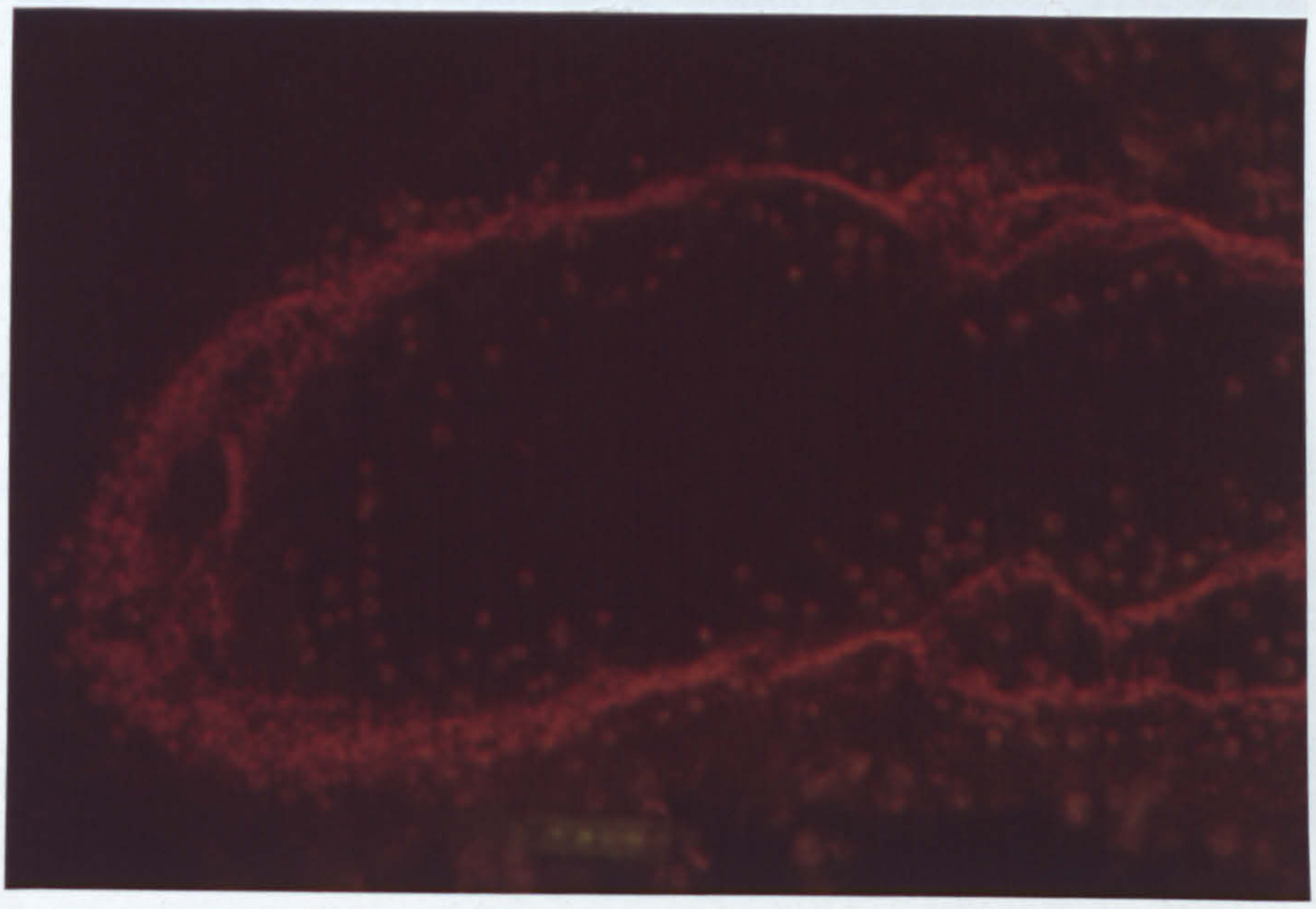


Plate 4.3 Fluorescent photomicrograph of murine ROS tumour 4h after intravenous injection of doxorubicin loaded, non-ionic surfactant vesicles composed of IV 47.5:CHOL 47.5:SA 5.0. (Magnification x25)

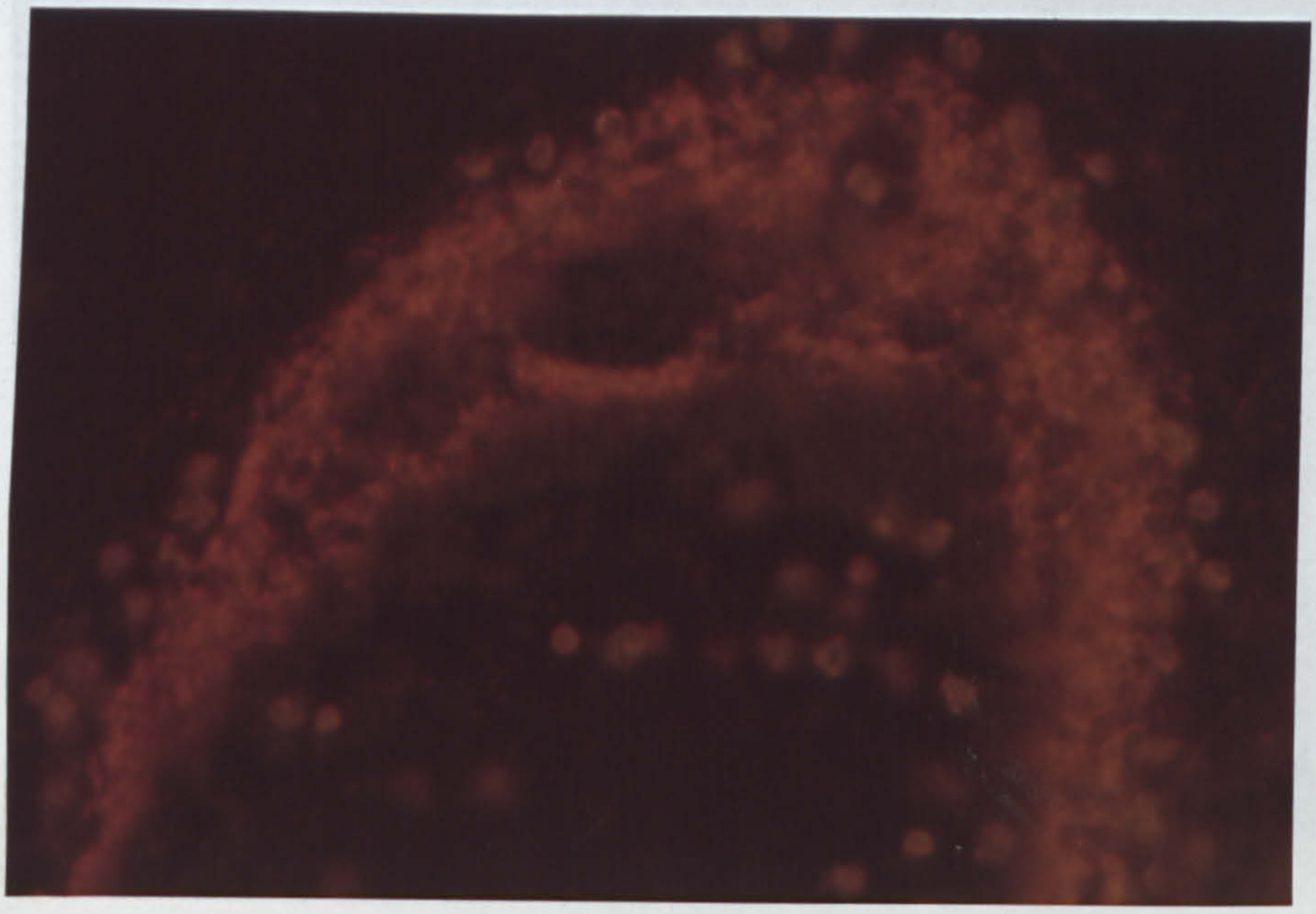


Plate 4.4 Fluorescent photomicrograph of murine ROS tumour 4h after intravenous injection of doxorubicin loaded, non-ionic surfactant vesicles composed of IV 47.5:CHOL 47.5:SA 5.0. (Magnification x50)

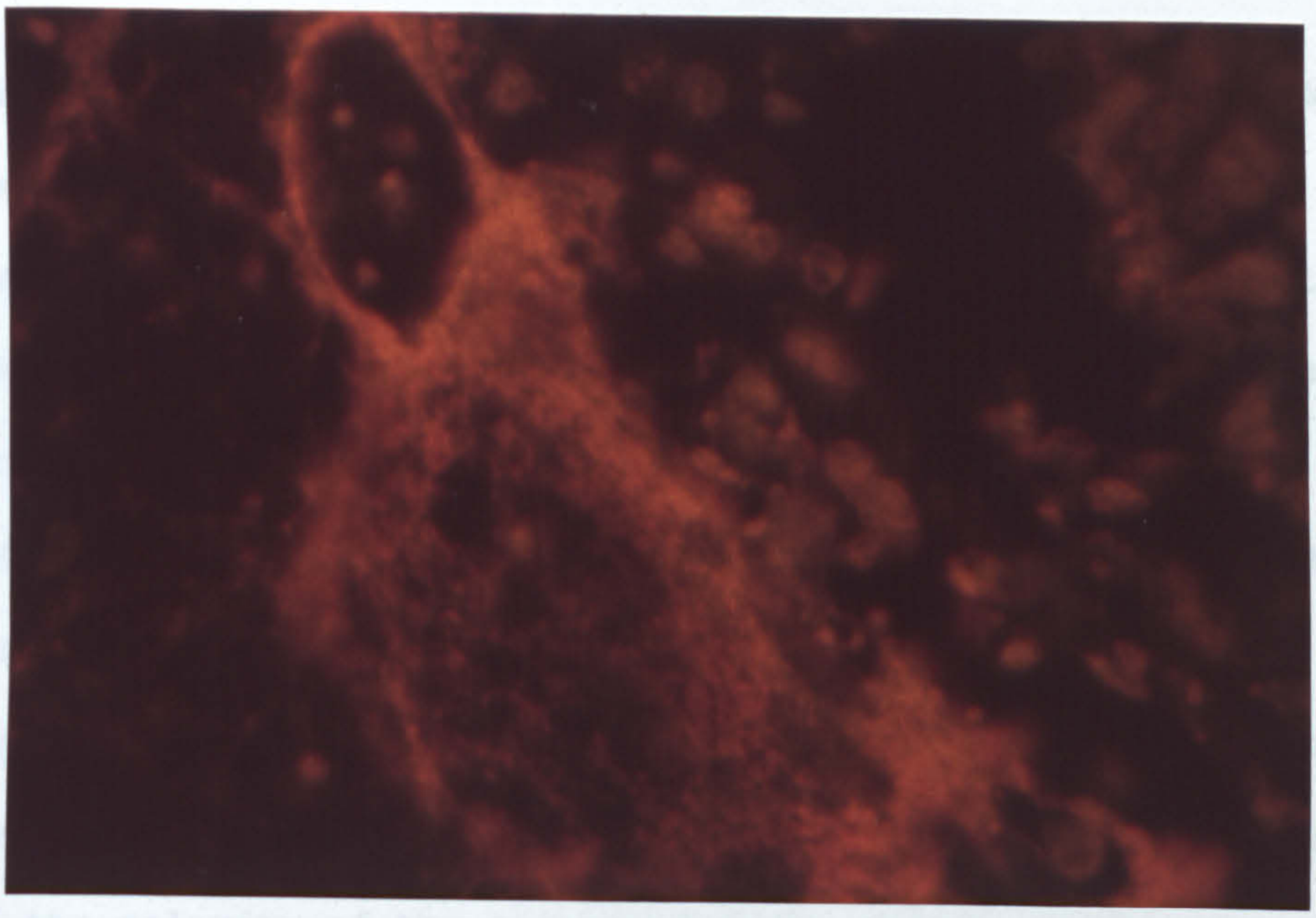


Plate 4.5 Fluorescent photomicrograph of murine ROS tumour 22h after intravenous injection of doxorubicin loaded, non-ionic surfactant vesicles composed of IV 47.5:CHOL 47.5:SA 5.0. (Magnification x50)

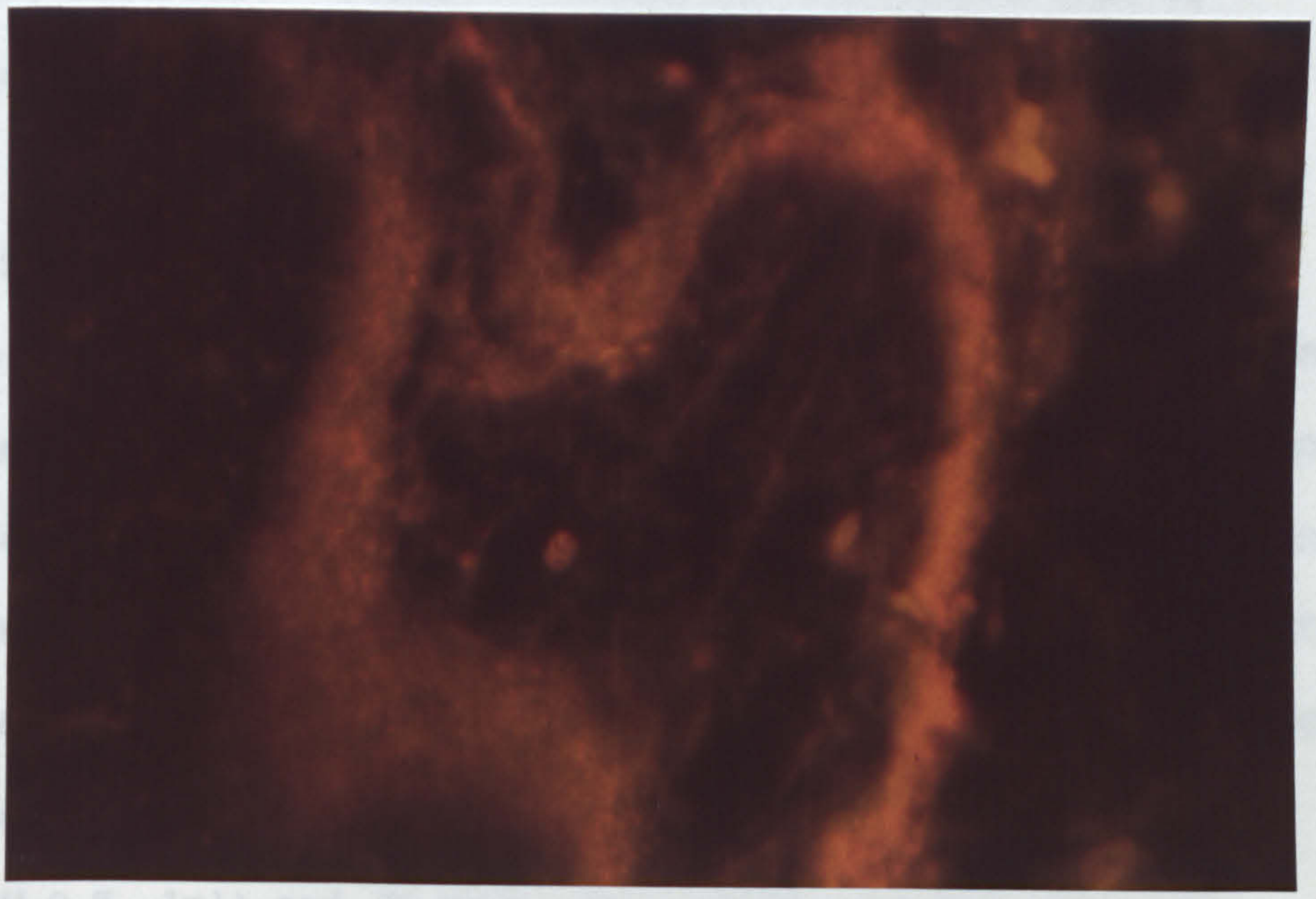


Plate 4.6 Fluorescent photomicrograph of murine ROS tumour 22h after intravenous injection of doxorubicin loaded, non-ionic surfactant vesicles composed of IV 47.5:CHOL 47.5:SA 5.0. (Magnification x50)

drug loaded vesicles in solution resulted in very little of the drug being measured in the extraction solvent, while much drug still remained in the aqueous phase, as seen by its red colour. Attempts to increase the amount of DOX extracted from the DOX loaded vesicles by increasing the length of time the extraction solvent was in contact with the vesicles, or by extracting the vesicle sample three times did not substantially increase the drug recovery.

Mixing aliquots (100μl) of the drug loaded sample with methanol, ethanol, propanol, chloroform, ethyl acetate or hexane was attempted to note if the polarity of the solvent had any effect on the recovery of DOX, but again the amount of DOX extracted was very low in all cases. Passing each of the solutions through a Bondelut C_{18} column resulted in much red material being retained on the top of the column, indicating the presence of DOX still associated with particulate material. Plate 4.7 shows the aggregated type of system obtained after the addition of chloroform to the vesicle sample. The aggregated material is red in colour and presumably contains DOX still associated with the bilayer material.

In a final effort to remove the entire quantity of DOX contained within the tissues, a hydrolysis method was attempted. This method had been used to remove daunorubicin which had been covalently bound to a polymer (Seymour, 1989) and involved adding 2M HCl (1ml) to the sample and heating at 85°C for 20min to produce the hydrolysed drug and metabolite aglycones. The sample was cooled, Tris-phosphate buffer (pH 9.5, 1ml) and 2M NaOH (1ml) added, and the resulting solution extracted in the normal manner.

HPLC analysis of the hydrolysed and extracted aliquots of drug loaded vesicles resulted in little drug being detected; the aqueous phase was still red after extraction. Increasing the time the acid

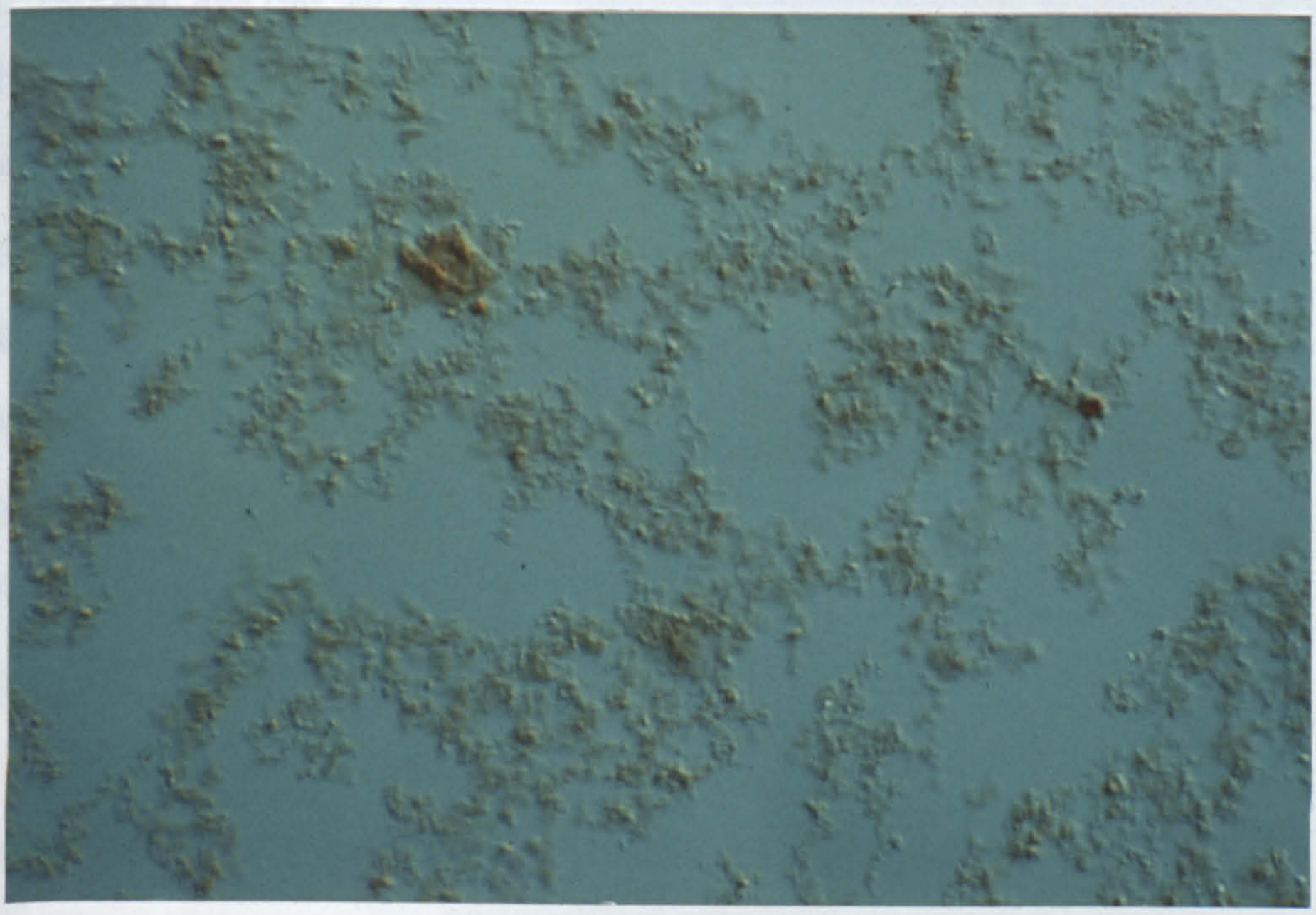


Plate 4.7 Photomicrograph showing the incomplete disruption of vesicles and aggregation of the resulting fragments after addition of chloroform to DOX loaded, non-ionic surfactant vesicles composed of IV 47.5:CHOL 47.5:SA 5.0. (Magnification x200)

was in contact with the sample from 10min to 200min had no effect on the amount of drug recovered.

Having failed to extract and recover all of the DOX which was entrapped within drug loaded vesicles in solution, it seemed very likely that in the tissues from the animals injected with the DOX loaded vesicles not all the DOX present would be extracted or recovered. Much of the DOX would remain associated with the vesicle in some way that does not allow easy recovery and measurement. The drug that was extracted and detected was likely to be drug which had been released from intact vesicles in vivo or possibly contained within the aqueous interior of the vesicles.

Dialysis of the drug loaded vesicle sample showed the release of drug to be very slow and this had also been reported for liposomes containing stearylamine with entrapped DOX (Forssen and Tokes, 1979) and daunorubicin (Juliano et al, 1978). Despite the fact that the DOX appeared to be in some way associated with the vesicles, only pure, intact DOX was released from the vesicles as shown in the dialysis studies (see section 3.2.7). Storm et al (1989) observed a remarkable colour change, from orange-red to red-purple, during storage of their negatively charged liposome samples. This colour change was shown to be caused by the liposomally associated drug and not the DOX which had been released from the vesicle and was free in solution, since this was still orange-red in colour.

Problems with recovery of DOX have not been reported for any liposomal compositions containing stearylamine and encapsulating DOX, although it was noted by van Hoesel (1984) that tissue levels of DOX after administration in positively charged, stearylamine containing liposomes were low compared to those obtained with free drug or negatively charged liposomes.

In the plasma, the drug loaded vesicle sample produced higher drug levels than free drug in solution and empty vesicle with free drug samples at later time points. It did not produce the higher initial peak normally seen after injection of vesicular systems containing DOX, when compared with free drug in solution (Rogerson et al, 1988; Rahman et al, 1986a). Positively charged or uncharged vesicles are generally cleared from the circulation less quickly than negatively charged vesicles (Juliano and Stamp, 1975) but in this study the measured plasma DOX concentration was likely to be only a measure of free drug in the plasma, with little contribution from any vesicularly associated DOX. It would seem likely, therefore, that the plasma concentration was being maintained by a slow release of DOX from the vesicles residing in the tissues at later times and possibly still in the plasma at the earlier time points, over the 48h of the study. This was suggested by Azmin et al (1985) as a possible mechanism for maintenance of the plasma methotrexate levels when drug loaded NSV's were administered intravenously. If it was possible to measure the plasma concentration due to DOX still entrapped within or associated with the vesicles, an exponential curve could be produced (Juliano and Stamp, 1975). Combining this curve with the trace actually measured would produce a drug profile which is likely to be similar to those obtained in previous studies i.e. combine the distribution curve for the DOX loaded vesicles with the plasma clearance half life of the DOX. A higher peak DOX concentration, compared with free drug, may still be produced due to the total DOX in the plasma, but is not detected using this particular method of drug extraction.

In this instance, the injection of empty vesicles with free drug has no effect on the plasma kinetics of the drug, since it produced

an almost superimposible curve to that of the free drug in solution sample.

Heart tissue is known to have low endocytotic activity (Trouet et al, 1974) so it may be expected that low uptake of vesicles by the heart will occur. Positively charged liposomes, containing stearylamine, were shown to reduce the cardiac DOX peak concentration to half that of free drug or drug entrapped in negative liposomes (Rahman et al, 1980), and also to reduce the extent and severity of the cardiac lesions produced (Rahman et al, 1982). They have been shown to reduce the peak cardiac concentration compared with free drug and reduce the half life of the drug in heart (Rahman et al, 1986a), while negatively charged liposomes reduced the heart DOX levels at 1h and 4h (Forssen and Tokes, 1979). The reduction of the peak cardiac concentration appears to be directly related to cardiomyopathy, since giving DOX as an infusion to reduce the peak level achieved in vivo reduced the possibility of cardiac damage (Unverferth et al, 1982). This may account for the apparent reduction in cardiotoxicity offered by the administration of vesicularly encapsulated DOX.

Unfortunately, in this study no real comments can be made about the peak levels produced by the drug loaded vesicle sample and direct comparisons with the free drug curve cannot be drawn. However, assuming that the amount of drug measured with the DOX loaded vesicles in tissues is free, released drug, this level will be related to the number of vesicles in the tissues. Thus, taking a broader view of the results produced in the tissues after injection of DOX loaded vesicles, an indication of the vesicular uptake by the various tissues can be inferred and compared with the free drug curve. In the heart very low levels of DOX are detected with the

DOX loaded sample, and the free drug and empty vesicle with free drug samples are again almost superimposible. This suggests that few vesicles are taken up by the heart, although nothing can be said about the peak concentration.

Removal of particulate material, such as vesicles, from the circulation by the lungs may take place by phagocytosis by the alveolar phagocytic cells in the basement endothelium or by becoming physically entrapped within the alveolar vasculature if the vesicles or vesicular aggregates are larger than about 7μm. Rogerson et al (1988) found that their NSV's tended to accumulate in the lungs, although these vesicles were much larger (circa 1μm) than those used in this study, and were uncharged, therefore more liable to aggregate to produce particles which were capable of becoming physically trapped. Rahman et al (1986a) found that the peak DOX concentration in the lungs was slightly less when the drug was encapsulated in positive liposomes rather than given as free drug.

The peak lung concentration was reduced and the half life of the drug in the tissue doubled (Rahman et al, 1986a) by encapsulation of DOX in positively charged liposomes. Forssen and Tokes (1979) found that anionic liposomes reduced the lung DOX concentration to less than half that of free drug after 4h.

In this study, very low levels of drug were detected with the drug loaded sample, compared with both the free drug and empty vesicles with free drug samples, suggesting few vesicles were removed by the lung, possibly due to their small size and the fact that they were charged and, therefore, less liable to aggregation. Again the free drug and empty vesicle with free drug traces were virtually identical.

Rahman et al (1980) found liposomally entrapped drug tended to

accumulate in lung, liver and spleen, and virtually all *in vivo* studies undertaken with vesicles have shown major uptake of the carriers by the liver and spleen, after intravenous injection.

Gabizon *et al* (1982) found that multilamellar and unilamellar liposomes of various compositions were taken up by the liver and spleen to a greater degree than free DOX but unilamellar liposomes were taken up to a lesser extent by the lung than the multilamellar vesicles. Most compositions also showed a reduction in the cardiac DOX concentration. 24h after injection of multilamellar, negatively charged liposomes containing ^{99m}Tc, vesicles were found to be localised in the organs of the reticuloendothelial system, with 44.5% of radioactivity located in the liver, 25.5% in spleen and 14.5% in lung. Activity was also recorded in the kidneys and bone marrow but could not be quantified (Lopez-Berestein *et al*, 1984). This preferential uptake of liposomes by these organs has been used to increase the therapeutic effect of DOX in the treatment of rat tumours which are metastatic to liver (Gabizon *et al*, 1983).

The positively charged vesicles used in this study appeared to be taken up by the liver to a greater degree than any of the other tissues examined. The liver showed a plateau concentration after 1h which actually became greater than the free drug concentration after 24h and 48h. The liver was the only tissue in which this was seen and suggested that many of the vesicles were residing in the liver. In all other tissues examined, the DOX concentration measured at 48h. for the DOX loaded vesicles was less than that measured for the other two sample types. Again the free drug and empty vesicle with free drug samples produced curves which were very alike.

Encapsulation of DOX in both negatively and positively charged vesicles increased the peak liver concentration compared with free

DOX (Forssen and Tokes, 1979: Rahman et al, 1986a). Encapsulation also increased the residence time of the drug in the liver and the area under the curve was increased more than 20 times (Rahman et al, 1986a). Van Hoesel et al (1984) reported that positively charged liposomes were taken up less by the liver and spleen compared to free drug and negative liposomes, although since all tissues tended to show lower uptake by positive liposomes it is difficult to attach too much significance to this observation. When methotrexate was administered intravenously to mice, significantly higher liver drug levels were produced, compared with free drug (Azmin et al, 1985). It has been shown that liposomes are taken up into liver cells intact, probably by endocytosis (Gregoriadis et al, 1974; Rahman and Wright, 1975) and it was thus proposed by Azmin et al (1985) that the NSV's were taken up intact by the liver and broken down by lysosomal lipases to release the free methotrexate which would re-enter the circulation and maintain the plasma drug concentration.

Spleen DOX concentrations for the drug loaded sample reached a peak after 4h and fell slowly thereafter, while the free drug and empty vesicle with free drug concentrations fell throughout the 48h. At all times, the DOX concentration measured in the spleen with the drug loaded vesicles was much lower than that measured with free drug. Uptake of DOX loaded liposomes into the spleen was reduced by incorporation in negatively charged liposomes (Forssen and Tokes, 1979), but was substantially increased if incorporated into positively charged liposomes (Rahman et al, 1986a). Injection of DOX containing non-ionic surfactant vesicles into mice resulted in no difference in spleen concentrations obtained for vesicular and free drug (Rogerson et al, 1988), a result which was similar to that seen after intravenous injection of liposomally entrapped actinomycin D

(Rahman et al, 1978). Uptake of NSV's by the spleen appeared to be greater than that in heart and lung, but less than in liver in this study.

Much of the benefit reported for the encapsulation of DOX has been the reduction of the cardiac drug levels and the degree of cardiac damage. However, for this property to permit liposomes and other vesicular carriers to become clinically acceptable, the antitumour activity of the encapsulated DOX must be at least as effective as the standard treatments available. Tumour cells have many different features compared with normal cells. Cancer cells may have a greater endocytotic capacity than normal cells (Busch et al, 1961; Sehested et al, 1987) which may permit increased particulate uptake by the tumour. Malignant tumours also have altered vasculature, with the blood vessels in the tumour being both newly formed vessels and those incorporated from the host tissue (Papadimitriou and Woods, 1974). The blood vessels of the tumour also tend to have fenestrated capillaries, resulting in increased permeability of the tumour vasculature, compared to normal vessels, although this does vary between tumours (Warren, 1970). Gabizon and Papahadjopoulos (1988) found that liposomes containing certain glycolipids, solid phase phospholipids and cholesterol, and with a size of about 100nm, had an increased residence time in the blood. As the blood residence time was increased, the uptake by the liver and spleen was reduced, and they were able to show a correlation between increased blood residence time and an increased uptake of the vesicles by implanted tumours in mice.

The DOX loaded vesicles used in this study produced a low, but consistent DOX concentration in the ROS tumour model, with both the free drug and empty vesicle with free drug samples producing much

higher DOX concentrations. The DOX concentrations measured in tumour correlated well with the antitumour effect of the various preparations. After injection of free DOX in solution or empty vesicles in conjunction with free DOX very little tumour growth was observed, while the DOX loaded vesicles produced a slight reduction in the rate of tumour growth, but was very much less effective than either of the other two sample types. Previous studies using NSV's of different compositions produced different results. Both Rogerson et al (1988) and Kerr et al (1988a) found that encapsulation of DOX increased tumour drug concentrations after intravenous injection of encapsulated drug. Kerr et al (1988a) found that the peak concentration was increased by injection of DOX in NSV's, while the terminal half life was longer for free drug in solution. In both cases above, the antitumour effect of the vesicular DOX was as good as, if not better than free DOX in solution. The differences in the antitumour effects of the various DOX loaded NSV's are probably related to the different rates at which DOX is released from the vesicles.

It would appear that the tumour model, the site of administration of the sample as well as the characteristics of the vesicle used to encapsulate the drug determine the final antitumour effect of the preparation.

Wolff and Rohdewald (1984) showed that, in vitro, free DOX solution caused greater cell damage than liposomally administered DOX. In vivo, van Hoesel et al (1984) saw the highest DOX concentrations in rat IgM immunocytoma tumour with free drug, with negatively charged vesicles producing slightly lower peaks but positively charged liposomes producing very low levels. This DOX concentration correlated well with the antitumour effect, with free

drug and negative liposomes having the same in vivo activity and the positive liposomes having much reduced activity. The opposite effect was seen using murine P388 leukaemia and Lewis lung carcinoma, where free drug and positive liposomes were equally effective, while negative liposomes were less effective (Rahman et al, 1980). Gabizon and Papahadjopoulos (1988) showed that intravenously injected, negatively charged, sonicated vesicles were more active than free drug in infiltrating tumours of the liver and spleen, but free drug was more effective against ascitic and subcutaneously implanted tumours. However, intraperitoneal injection of drug loaded liposomes was more effective than free drug in treating ascitic tumours.

Solid liposomes were shown to have a delayed antitumour effect in vivo in rats bearing a solid tumour model (Storm et al, 1987). This was shown to correlate with their relatively slow degradation and it was suggested that the rate of degradation of liposomes within mononuclear phagocytes of the MPS may influence antitumour activity, since differential uptake of solid or fluid liposomes by tumours does not appear to occur.

Metabolism of the free drug in solution or empty vesicle with free drug samples was seen to some degree in liver and lung where they both gave very similar traces, showing initial high levels which rapidly fell. The DOX loaded vesicles showed a similar pattern to the parent drug levels seen in all the tissues, with a low level detected which was maintained throughout the experiment. This gives further evidence that the DOX concentrations measured in the plasma and tissues are actually drug released from the vesicles and not the total drug contained. The previous study with vesicles composed of I 50:CHOL 25:SOL24 25 showed that encapsulation gave some degree of

protection to the metabolism of the DOX and it was suggested that only drug that was free in the tissues i.e. had been released from the vesicles, was capable of being metabolized. This reduction in metabolism has also been noted with encapsulated methotrexate (Kimelberg and Atchison, 1978; Azmin et al, 1985; Kimelberg et al, 1977) and DOX (Rogerson et al, 1986) although increased hepatic metabolism was observed with the DOX containing vesicles used by Kerr et al (1988a).

In general, and unlike the previous study using the SOL24 containing vesicles, the sample containing the empty vesicles with free drug appears to behave in an identical manner to the free drug. This is despite the fact that in vitro dialysis showed that an association between the DOX and the empty vesicles did occur (see section 3.2.8). The absence of any association observed after injection in vivo is probably due to the short time that the vesicles and drug are in contact before injection, since dialysis had shown the association only became noticeable after a few hours.

Although empty, negatively charged liposomes were non toxic to cells in vitro (Wolff and Rohdewald, 1984) and positively charged liposomes were toxic in vitro (Olson et al, 1982), van Hoesel et al (1984) found that neither negatively nor positively charged empty vesicles, containing stearylamine, produced an antitumour effect in rats. Injection of empty NSV's was shown to have some effect in reducing the liver parasite burden of Leishmania donovani amastigotes (Hunter et al, 1988).

Fluorescence photomicrographs taken of the various tissues after injection of drug loaded vesicles showed uptake into the liver and spleen with little seen in any other tissue except the tumour. The tumour uptake appeared to be much greater than that in the liver and

spleen and was somewhat at odds with the DOX concentrations measured in the tumour and the antitumour effect observed after injection of the DOX loaded vesicle sample. The vesicles taken up by the liver and spleen appeared to be residing in sinusoids or Kupffer cells while the vast majority of those in the tumour did not appear to have penetrated the tumour mass but were still retained within the vasculature. The diffuse orange colour seen around the tumour vessels at 22h was thought to be DOX which had been liberated from the vesicles and had diffused into the surrounding tissue.

As discussed above, the blood supply in tumours is different to that of normal tissue. After intravenous injection of a dye, lissamine green, the centre of a solid, subcutaneously grown, murine tumour contained no dye (Goldacre and Sylven, 1962). Thus, the intratumoural drug concentration will depend on the vascular supply of the tumour and the ability of the drug to penetrate the central regions of the tumour after it leaves the blood vessel.

Doxorubicin, injected intravenously as a free drug solution, appears to enter cells by diffusion of the neutrally charged, monomer molecule through the lipid domain of cell membranes (Dalmark and Storm, 1981). However, within the tumour mass there may be areas of necrosis and hypoxia which may alter the pH of areas within the tumour (Vaupel et al, 1981) and may affect the drug uptake.

For liposomal carriers, positively charged or uncharged unilamellar vesicles tended to be removed more slowly from the bloodstream than negatively charged vesicles (Juliano and Stamp, 1975) and the smaller the liposome the longer its blood residence time (Magin et al, 1986). The composition of the vesicles, as well as the in vivo tumour model used may affect the tumour uptake of the vesicles. Magin et al (1986) found that by increasing the blood

residence time of vesicles tumour uptake was not significantly affected, while Gabizon and Papahadjopoulos (1988) found that by altering the vesicular composition, an increased blood residence time and increased tumour uptake could be achieved.

It was suggested that positively charged vesicles initially interacted with negatively charged cells by electrostatic adsorption with subsequent internalization by fusion or, more probably, by endocytosis (Magee et al, 1974). This liposome-cell adsorption was increased with positively charged rather than negatively charged or uncharged liposomes (Roozemond and Urli, 1982; Jansons et al, 1978). Martin and MacDonald (1976) found that stearylamine was essential for liposomes to bind to erythrocytes, although this produced haemolysis at stearylamine concentrations greater than 0.5mol parts in electrolyte free systems. The efficiency of liposome-macrophage interaction in vitro can be increased by incorporation of stearylamine, although cell viability was significantly reduced at concentrations of stearylamine greater than 20mol% (Schwendener et al, 1984).

In the ROS tumour model used in this study, the positive charge combined with a size of 194nm may help extend the time that the vesicles reside in the bloodstream after intravenous injection. From the tissue photomicrographs, it appears that many of the vesicles reside on the walls of the blood vessels of the tumour. This may be due to an electrostatic interaction with the walls of the blood vessel of the tumour or may be due to removal of vesicles from the circulation due to the leaky capillary walls of the tumour vasculature. However, few vesicles seem to penetrate the bulk of the tumour and most remain at the wall of the blood vessel even after 22h. These vesicles will release their contents, presumably very

slowly as was suggested by <u>in vitro</u> dialysis, at the vessel wall where some of the free DOX will enter the tumour, as shown by the diffuse orange colour of the tissue after 22h, and the rest will be introduced into the bloodstream for distribution. This may help to explain why the measured tumour levels of DOX were so low and the apparent lack of antitumour effect despite the fact that there appear to be many of the vesicles delivered to the tumour.

The results obtained in this part of the work are difficult to interpret and discuss due to the inability to recover all the drug located in the tissues, and this limits the conclusions that can be drawn. However, these vesicles do have some encouraging features, notably the apparent ability of the vesicles to accumulate within the tumour and with further modification it may be possible to increase the DOX released in the tumour by changing the composition of the vesicles, and may increase the antitumour activity of the vesicles.

Alternatively, these vesicles with their slow release characteristics may be of more use in inhibiting the growth of slower growing tumours, rather than the relatively fast growing tumour used in this study.

Conclusions

Throughout this work it has been obvious that many of the non-ionic surfactant vesicles (NSV's) prepared had similar physicochemical properties to liposomes. Furthermore, important differences that gave each non-ionic surfactant vesicular composition its own set of unique properties were observed.

In general, for all vesicles the method of preparation largely determined the size and entrapment characteristics of the vesicular dispersions, with the two properties seeming to be interrelated. These properties could be altered to some degree by incorporation of a charged molecule in the vesicular bilayer or by the inclusion of cholesteryl polyoxyethylene ethers of varying chain length.

The formation of disc-like structures by equimolar concentrations of monoalkyl non-ionic surfactants and SOL24 was somewhat unexpected. From the information gathered so far on these discs, they appeared to be two-dimensional, measuring about 25μm in diameter and were fairly rigid since they shattered when briefly sonicated.

Sonication of multilamellar vesicles containing SOL5 (25 or 50mol%) and a monoalkyl surfactants resulted in the system breaking down. The system then reformed, producing multivesicular vesicles rather than SUV's.

The discs and the multivesicular structures were not formed if the dialkyl non-ionic surfactant was used. This suggested that the properties and dimensions of the surfactant were one important factor in determining how the vesicular components interacted to produce the final system.

The addition of cholesteryl polyoxyethylene ethers of increasing polyoxyethylene chain length to vesicles was found to reduce their

electrophoretic mobility (EM) and zeta potential over a wide pH range. Encapsulation of doxorubicin (DOX) in SOL24 containing vesicles had little effect on the EM when compared with empty vesicles encapsulating only buffer. When DOX was encapsulated in positively charged vesicles, an almost constant EM was measured over the pH range examined. Empty vesicles behaved differently, with the EM falling constantly with increasing pH. The difference in electrophoretic behaviour of the drug loaded and empty vesicles was likely to be related to the ionization of the DOX and the stearylamine molecules, at a given pH. The eventual charge at the vesicular surface would be determined by the pH at the vesicular surface, how ionized the molecules were at that pH and to what extent the molecules affected each others ionization state.

Calorimetric studies showed that the NSV's behaved very similarly to liposomes. As the cholesterol content of the NSV's was increased, the size of the endothermic peak was reduced, and the peak was eventually abolished by the incorporation of 50mol% cholesterol. The addition of a charged molecule had little effect on the thermograms obtained. Any slight peak temperature shift or change in peak shape was thought to be due to the insertion of the alkyl chain of the charged molecule into the vesicular bilayer.

The formation of sonicated dispersions in either water or 300mM glucose solution appeared to produce stable dispersions with little aggregation noted after 30days. When phosphate buffered saline was used as the hydrating solution, aggregation was observed. NSV's were shown to be prone to aggregation when exposed to NaCl. However, the inclusion of a charged molecule or of cholesteryl polyoxyethylene (24) ether (SOL24) in the vesicular composition overcame this aggregation.

Data from dialysis studies were difficult to interpret due to the complex nature of the processes occurring. However, all the DOX loaded vesicles produced a biphasic release with an initial fast phase followed by a much slower release. Incorporation of SOL24 had little effect on the drug release compared with vesicles containing only surfactant and cholesterol. When stearylamine was included in the vesicular composition, the DOX releases were much slower. However, by reducing the cholesterol or stearylamine content of the vesicles, a more rapid drug release could be obtained. Dialysis studies carried out with empty vesicles combined with a free DOX solution gave some interesting results. Empty vesicles containing SOL24 appeared to reversibly bind DOX, with this binding seeming to take place as soon as the vesicles and drug came into contact. When stearylamine containing empty vesicles were mixed with DOX solutions, no immediate binding of drug to the vesicles occurred. However, after 3–4h, binding of drug began to occur with the drug appearing to become tightly bound to the vesicle. As the number of vesicles was increased, the amount of DOX bound increased.

The intravenous injection of DOX loaded, SOL24 containing vesicles into mice resulted in tissue distributions and plasma profiles similar to those reported previously for NSV's and liposomes. The plasma drug profile remained higher than that of free drug throughout the duration of the experiment and cardiac and kidney peak drug concentrations were reduced when the drug was in encapsulated form. Peak liver levels were similar for both the free DOX solution and the encapsulated drug although the encapsulated drug levels were higher at later times. This type of plasma profile and tissue distribution of vesicles would appear to support the theory that the vesicles act as a "depot" for DOX after their removal from

the blood. Drug metabolism in the liver was reduced by encapsulation. This suggested that only free, non-encapsulated DOX was capable of being metabolised while the drug remaining in the vesicles was protected.

The co-administration of a free drug solution with empty vesicles produced some interesting results. Peak drug concentrations in the heart and kidney were higher than that of free drug in solution alone and was thought to be caused by increased penetration of the DOX into these tissues by the polyoxyethylene chains of the SOL24 protruding from the vesicle surface. The plasma and liver profiles generally lay between those of the encapsulated drug and free drug and may be due to binding of some of the drug to the vesicles and thus acting as a small "depot" of drug. Drug metabolism in this case was similar to that measured for the free drug solution.

As previously discussed, the distribution study using the stearylamine containing, DOX loaded vesicles was hampered by the inability of the extraction method to recover all the drug present in the various tissues. Very low concentrations of drug were recovered from the tissues, and this was thought to be DOX which had been released from the vesicles in vivo, while any DOX still associated with the vesicles was not recovered. If this is the case, it may be possible to calculate an in vivo drug release and compare this with the release measured in vitro. The in vivo release appeared to be very slow, which relates well with the drug release measured from the vesicles in vitro.

The metabolic profiles obtained showed that encapsulation of the drug reduced metabolism when compared to that measured for free drug solutions.

Unlike the previous study, injection of free drug solutions or free drug with empty vesicles produced virtually identical drug levels and profiles in all tissues and similar metabolite profiles.

Furthermore, results from the tumour growth delay study showed the drug loaded vesicles produced a slight growth delay which would appear to be consistent with a slow in vivo drug release. The antitumour activity of free drug solutions and free drug solution with empty vesicles was almost identical.

Fluorescence photomicrographs showed DOX loaded, stearylamine containing vesicles were taken up by liver Kupffer cells and into spleen sinusoids. However, many of the vesicles appeared to be attached to the walls of the tumour blood vessels with a few seeming to penetrate the tumour mass. Drug appeared to be slowly released from these vesicles at later times and appeared to contradict some of the evidence above.

Pharmacokinetic modelling of the fate of vesicles and the release of their encapsulated drug may provide one way of ascertaining what is actually happening in vivo.

The work carried out in this thesis has shown that the surface properties of non-ionic surfactant vesicles can be modified by incorporation of suitable molecules. However, until the precise parameters which affect the in vivo fate of vesicles are known, custom made vesicles with the desired in vivo characteristics cannot be formed.

Suggestions for Further Work

The most pressing piece of further work is to attempt to obtain total DOX concentrations in murine tissues and plasma after intravenous injection of DOX loaded. stearylamine containing NSV's. Efforts thus far to increase DOX recovery from the tissues by modifying the extraction method have failed and it may be that to obtain this information. radiolabelled vesicles must be used. Ideally. the vesicles would contain a dual label i.e different labels being used on the drug and surfactant molecules such that they could be detected independently. This dual label would enable estimates of the amount of DOX which was still vesicularly entrapped to be made. Armed with evidence about the total distribution of the vesicularly encapsulated drug. more definite conclusions could be drawn about the pharmacokinetics of these vesicles and how this relates to the results from the tumour growth delay study and fluorescent photomicrographs.

It would be possible to go on ad infinitum modifying the composition of vesicles and the methods of preparation and measuring the changes in the physicochemical properties. This would be time consuming and of little benefit in increasing the pool of information already known about the properties of NSV's. However, several vesicular parameters appear to be more important than others in determining their in vivo fate. For this reason it may be that methods of vesicular preparation capable of producing vesicles which have a diameter smaller than about 100nm. such as extrusion methods should be pursued. The incorporation of poloxamer/poloxamine polymers or other long chained hydrophilic molecules into vesicular bilayers may enable the in vivo distribution of the injected vesicles to be altered and incorporation of specific molecules such as

monoclonal antibodies may allow some degree of targeting to particular tissues of interest.

X-ray diffraction studies of the vesicles may help provide information about the interbilayer distances and how changing the composition of the vesicles affects this distance.

Many interesting structures were produced during this work and may be worth investigating further. The release characteristics of the multivesicular vesicles formed are likely to be different to that of multilamellar vesicles and identification of the actual form and structure of the discs produced would also be of interest.

Many arguments still rage as to exactly what happens to vesicles, be they liposomes or NSV's, after they are injected into animals. Perhaps computer pharmacokinetic modelling programs will be able to shed some light on where vesicles are finally located, the release characteristics of the vesicles in these tissues and the ways in which the released drug further affects the drug concentrations in the plasma and tissues.

The way in which vesicles interact with various tissues in vitro could be examined and may help to decide the most suitable vesicular composition to give the desired distribution in vivo.

The ultimate aim of much of this work on vesicular carriers is to produce a system which will be useful in the treatment of various human conditions. However, before this can be attempted, methods of producing vesicles with consistent properties and which can be made sterile and pyrogen free must be devised. More information on the potential toxicity of vesicular carriers needs to be obtained as does the effect of their chronic administration.

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